The binding of Varp to VAMP7 traps VAMP7 in a closed, fusogenically inactive conformation

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SNAREs provide energy and specificity to membrane fusion events. Fusogenic trans-SNARE complexes are assembled from glutamine-contributing SNAREs (Q-SNAREs) embedded in one membrane and an arginine-contributing SNARE (R-SNARE) embedded in the other. Regulation of membrane fusion events is crucial for intracellular trafficking. We identify the endosomal protein Varp as an R-SNARE-binding regulator of SNARE complex formation. Varp colocalizes with and binds to VAMP7, an R-SNARE that is involved in both endocytic and secretory pathways. We present the structure of the second ankyrin repeat domain of mammalian Varp in complex with the cytosolic portion of VAMP7. The VAMP7–SNARE motif is trapped between Varp and the VAMP7 longin domain, and hence Varp kinetically inhibits the ability of VAMP7 to form SNARE complexes. This inhibition will be increased when Varp can also bind to other proteins present on the same membrane as VAMP7, such as Rab32–GTP.

SNAREs are at the core of the vesicle-organelle and organelle-organelle membrane fusion machinery as a consequence of their ability to provide much of the mechanical energy and specificity to these events. They are small membrane proteins that contain at least one 60-residue SNARE motif. Four SNARE motifs come together to form a four-helix bundle called the trans-SNARE complex. One SNARE (an R-SNARE, previously called a vesicle SNARE or v-SNARE) is on one of the membranes to be fused, and the other SNAREs (Q-SNAREs, previously called target SNAREs or t-SNAREs) are on the other. The ‘zippering up’ of the SNARE motifs pulls the two membranes into sufficiently close apposition to allow fusion to proceed (reviewed in refs. 1,2). Only certain combinations of the 38 mammalian SNAREs are able to form fusogenic trans-SNARE complexes, and this ability has a major role in conferring specificity to the fusion process3,4. Both the fusogenic activity of SNAREs and their localization to specific intracellular membranes must be carefully controlled if the vesicle transport system is to function correctly. The subcellular distribution of the SNAREs is determined by incorporation into or exclusion from the various types of route-specific transport vesicles and tubules within the cell.

In most SNAREs, the SNARE motif is preceded by either a short unstructured peptide of 10–30 residues or a folded domain of 100–150 residues5. The syntaxins and Vti1 (VPS10 tail interactor 1) SNAREs have three-helix Habc domains, which, in some cases, bind back onto the SNARE’s own SNARE motif and so can inhibit their ability to participate in SNARE complex formation (reviewed in ref. 5). In the Qa-SNAREs, regions of the Habc domain interact with proteins of the Munc18 family to regulate Qa-SNARE incorporation into SNARE complexes, with both inhibitory and activating roles having been proposed6–9.

In mammalian cells, the major post-Golgi R-SNAREs are the VAMPs, of which only VAMP7 has a folded N-terminal region: a 120-residue mixed α and β longin domain that is also present in the R-SNAREs Sec22b and Ykt6 (ref. 10). VAMP7 is highly conserved across eukaryotes, is ubiquitously expressed and has been reported to be involved in a variety of membrane trafficking events. VAMP7 is also important for heterotypic fusion events both between terminal endocytic compartments (late endosomes and lysosomes) by complexing with syntaxin7, syntaxin8 and vti1b11 and between these organelles and other cellular membranes, including autophagosomes12,13 and, by complexing with syntaxin3 or syntaxin4 and SNAP23, the limiting membrane of the cell14. The latter complex allows VAMP7 to have a role in wound healing14,15, metastasis through secretion of the endolysosomal membrane type 1 matrix metalloproteinase protein16 and the expansion of the plasma membrane during mitosis17. VAMP7 also has an important role in secretion in specialized cells (reviewed in ref. 15), neurite outgrowth18 and dendrite formation in melanocytes19 and has been implicated in more generalized secretion to the plasma membrane from the trans-Golgi network15. Because of its involvement in such a diverse set of important membrane fusion events, it is logical that both the organelle-localization and fusogenic activity of VAMP7 should be tightly controlled.

To identify binding partners for VAMP7 that could regulate its localization and ability to form fusogenic SNARE complexes, we20 and others21–23 used yeast two-hybrid screening (Y2H) for both the entire cytosolic region and the isolated N-terminal longin domain
of VAMP7. Screening with the longin domain identified two vesicle-coat components, the heterotetrameric AP3 adaptor complex δ-adaptin subunit and Hrb, a clathrin adaptor and member of the ArfGAP family, both of which direct the trafficking of VAMP7 along the endocytic pathway.20,22–24 Here we demonstrate that the widely expressed, multidomain protein Varp (Vps9 and ankyrin repeat–containing protein), which is an endosomal Rab21 guanine nucleotide exchange factor (GEF)25 and effector for Rab32 and Rab38 (refs. 26,27), is a binding partner for the full cytosolic domain of VAMP7 but not for the isolated longin domain nor the SNARE motif alone. We have solved the structure of the complex containing portion of VAMP7 but not for the isolated longin domain nor the SNARE motif alone. We have solved the structure of the complex

Figure 1 Mapping and confirming the binding site of Varp on VAMP7. (a) Coomassie-stained (top) and western blots developed with HisProbe–horseradish peroxidase conjugate (bottom) from SDS-PAGE of GST pulldown assays using the baits indicated. Full-length Varp (Varp control) interacts with GST-VAMP7CD (GST-V7CD) but not with GST or GST-VAMP7LD (GST-V7LD). (b) Coomassie-stained SDS-PAGE showing the effects of digesting recombinant Varp-His10 with chymotrypsin (10 min) and trypsin (20 min). N, proteolytic products of Varp with an N terminus identical to that of the full-length protein; the asterisk indicates the C-terminal chymotryptic fragment of Varp (residues 653–1050) with an intact C-terminal His10 tag. Western blotting of the SDS-PAGE (right) showed that, of the proteolytic fragments produced, the only detectable interaction was between the C-terminal chymotryptic fragment of Varp (residues 653–1050) and GST-VAMP7CD. Chym, chymotryptic digestion products; tryp, tryptic digestion products. (c) Panel order as in a. His10-tagged residues 653–1050, 658–921, but not 718–921, of Varp can bind to GST-VAMP7CD, whereas no Varp constructs bind to GST-VAMP7LD. (d) Domain organization of human Varp (left) and mouse VAMP7 (right) as indicated by pfam30 and described previously25. The color scheme used is replicated in all subsequent figures. A summary of the mapping of the interaction determined using the constructs indicated is shown. (e) Panel order as in a. Varp658–921–His6 is able to bind GST-VAMP7CD but not the GST-VAMP7–SNARE motif or GST-VAMP7LD. (f) Panel order as in a. Varp658–921–His6 cannot bind to the GST-tagged full cytosolic domains of the mammalian longin domain–containing SNAREs Sec22b (GST-Sec22bCD) and Ykt6 (GST-Ykt6CD). (g) Panel order as in a. Truncating GST-VAMP7 before residue 160 abolishes binding to Varp658–921–His6.

RESULTS

Varp binds only to the whole cytosolic domain of VAMP7

Our parallel Y2H screens of complementary DNA libraries using the VAMP7 longin domain (VAMP7LD) and the full VAMP7 cytosolic domain (VAMP7CD) gave completely nonoverlapping results. Screening with the longin domain resulted in 37 identifications of δ-adaptin and 1 identification of Hrb as binding partners. Our screen with the full-length cytosolic domain of VAMP7 showed no interaction with Hrb or δ-adaptin but did show interaction with SNAP29 (1 identification) and Varp (11 identifications) as binding partners (Supplementary Fig. 1). These data are in broad agreement with previously published studies.21–23 We cloned the open reading frame of the full-length human Varp gene from the IMAGE clone IMAGE:6067580 and confirmed the interaction with VAMP7 by Y2H (Supplementary Fig. 1) and glutathione S-transferase (GST) pulldowns using both inhibitory effect. Potential roles of this inhibition in vivo are discussed in the light of the other functions of Varp.
endogenous Varp from MNT-1 cells (Supplementary Fig. 1) and Varp expressed in a C-terminally His10-tagged form in Escherichia coli (Fig. 1). However, in both Y2H and the GST pulldown experiments, only the full-length cytosolic portion of VAMP7, but neither the isolated longin domain nor the isolated SNARE motif (Fig. 1), interacted with Varp. This is consistent with our subsequent structural work but not with previously published data21.

Mapping the VAMP7-interacting domain of Varp

We then used domain analysis and structure prediction28,29 in combination with N-terminal sequencing of limited tryptic and chymotryptic proteolytic digests of full-length Varp to identify stable domains of Varp (Fig. 1). The smallest of the designed stable domains that still bind strongly to VAMP7 consisted of residues 658–921 of Varp, which includes the second predicted set of four adjacent ankyrin repeats30 and part of the linker between these and the first predicted set of ankyrin repeats. This domain also contains the smallest portion of Varp641–707 that is common to all VAMP7-interacting clones identified in another study22 and the smallest region (residues 671–731) found in a single interacting clone in our own Y2H screen (Supplementary Fig. 1). As in the full-length protein, residues 658–921 of Varp bound only to full-length VAMP7 and not the isolated SNARE motif or the longin domain and were also unable to bind the full-length cytosolic portions of the other longin domain–containing SNAREs Ykt6 and Sec22 (Fig. 1 and Supplementary Figs. 1 and 2).

Residues 658–921 of Varp showed robust binding to the VAMP7 full-length cytosolic domain (residues 1–188). We measured the dissociation constant (Kd) and the stoichiometry of the interaction by isothermal titration calorimetry (ITC) as 2.3 ± 0.6 μM (mean ± s.d.) and 1:1, respectively, the latter of which we confirmed by gel filtration (Supplementary Fig. 2e). ITC gave a similar Kd for full-length Varp binding to VAMP7 (Kd ~3 μM; Supplementary Fig. 2), indicating that no additional VAMP7 binding sites were present. In addition, residues 1–650 of Varp showed no binding to VAMP7 (Supplementary Fig. 2), and we did not identify any Varp clones in our Y2H screen that did not contain residues 671–731 (Supplementary Fig. 1). Using C-terminal truncations of VAMP7, we also found that only residues 1–160 (containing the longin domain and the N-terminal portion of the SNARE motif) were necessary for binding of VAMP7 to Varp (Fig. 1).

Varp and VAMP7 colocalize in the endocytic pathway

In the absence of an antibody that detects endogenous Varp in cells, we expressed Varp with a hemagglutinin (HA) tag appended at its C terminus (Varp-HA) in NRK cells and showed good, although not complete, colocalization with endogenous VAMP7 and also with Rab7, a marker of late endosomes, using immunofluorescence microscopy (Fig. 2). Varp-HA also partially colocalized with lgp110 (also known as LAMP2), a marker of terminal endocytic compartments, but localized poorly with the trans-Golgi network (TGN) marker TGN38 (Supplementary Fig. 3). We were also able to detect faint Varp-HA staining at the cell periphery. Immunogold electron microscopy of NRK cells expressing Varp-HA showed the presence of Varp on organelles with the characteristics of endosomes, vesicular tubular elements, lysosomes and the plasma membrane (Fig. 2 and Supplementary Fig. 3). This localization pattern was also consistent with sites at which VAMP7 has previously been shown to localize and suggested to function23,31. The localization of Varp is consistent with it being an effector of the two highly similar Rabs Rab32 and Rab38 (67% sequence identity26,27), which, together with Rab7, Rab7L1, Rab29 and Rab23, are classified as the late endocytic group III Rabs32. Rab32 is expressed at low levels in a wide variety of cell types33–35, including the NRK cells used in this study.

**Figure 2** Varp-HA is localized to vesicular-tubular elements of the endocytic pathway. (a,b) Confocal immunofluorescence of NRK cells stably expressing Varp-HA stained for the HA tag and either endogenous VAMP7 (a) or antibody to monomeric red fluorescent protein (mRFP) after transfection with mRFP-Rab7 (b). The bottom row shows enlarged images of the boxed areas in the top row. The arrowheads indicate examples of structures positive for both markers. Scale bar, 20 μm. (b) The Pearson R correlation coefficient for Varp-HA and VAMP7 colocalization was 0.67 and for Varp-HA and mRFP-Rab7 was 0.51. The bottom row shows enlarged images of the boxed areas in the top row. The arrowheads indicate examples of structures positive for both markers. Scale bar, 20 μm. (c,d) Immunogold electron microscopy of NRK cells stably expressing Varp-HA and that had taken up dextran conjugated to Texas Red were labeled for Texas Red (5 nm gold), to lgp110 (10 nm gold) or to the HA tag (15 nm gold). Anti-HA immunoreactivity (large arrowheads show representative examples) associated with vesicular-tubular clusters (c) and dense core lysosomes (d) immunolabeled with anti–Texas Red and anti-lgp110 (small arrowheads show representative examples, and large arrowheads are as in c) on ultrathin cryosections. Scale bar, 200 nm. (e) Quantification of the labeling density of HA associated with membranous compartments: 62% of the immunogold (Au) labeling was associated with cytosol. The relative distributions of membrane-associated gold label with the plasma membrane (PM), lysosomes, endolysosomes and hybrid organelles (Lys-hyb), endosomes and vesicular-tubular clusters (E-VTC) and the TGN are shown. Data shown as the mean of four independent immunolabeling experiments ± s.e.m.
(Supplementary Fig. 3), and has previously been detected on late endocytic pathway organelles and, along with Rab38, on melanosomes26,36–38. Thus, the recently reported physiological roles of the interaction of Varp with VAMP7, including the Rab32- and Rab38-dependent Tyrp1 trafficking in melanocytes19, are consistent with this localization of Varp. We also found that a 75% depletion of Varp

| Table 1 Data collection and refinement statistics |
|-------------------------------------------------|
| **Native** | EMTS1 | EMTS2 | PCMBS1 | PCMBS2 |
| **Data collection** | | | | |
| Space group | C222₁ | C222₁ | C222₁ | C222₁ | C222₁ |
| Cell dimensions | 69.6, 122.7, 158.6 | 69.1, 123.2, 158.6 | 69.7, 123.5, 158.7 | 69.5, 123.1, 158.8 | 69.7, 123.0, 158.6 |
| Resolution (Å) | 2.00 (2.05–2.00) | 2.45 (2.55–2.45) | 2.09 (2.15–2.09) | 2.29 (2.37–2.29) | 2.30 (2.38–2.30) |
| Resolution (Å) along a, b, c | 2.69, 2.51, 2.00 | 3.37, 2.83, 2.45 | 2.86, 2.95, 2.09 | 3.07, 2.81, 2.29 | 3.04, 2.63, 2.30 |
| Rmerge | 0.103 (0.96) | 0.131 (1.50) | 0.117 (1.37) | 0.105 (1.09) | 0.104 (1.57) |
| Rmerge-c | 0.134 (1.50) | 0.181 (1.88) | 0.147 (1.79) | 0.129 (1.39) | 0.135 (2.02) |
| CC₁/₂ | 0.994 (0.59) | 0.994 (0.50) | 0.995 (0.42) | 0.996 (0.65) | 0.996 (0.77) |
| Completeness (%) | 99.7 (99.5) | 96.6 (98.4) | 99.5 (97.9) | 99.1 (97.2) | 99.4 (99.7) |
| Multiplicity | 4.0 (3.4) | 4.1 (4.1) | 4.4 (3.8) | 4.8 (4.3) | 4.0 (4.1) |
| Wilson B-factor (Å²) | 33 | 55 | 38 | 47 | 44 |

| **Refinement** | | | | | |
| Resolution (Å) | 2.00 |
| No. reflections | 43,692 |
| Rwork / Rfree | 0.213 / 0.251 |
| No. atoms | 2,924 |
| Protein | 2,715 |
| Water | 209 |
| R.m.s. deviations | | |
| Bond lengths (Å) | 0.018 |
| Bond angles (°) | 1.8 |

Values in parentheses are for the highest-resolution shell. Resolution estimates are based on half data set correlation coefficients (CC₁/₂ >0.5, calculated in cones along a*, b*, c* of semi-angle 20°). Rmerge = Σh–I(h)–1/ΣhI(h), where n is the number of observations for unique reflection h with mean intensity <I(h), summed over all reflections for each observed intensity Ih. CC₁/₂ is the correlation coefficient on <I> between random halves of the data set. EMTS, ethylmercurythiosalicylate; PCMBS, p-chloromercuribenzenesulfonate. Δanom, anomalous difference I⁺ – I⁻.

**Figure 3** Molecular details of the interaction between Varp and VAMP7. (a–c) Structure of the Varp–VAMP7 complex. VAMP7 (longin domain, purple; SNARE motif, green) binds in a closed conformation to the top of the ankyrin stack of Varp. The N-terminal ankyrin repeat is in cyan, the four predicted ankyrin repeats are in yellow, and the C-terminal ankyrin repeat is in red. (d–f) Comparisons with other VAMP7 complex structures. Superposition of VAMP7CD with the VAMP7–Hrb20 and VAMP7–δ-adaptin (AP3-δ) complexes24. The VAMP7–SNARE motif (green), the Hrb peptide (gold) and the δ-adaptin peptide (cyan) all bind to the same groove of VAMP7LD (purple). Hydrophobic residues that slot into the hydrophobic groove of VAMP7LD are shown. (e) Structure-based alignment of the three VAMP7LD-binding sequences showing that they share a pattern of hydrophobic side chains (yellow shading), but the key residues are separated by distinct sequences. (f) Ribbon representations of the three complexes in e superimposed on the basis of the longin domains.
by short interfering RNA (siRNA) treatment in NRK cells showed no readily detectable alteration in the trafficking of VAMP7 and only a subtle increase in the steady-state colocalization of VAMP7 with a terminal endocytic compartment marker (Supplementary Fig. 3), which is consistent with an increase in the amount of fusion between the late and terminal endocytic compartments.

The structure of the Varp–VAMP7 complex

The robust interaction between the stable domain consisting of residues 658–921 of Varp and the VAMP7 cytosolic domain ($K_d \sim 2 \mu M$) allowed us to purify a complex of the two individually expressed proteins by gel filtration (Supplementary Fig. 4). We crystallized the complex between the two proteins and solved its structure by multiple isomorphous replacement using mercury derivatives (Table 1). The structure of VAMP7LD was as expected from previous studies, but we found that Varp 658–921 forms a stack of six ankyrin repeats rather than the four that were predicted (Fig. 3 and Supplementary Fig. 4). The first ankyrin repeat, although separated from the next five repeats by a disordered region (residues 694–729) for which we found no electron density, contacts the second ankyrin repeat through an extensive and tightly packed hydrophobic interface. The VAMP7 binds on top of the ankyrin repeat stack, binding almost exclusively to the first repeat, and this represents a new mode of ligand binding by ankyrin repeats (Fig. 3 and Supplementary Fig. 5).

Figure 4 Analysis of the Varp–VAMP7 complex interface. (a) Semitransparent surface representation of the Varp–VAMP7 complex with the underlying backbone structure showing. Residues involved in the interface are shown in ball and stick representation, and these residues and the parts of the protein surfaces they map to are highlighted in color (VAMP7LD, purple; VAMP7–SNARE motif, green; Varp, cyan). (b) The ‘flipped open’ interaction interface of the Varp–VAMP7 complex. VAMP7 (V7) and Varp have been rotated and separated as indicated by the arrows. Side chains involved in the interaction are shown and colored as in a with only the VAMP7–SNARE motif backbone shown for clarity. (c) VAMP7–SNARE motif and the longin and cytosolic domains rotated and separated as indicated by the arrows. Residues involved in the binding to Varp are shown in darker purple (on the longin domain) or green (on the SNARE motif) and labeled accordingly. Residues involved in the interaction between VAMP7LD and the SNARE motif are shown in lighter purple or green and labeled in black.

Similarly, residues 121–128, which link the longin domain to the SNARE motif (Supplementary Fig. 6), also have no electron density. The complex structure shows that, when bound to Varp, VAMP7 adopts a closed conformation whose existence has been previously suggested for the isolated protein free in solution. In this closed state, an ordered part of the SNARE motif (residues 129–163) binds back in an extended conformation in a groove stretching half-way around globular VAMP7LD (Fig. 3). The interaction between the longin domain and the SNARE motif is mediated largely by the hydrophobic side chains (Figs. 3 and 4), burying ~2,300 Å$^2$ of accessible surface area (calculated with PISA), which explains the stability of this interaction in solution. A subset of these longin domain–interface residues are protected in NMR experiments, confirming that the intramolecular ‘bound-back’ conformation of VAMP7 in the complex structure presented here is preformed in solution and trapped by Varp. Comparison with the structures of VAMP7–Hrb and VAMP7–δ-adaptin shows a similar binding mode in the same groove on VAMP7LD for fragments of Hrb, δ-adaptin and the VAMP7–SNARE motif, with the same longin domain residues being important to all three interactions (Fig. 3). Thus, the interactions of VAMP7LD with Hrb or δ-adaptin and the SNARE motif are mutually exclusive. There is, however, little homology between the three longin domain ligands: their key residues are differently spaced, and, therefore, we could not predict their mechanisms of binding without detailed structural information. The major difference between the three interactions is that the interaction between VAMP7LD and its SNARE motif is intramolecular and the other two interactions are intermolecular;
Figure 5 Mechanistic details and quantification of the Varp–VAMP7 interaction. (a) The interaction interface between VAMP7 and Varp is comprised of residues belonging to the first binding ankyrin repeat of Varp and both the longin domain and SNARE motif of VAMP7. Residues targeted by mutagenesis that were used to confirm the interface in solution are highlighted by a box of the same color as the corresponding ITC traces. (b,c) ITC analysis of the VAMP7–Varp interaction. Mutations in the Varp–VAMP7 interface abolish the binding of Varp to VAMP7. The $K_d$ for the wild-type (WT) proteins was $2.3 \pm 0.6 \mu M$ (mean ± s.d.), and all four mutations, which do not affect folding as judged by circular dichroism (data not shown), reduce binding to below detectable levels ($K_d > 300 \mu M$).

Effect of mutating residues in the Varp-VAMP7 interface

The longin domain and the SNARE motif make approximately equal contributions to VAMP7 binding to Varp (Figs. 3–5). Because the Varp binding site on VAMP7 can only be formed when the extended SNARE motif passes through the groove on the longin domain, the complete cytosolic domain of VAMP7 is required for Varp binding, and neither the longin domain nor the SNARE motif on their own are sufficient for the interaction (Fig. 1). Thus, our structural data are entirely consistent with our interaction data determined by both Y2H and GST pulldowns.

The interface buries a total of 1,200 Å$^2$ of solvent-accessible surface area, a figure that, although not inconsistent with the $K_d$ of $\sim 2 \mu M$ for the interaction, is on the low side of what could be expected for this $K_d$ value. This may be explained by the network of charged hydrogen bonds centered on Arg72 of the longin domain, which is 90% buried, with most of the hydrogen bonds consequently protected from solvent (Fig. 5 and Supplementary Fig. 6).

Regions of contact involve contributions from all three components of the complex (Fig. 5). Using ITC experiments, we demonstrated that mutations in residues that are crucial to the interaction between all three components, M684D Y687S and D679A D681A in Varp and R72A and D69A E71F S73D in VAMP7, abolished complex formation (Fig. 5) but did not affect protein folding, as judged by behavior during purification and circular dichroism (data not shown). The effects of these mutations confirm that the complex identified in the crystal structure is the same as that in solution. Most of the residues in VAMP7 that are involved in Varp binding are not conserved in the other longin domain–containing SNAREs Sec22 and Ykt6 (Supplementary Fig. 6), which explains why these SNAREs have no binding to Varp (Fig. 1f).

Varp inhibits the ability of VAMP7 to form SNARE complexes

The intermolecular Hrb and AP3 β-adaptin interactions with VAMP7 direct the trafficking of inactive cis-SNARE complexes containing VAMP7, suggesting that the intramolecular interaction of Varp with uncomplexed VAMP7 has a different cellular role. The complex structure indicates that Varp traps a closed conformation of VAMP7 in which the SNARE motif is unavailable for cognate trans-SNARE complex formation. In VAMP2, VAMP3 and VAMP8, a trigger-site sequence initiates SNARE complex formation$^{41}$. The equivalent trigger sequence of VAMP7, residues 136–142 (LKGIMVR), is sandwiched in the VAMP7–Varp interface, with the crucial isoleucine and methionine residues buried in the center of the interface (Fig. 6).

To test the hypothesis that Varp binding inhibits the ability of VAMP7 to form SNARE complexes, we analyzed the formation of the syntaxin7–syntaxin8–Vti1b–VAMP7 SDS-stable SNARE complex (Supplementary Fig. 7) as a function of Varp$_{658–921}$–His$_6$ concentration. We found that the extent of SNARE complex formation is inversely correlated with the concentration of Varp$_{658–921}$–His$_6$ present in the reaction, whereas the VAMP7 nonbinding mutant of Varp$_{658–921}$–His$_6$ (M684D Y687S) did not inhibit SNARE complex formation (Fig. 6).

In a related set of experiments, we analyzed the time dependence of SNARE complex formation on the full-length cytosolic portion of GST-VAMP7 and on the GST-VAMP7–SNARE motif (which is missing the putative inhibitory longin domain) in the presence of wild-type and M684D Y687S mutant Varp$_{658–921}$–His$_6$, and in the absence of Varp$_{658–921}$–His$_6$ (Fig. 6 and Supplementary Figs. 7 and 8). In assays with the full GST-VAMP7 cytosolic domain, wild-type Varp$_{658–921}$–His$_6$, but not the M684D Y687S mutant, reduced the rate of SNARE complex formation to about 30% of the rate when no Varp was present (notably, however, after 24 h, the level of SNARE complex attained in the presence of wild-type Varp$_{658–921}$–His$_6$ was still only 80% of that achieved when the M684D Y687S VAMP7 nonbinding mutant was present).
The SNARE complex formation rates were the highest when using the GST-VAMP7–SNARE motif (Fig. 6d and Supplementary Fig. 8), thus confirming the proposed inhibitory role of the longin domain. In this case, the presence of wild-type or M684D Y687S mutant Varp had no observable influence on complex formation, which again is in line with our observations that the binding of Varp to VAMP7 occurs only to the full-length VAMP7 cytosolic domain. Thus, the presence of the longin domain on VAMP7 and the addition of Varp together resulted in a rate of SNARE complex formation that was 60–70 times slower than that with the isolated VAMP7–SNARE motif (in other words, only 1.5% of the rate seen with the isolated motif), demonstrating that, at least in vitro, this two-component system functions as an effective kinetic inhibitory system for VAMP7-mediated SNARE complex formation.

Varp does not inhibit VAMP8’s ability to form SNARE complexes

VAMP8, which is also found on compartments of the endocytic pathway, is closely related to VAMP7 (43% sequence identity and 68% similarity) in their SNARE motifs, and both can form complexes with the same Q-SNAREs (syntaxin7, syntaxin8 and Vti1b), but VAMP8 has no longin domain. We therefore investigated the rate of complex formation with syntaxin7 and syntaxin8 (SNARE motifs only) on GST-Vti1b for VAMP7 and thioredoxin (Trx)-VAMP8 and tested whether they were sensitive to the presence of Varp658–921–His6. The rate of SNARE complex formation for VAMP8 was faster than for VAMP7, with around six times more SNARE complex formed with VAMP8 than with full-length VAMP7 after 2.5 h, and was similar to the rate with the isolated VAMP7–SNARE motif (Figs. 6 and 7). Again, as with the VAMP7–SNARE motif only (Fig. 6), the presence of Varp658–921–His6 at either an equimolar or a ten-fold excess of the amount of VAMP caused no obvious reduction in the amount of VAMP8 incorporated into SNARE complexes but markedly reduced the amount of VAMP7 that incorporated. In fact, to achieve approximately equal amounts of VAMP7 and Trx-VAMP8 incorporation into SNARE complexes when they were both present in the same reaction and when Varp was absent, we needed to use ten times as much VAMP7 as Trx-VAMP8. Under these conditions, the presence of Varp658–921–His6 at an equimolar and a ten-fold excess of the amount of VAMP caused a 74% and a 95% reduction, respectively, in the amount of VAMP7 incorporated into SNARE complexes compared to the reaction without Varp, whereas the amount of Trx-VAMP8 incorporated in these conditions increased by 20% and 27%, respectively.

Varp binds simultaneously to VAMP7 and Rab32 or Rab38

It has been demonstrated that residues 378–717 of Varp, which are predicted to contain another set of ankyrin repeats, interact with the GTP-bound form of the closely related endosomal Rabs Rab32 and Rab38 (ref. 27). Because avidity effects through a single protein molecule binding multiple ligands on the same membrane can greatly increase the apparent affinity of a protein for a membrane, we investigated whether Varp could bind to both VAMP7 and Rab32 or Rab38–GTP. We pulldowns on GST-Rab32–GTP and Rab38–GTP with either GST-VAMP7 or GST-VAMP8 to investigate whether Varp could bind to both VAMP7 and Rab32 or Rab38–GTP. We only detected VAMP7CD incorporated in pulldowns on GST-Rab32–GTP or Rab38–GTP at the same time. We only detected VAMP7CD incorporated in pulldowns on GST-Rab32–GTP Q69L in the presence of Varp658–921–His6, which contains both the VAMP7 and the Rab binding site.

We obtained identical results using Rab32 instead...
of Rab38, and neither Rab was able to bind directly to VAMP7 (data not shown). These data therefore establish that Varp can bind simultaneously to both VAMP7 and Rab32–GTP or Rab38–GTP.

**DISCUSSION**

If VAMP7-mediated fusion events of various organelles with endolysosomes (reviewed in refs. 47,48) took place at the wrong time and/or place, there would be serious consequences for the cell, with inappropriate luminal content digestion occurring. It therefore seems logical that VAMP7-mediated fusion events should be tightly and accurately controlled in both a spatial and temporal manner, and that inappropriate fusogenic behavior of VAMP7 must be effectively inhibited. This regulation must, however, occur in the context of low-nanomolar K_d values for adding the R-SNARE to a preformed Q-SNARE complex.  

In this study, we show how the folded longin domain of VAMP7 binds back onto the unstructured trigger sequence of its own SNARE motif, thus inhibiting the SNARE motif from participating in SNARE complex formation, and how this intramolecular interaction can be stabilized by the subsequent binding of Varp. The binding site for Varp on VAMP7 is only created when the longin domain and the SNARE motif bind to each other, which explains why binding to Varp only occurs with the full-length cytosolic domain of VAMP7. The result of this two-component inhibitory system (longin domain and Varp) is that the rate of SNARE complex formation is 60–70 times slower than that when a VAMP7 form lacking its longin domain is present. This can be explained by the GAP reducing the amount of this two-component inhibitory system (longin domain and Varp), thus inhibiting the SNARE motif from participating in SNARE complex formation. The effective K_d for the intramolecular VAMP7 interaction is difficult to determine, but an upper limit to the effective K_d for intact VAMP7 can be estimated from the K_d of 10 µM for the interaction of the longin domain with Hrb that does not compete with the intramolecular binding between the VAMP7–SNARE motif and its longin domain.  

In *vivo*, the capacity of Varp to inhibit the participation of VAMP7 in SNARE complex formation and, consequently, inhibit VAMP7-dependent fusion events can be enhanced over its ability to do so in *vitro* by an increase in its effective local concentration. This may be achieved through coincidence detection by the simultaneous binding of Varp to VAMP7 and other factors that are present on the same membrane as VAMP7 or on a membrane to which the VAMP7-containing organelle or vesicle has been tightly tethered, such as GTP-bound Rab32 or Rab38 (Fig. 8). Similar to Varp, Rab32 is present on late endocytic compartments, as well as early and late stage autophagosomes, and both Rab32 and VAMP7 have been shown to be involved in autophagy.  

Rab32, its close homolog Rab38 and Varp are all enriched in cell types containing lysosome-related organelles such as platelets and, especially, melanocytes, where they show good colocalization with VAMP7 (ref. 38). This probably reflects the importance of preventing...
VAMP7 has been proposed to be involved in fusion events later in the pathway to the degradative endolysosome, which would switch the endosome to a state in which fusion with dense core lysosomes and endolysosomes would predominate and, thus, the luminal contents of the endosomes would be degraded (reviewed in refs. 47,48). Reduction in the levels of Varp on an endosomal membrane could be brought about by hydrolysis of GTP on Rab32 or Rab38 or binding Varp to a non–endosome localized protein.

In conclusion, the structural and biochemical studies described here demonstrate that, in vivo, Varp is a kinetic inhibitor of SNARE complex formations uniquely involving VAMP7. We predict that in vivo, Varp should sequentially inhibit but then, at the correct time, effectively promote the fusion of late endocytic organelles (where Varp is predominantly found) with target membranes. The switching from inhibiting to potentiating VAMP7-dependent fusion depends on the complement of other proteins that can bind directly or indirectly to Varp and/or the presence of SNAREs that can compete with VAMP7 for inclusion into SNARE complexes on the surface of an endosome. Varp could also function as a ‘chaperone’, in the Victorian sense of the word, for VAMP7 in that it could inhibit ‘improper’ interactions that VAMP7 could indulge in, such as off-path VAMP7-mediated fusion events. That this is important for the cell is suggested by the observation that during its trafficking down the endocytic pathway from the plasma membrane mediated first by Hrb and then by AP3, VAMP7 is kept in an inactive state as part of cis-SNARE complexes. At the physiological level, the list of cellular processes in which Varp functions is probably not complete, as the previously published studies focused on specialized cell types. Nonetheless, in all the processes that have been characterized, the ability of Varp to bind VAMP7 is essential. The work presented here suggests that this is because of Varp’s ability to regulate the participation of VAMP7 in membrane fusion events.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Coordinates and structure factors have been deposited in the Protein Data Bank with accession code 4B93.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
I.B.S. performed biochemistry. I.B.S. and P.R.E. determined structures. G.G.H. performed cell biology, and N.A.B. performed electron microscopy, S.R.G. and P.R.P. performed Y2H. D.J.O., I.B.S. and J.P.L. conceived and designed the study.

COMPETING FINANCIAL INTERESTS
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independent ITC measurements that showed clear signs of saturation of binding VARP658–921-His6 (2 ml total reaction volume and ~20 mg total protein). The V AMP7CD were purified separately, and the complex was reconstituted in a MgCl2 at concentrations of at least 10^5 purified in a similar way as described for V AMP7CD with the difference that reconstitution buffer (500 mM NaCl, 20 mM HEPES, pH 7.4, 10 mM DTT and
on their affinity tag. All SNARE proteins were gel filtered into SNARE complex
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Cells were initially grown at 37 °C until an optical density at 600 nm (OD 600)
using 2× TY medium supplemented with the suitable combination of antibiotics. The
DNA constructs and antibodies. For a comprehensive description of the DNA
structures and antibodies used in this study, see the Supplementary Note.

Protein expression and purification. The expression of all the constructs
described was performed in the E. coli strain Rosetta 2 (DE3) pLysS (Novagen)
using 2× -TY medium supplemented with the suitable combination of antibiotics. Cells were initially grown at 37 °C until an optical density at 600 nm (OD600) of 0.6–1.0 was reached. The temperature was then lowered to 16 °C, and the culture was cooled down for at least an hour before the induction of protein expressions with 500 µM IPTG. After induction, cells were grown for 20–24 h at 16 °C. Cells were lysed at 4 °C using either six 30-s bursts of sonication or a continuous-flow French press, and the insoluble material was removed by centrifugation. Supernatants were filtered through 0.45-µm cutoff syringe filters. Proteins tagged only with His tag (for example, Varp-His10) were purified on
nickel–nitrilotriacetic acid (Ni–NTA) agarose (Qiagen) using an imidazole step gradient for elution. The protein was further purified by anion exchange chromatography on a Sepharose Q column, and a Superdex 200 gel filtration was then performed (the column was equilibrated in 500 mM NaCl, 20 mM Tris, pH 8.5, 1 mM EDTA and 10 mM DTT).

For the reconstitution of the Varp–VAMP7 complex, VARP58–921-His6, and VAMP7CD were purified separately, and the complex was reconstituted in a final gel filtration step. GST-VARP58–921-His6 was purified on glutathione (GSH)-Sepharose, and the protein was released from the GST tag by proteolytic cleavage with human thrombin 3C protease. The VARP58–921-His6 was further purified using Ni–NTA agarose and Superdex 75 gel filtration. The protocol used for the purification of VAMP7CD188 is similar to that described for VARP58–921-His6, except that the protein was not purified using a Ni–NTA column but instead was immediately run over the size-exclusion column after cleaving the GST tag with PreScission protease. VAMP7CD188 was mixed in a molar excess of 1:2.5:1 with VARP58–921-His6 (2 ml total reaction volume and ~20 mg total protein). The reaction was incubated for 1 h at 4 °C on a roller incubator to allow the complex to form. The sample was then applied on a Hi Load Superdex 75 16/60 prep-grade gel filtration column (the column was equilibrated in 200 mM NaCl, 20 mM HEPES, pH 7.4, and 5 mM Tris–(2-carboxyethyl)phosphine). The presence of a 1:1 complex was assessed by SDS-PAGE gel electrophoresis (Supplementary Fig. 4).

SNARE proteins were purified in a similar fashion as outlined above depending on their affinity tag. All SNARE proteins were gel filtered into SNARE complex reconstitution buffer (500 mM NaCl, 20 mM HEPES, pH 7.4, 10 mM DTT and 1 mM EDTA) after the affinity chromatography step. Rab38 constructs were purified in a similar way as described for VAMP7CD with the difference that all buffers contained the matching nucleotide (for example, GDP for T23N) and MgCl2 at concentrations of at least 10 µM and 0.5 mM, respectively. GST-tagged bait proteins for pulldown experiments were purified as described above with the exception that the GST tag was not removed and the proteins instead were eluted off the GSH–Sepharose resin using a suitable buffer containing 20 mM reduced GSH and then run over a Superdex 75 gel filtration column (equilibrated in 500 mM NaCl, 20 mM HEPES, pH 7.4, and 1 mM DTT).

Limited proteolysis of VARP1-1050-His10. VARP1-1050-His10 was digested with chymotrypsin (mass ratio of 5,000:1) at 23 °C for the indicated times. Samples were blotted on PVDF membrane and N-terminally sequenced. For a full description of this process, see the Supplementary Note.

ITC and pulldowns. GSH–Sepharose pulldowns were conducted according to commonly established protocols, for example, those described in ref. 20. Briefly, 50 µl of the GST-tagged bait was incubated with 300 µg of theuntagged prey protein and 60 µl of a 50% (v/v) GSH–Sepharose bead suspension (GE Healthcare) for 1 h at 4 °C. The beads were washed four or five times with 1 ml of pulldown buffer. The proteins were eluted off the beads using 30 mM reduced GSH in the pulldown buffer. Binding was assessed by SDS-PAGE followed by Coomassie staining or western blotting with HisProbe–HRP conjugate and quantification using ImageJ (http://rsbweb.nih.gov/ij/). For ITC measurements, all proteins were gel filtered into the appropriate ITC buffer before the measurements. All measurements were performed with a VP-ITC isothermal titration calorimeter (MicroCal) at 4 °C. To determine the Kd of a binding reaction, the mean of four independent ITC measurements that showed clear signs of saturation of binding by the end of the run were used, and the s.d. was calculated. For a full description of the process, see the Supplementary Note.

Crystallization and structure determination. The best quality crystals of the Varp–VAMP7 complex grew at a concentration of 10 mg per ml of complex from wells containing 5–7.5% (v/v) isopropanol and 0.1 M imidazole, pH 6.5–7.8, in hanging and sitting drops at 4 °C. Crystals were cryoprotected in mother liquor containing additional 25% (v/v) ethylene glycol. Mercury derivatives were prepared by soaking crystals in mother liquor containing additional 0.2–0.5 mM EMTS or PCMBs for 5–30 min and then cryoprotected. Data sets were collected at beamline ID23 of the European Synchrotron Radiation Facility light source at a 1.0 Å wavelength. Data were indexed and integrated using Mosflm52 and scaled with Scala53. Heavy-atom sites were located with ShelxD54 and Shelxl55. The final refined R factor (Rfree) was 0.213 (0.25), with one Ramachandran outlier (Varp Asp743, which is on the edge of the acceptable region) and a Molprobity clashscore of 10.5 (79th percentile)56.

Pictures of the refined structure were prepared with PyMOL (http://www. pymol.org/) and CCP4mg (ref. 60).

SNARE complex inhibition assay. GST-tagged SNARE proteins were incubated with their SNARE partners in a molar ratio of 1:4–4/4 (plus 4 in the experiments in which VAMP8 was also expressed present). Varp was added in the concentrations described. One hour before the end of the experiments, the reactions were supplemented with 1/3 volume of 50% (v/v) GSH–Sepharose beads if not stated otherwise. After washing the beads five or six times with reconstitution buffer containing 1% (v/v) NP-40, bound proteins were eluted using wash buffer supplemented with 30 mM GSH. Samples were analyzed on 4–12% NuPAGE gradient gels (Invitrogen). The SDS stability of the complexes was determined (Supplementary Fig. 7). For a full description of the process, see the Supplementary Note.

Immunofluorescence confocal microscopy examining colocalization of Varp–HA and endogenous markers. NRK cells stably expressing Varp–HA were created using the pLXIN retroviral system (Clontech). For immunofluorescence, stable cells seeded on glass coverslips were fixed with 3.7% formaldehyde in PBS for 20 min. Cells were then permeabilized by incubation with 0.1% Triton X-100 in Tris-buffered saline and 0.1% (v/v) Tween 20 (TBST) for 20 min and incubated with blocking solution (5% nonfat dry milk powder in TBST) for 1 h. Cells were incubated with primary antibody and then fluorescently conjugated secondary antibodies, and images were captured using a confocal microscope. For information about the antibodies used, please see the Supplementary Note.

siRNA mediated gene knockdown in NRK cells. Endogenous genes (Varp and Rab32) were knocked down in wild-type NRK cells using siRNA oligonucleotides at 100 nM transfected with DharmaFECT 2 (Dharmacon, Thermo) reagent according to the manufacturer’s instructions. Levels of knockdown were compared to those of cells transfected with a nontargeting control siRNA oligonucleotide at 100 nM.

Electron microscopy. For immunogold labeling of cryosections, NRK cells stably expressing Varp–HA were incubated with dextran conjugated to Texas Red for 4 h followed by a 20-h chase, as previously described61. Cells were fixed with 0.1% glutaraldehyde and 4% paraformaldehyde in 0.1 M Na cacodylate, pH 7.2, at 20 °C, scraped, pelleted, embedded in 10% gelatin and infused with 1.7 M sucrose and 15% polyvinylpyrrolidone before being frozen on aluminum stubs in liquid nitrogen. Ultrathin frozen sections were cut using an ultramicrotome equipped with a cryochrome attachment (Leica, Milton Keynes, UK) and collected from the knife edge with 2% methyl cellulose and 2.3 M sucrose in a ratio of 50:50 (ref. 62). The sections were immunolabeled using rabbit anti–Texas Red, rabbit anti-lgp110 (LAMMP) and mouse monoclonal anti-HA and detected using protein A conjugated to 5 nm diameter colloidal gold (Department of Cell Biology, University of Utrecht, Netherlands), protein A conjugated to 10 nm diameter colloidal gold and rabbit anti-mouse immunoglobulin followed by protein A conjugated to 15 nm diameter colloidal gold, respectively, using a sequential immunolabeling protocol. The immunolabeled sections were contrasted, dried and observed in a Philips CM100 transmission electron microscope at 80 kV.
Quantification of the degree of immunolabeling was performed by counting gold particles overlying defined cellular components by scanning sections sequentially and recording images with a charge-coupled device camera (MegaView III). The labeling was repeated four times, and the distribution of 200 gold particles for each labeling experiment was determined.

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