acceptor liposomes (45 μl) and donor liposomes (5 μl) in a 96-well FluoroNunc microtitre plate (Nunc). In some cases a Sec17/Sec18 cocktail was added to the fusion reactions consisting of 15 μg ml^{-1} Sec17, 87 μg ml^{-1} Sec18, 0.97 mg ml^{-1} ATP, 9.7 mM creatine phosphate (Boehringer Mannheim), 0.1 mg ml^{-1} creatine phosphate kinase (Boehringer Mannheim), 1.3 mM Mg_2Cl_2 (or 1.3 mM EDTA). Microtitre plates were then placed in a Fluoroscan II (Labsystems) equilibrated at 37 °C and NBD fluorescence was followed. Conversion to a measure of rounds of fusion was done as described[14].

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Functional architecture of an intracellular membrane t-SNARE

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Lipid bilayer fusion is mediated by SNAREs (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors) located on the vesicle membrane (v-SNAREs) and the target membrane (t-SNAREs)[1,2]. The assembled v-SNARE/t-SNARE complex consists of a bundle of four helices, of which one is supplied by the v-SNARE and the other three by the t-SNARE[3]. For t-SNAREs on the plasma membrane, the protein syntaxin[4] supplies one helix and a SNAP-25 protein[5] contributes the other two. Although there are numerous homologues of syntaxin on intracellular membranes[6], there are only two SNAP-25-related proteins in yeast, Sec9 and Spo20, both of which are localized to the plasma membrane and function in secretion[7] and sporulation[8], respectively. What replaces SNAP-25 in t-SNAREs of intracellular membranes? Here we show that an intracellular t-SNARE is built from a 'heavy chain' homologous to syntaxin and two separate non-syntaxin 'light chains'. SNAP-25 may thus be the exception rather than the rule, having been derived from genes that encoded separate light chains that fused during evolution to produce a single gene encoding one protein with two helices.

To test our model of the functional architecture of the t-SNAREs on intracellular membranes (Fig. 1), we studied the SNAREs involved in the well characterized process of fusion of yeast vacuoles[9–12]. Homotypic fusion of yeast vacuoles is the final step in the inheritance of vacuoles from a mother cell to a daughter cell after bud formation in *Saccharomyces cerevisiae*. The SNARE proteins Vam3, Vam7, Vti1, Nyv1 and Ykt6 have been identified

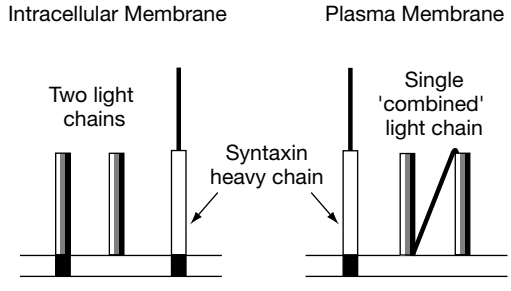

Figure 1 The model for the architecture of intracellular t-SNAREs. The plasma membrane t-SNARE is composed of one syntaxin heavy chain, which contributes one α-helix, and one combined light chain, SNAP-25, which contributes two α-helices. In comparison, most intracellular t-SNAREs are composed of one syntaxin heavy chain and two associated light chains. In the case of vacuolar homotypic fusion, Vam3 functions as a syntaxin heavy chain, and Vti1 and soluble Vam7 act as two light chains. In contrast to Vam7, most light chains are probably integral membrane proteins.

as being involved in this fusion process. Vam3 is a syntaxin family member; the others are non-syntaxin SNAREs. Vti1 has been found to be present in cells in a complex with the syntaxin Vam3, and also in distinct complexes with all but three of the other yeast syntaxins[6,13]. Although Vti1 was originally classified as v-SNARE[14], from the perspective outlined above it could just as easily be a shared light chain of many intracellular t-SNAREs, including a vacuolar t-SNARE. The function of Ykt6 in vacuolar fusion is not well defined, and this protein has been implicated in a variety of transport steps[6,13]; it has also been proposed to be a fifth member of a single vacuolar SNARE complex[11], along with Vam3, Vam7, Vti1 and Nyv1. If this proposal is correct, then the vacuolar SNARE complex would have to be based on a different packing principle than the exocytic/neuronal SNARE complex[11].

We expressed recombinant Vam3, Vam7, Vti1, Ykt6 and Nyv1 as His_6- or glutathione-*S*-transferase (GST)-tagged proteins in *Escherichia coli*. Various combinations of purified SNAREs were incubated together to identify direct SNARE–SNARE interactions and higher order complexes. All binding studies were done under high stringency conditions in high salt (≥ 500 mM KCl) using full-length proteins in detergent solution. We analysed bound products by SDS–polyacrylamide gel electrophoresis (PAGE) and Coomassie blue staining (Fig. 2).

Immobilized GST–Vam3 (the syntaxin) efficiently binds Vti1 (Fig. 2a, lane 3) but not Vam7 (Fig. 2a, lane 2), which were added in a 5- and 25-fold excess, respectively. However, Vam7 was efficiently bound to the Vam3–Vti1 complex in roughly the same molar amount as Vti1 (Fig. 2a, lane 4). Binding of Vti1 and Vam7 to the

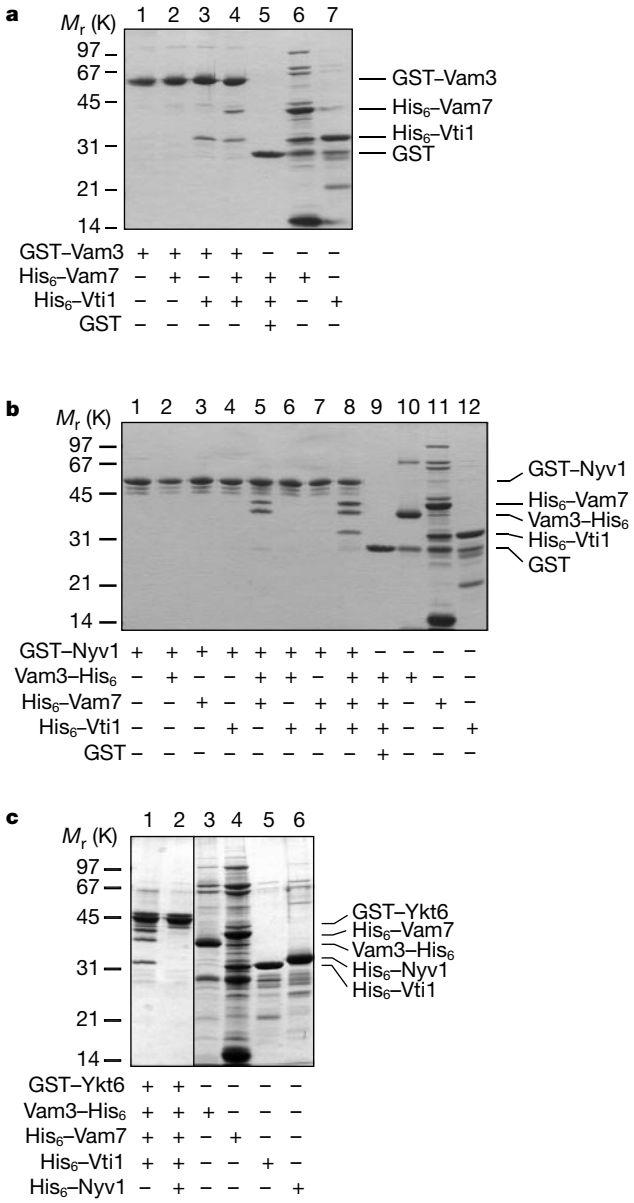

Figure 2 Vacuolar v-SNARE/t-SNARE complex. **a**, t-SNARE complex formation. Purified GST–Vam3 (12 μg), or GST (5.5 μg) immobilized on glutathione–agarose was incubated with purified His_6–Vam7 (207 μg) and/or His_6–Vti1 (29 μg) in the presence of 1% Triton X-100 in a final volume of 100 μl. After overnight incubation at 4 °C, the beads were washed. One eighth of the reactions was analysed by SDS–PAGE, and proteins were stained with Coomassie Blue R 250. Lanes 6 and 7 correspond to 10% of input proteins. **b**, v-SNARE/t-SNARE complex formation. Purified GST–Nyv1 (11.2 μg) or GST (5.5 μg) immobilized on glutathione–agarose were incubated with purified Vam3–His_6 (32 μg), His_6–Vam7 (207 μg) and/or His_6–Vti1 (29 μg) as described in **a**. Lanes 10–12 correspond to 10% of input proteins. **c**, SNARE complex formation with Ykt6. Purified GST–Ykt6 (10 μg) immobilized on glutathione–agarose was incubated with purified Vam3–His_6 (98 μg), His_6–Vam7 (621 μg) and His_6–Vti1 (86 μg) in the presence or absence of His_6–Nyv1 (86 μg), in a final volume of 200 μl. Lanes 3–6 correspond to 0.33% of input proteins.

syntaxin Vam3 was stoichiometric (data not shown) and specific in that neither Vam7 nor Vti1 interacted with GST, and protein impurities in the SNARE preparations did not bind to GST or GST–Vam3 (Fig. 2a, lanes 5 and 6–7, respectively).

These data suggest that the syntaxin (Vam3)-containing vacuolar t-SNARE is a ternary complex consisting of a syntaxin heavy chain (Vam3) and two non-syntaxin light chains (Vti1 and Vam7), along the lines we proposed. The t-SNARE would then be expected to bind of the v-SNARE Nyv1 (Fig. 2b), and indeed, immobilized GST–Nyv1 efficiently binds the ternary t-SNARE complex (Fig. 2b, lane 8). Binding of the t-SNARE complex to the v-SNARE Nyv1 was saturable and stoichiometric (data not shown). Immobilized GST–Nyv1 does not bind efficiently to any of the individual t-SNARE subunits Vam3 (heavy chain), or Vam7 or Vti1 (the light chains) (Fig. 2b, lanes 2–4). Nor does Nyv1 bind the binary complex of Vam3–Vti1 (Fig. 2b, lane 6), which is stable (Fig. 2a, lane 3). However, Nyv1 does stabilize a complex of Vam3 and Vam7 (Fig. 2b, lane 5), which does not form appreciably in the absence of Nyv1 (Fig. 2a, lane 2), clearly illustrating the cooperativity of the interactions among the SNAREs.

As Ykt6 has been suggested to be a fifth component of the vacuolar SNARE complex[11], we determined whether Ykt6 binds the above tetrameric SNARE complex of Vam3–Vam7–Vti1–Nyv1. GST–Ykt6 binds the ternary t-SNARE (Vam3–Vam7–Vti1) (Fig. 2c, lane 1), but binding is blocked by equimolar amounts of Nyv1 (Fig. 2c, lane 2). In other words, Nyv1 and Ykt6 cannot bind to the t-SNARE complex at the same time. This directly establishes that the five vacuolar SNAREs can assemble into either of two alternative quaternary complexes, but not into a pentameric complex. On the basis of inhibition studies using antibodies against Ykt6 (ref. 11), Ykt6 appears to be involved in vacuolar fusion; and co-immunoprecipitation experiments from lysates showed that a pentameric SNARE complex containing all five SNAREs—Ykt6, Nyv1, Vam3, Vam7 and Vti1—can be isolated from membranes[11]. Our results are not necessarily inconsistent with these data[11], as other unidentified proteins might be present in the immunoprecipitates characterized by western blotting using antibodies to known SNARE proteins. Alternatively, oligomeric complexes containing both of the tetrameric complexes that we identified might have been precipitated.

To test the functional capacity of the quarternary vacuolar SNARE complex, we reconstituted its v- and t-SNARE portions into lipid bilayer vesicles, by using established procedures[2]. Recombinant vacuolar t-SNARE subunits (the syntaxin Vam3 and the light chains Vti1 and Vam7) were reconstituted into 'acceptor' liposomes, whereas the v-SNARE Nyv1 was reconstituted into fluorescent 'donor' liposomes[2] (Fig. 3a). Typical molar lipid-to-SNARE ratios for the reconstituted donor (v-SNARE) and acceptor (t-SNARE) liposomes were about 60 and 470, respectively. Like

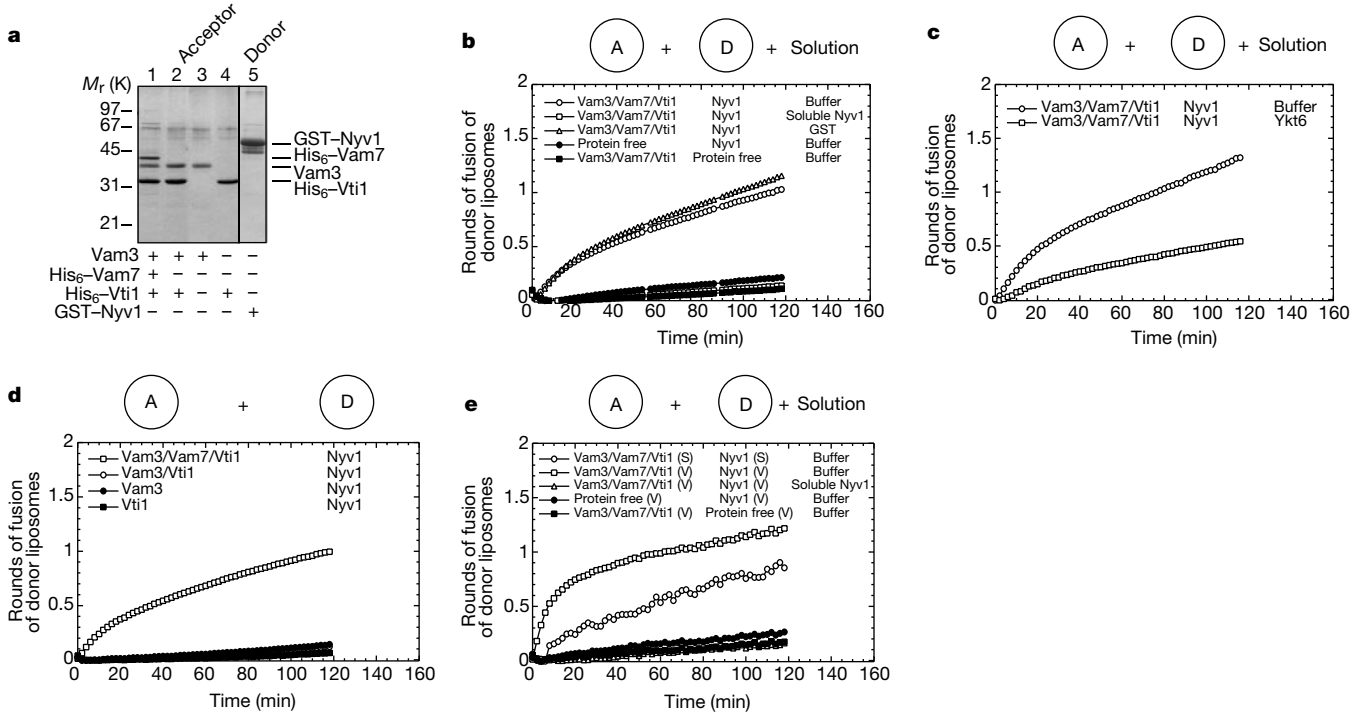

Figure 3 Fusion between vesicles containing vacuolar v- and t-SNAREs. **a**, Acceptor vesicles containing t-SNAREs and donor vesicles containing v-SNAREs. Protein patterns of purified acceptor liposomes containing various t-SNARE subunits and of donor liposomes containing the v-SNARE GST–Nyv1 were analysed by SDS–PAGE and Coomassie Blue R 250. Lanes 1–4 correspond to ~10% of acceptor vesicles; lane 5 to 100% of donor vesicles used in a typical fusion reaction. **b**, Donor vesicles (5 μl; 4.3 nmol phospholipid) containing the v-SNARE and acceptor vesicles (45 μl; 107 nmol phospholipid) containing t-SNAREs were mixed with 15 μl of either reconstitution buffer, or GST–cytoplasmic domain of Nyv1 (soluble Nyv1; 2.86 mg ml^{-1}), corresponding to a fourfold molar excess over t-SNAREs or GST (1.48 mg ml^{-1}). Fusion of protein free vesicles was also tested with either donor v-liposomes or with acceptor t-liposomes. The temperature was shifted to 37 °C and NBD fluorescence monitored at 538 nm. After 2 h at 37 °C, *n*-dodecylmaltoside was added to solubilize liposomes and to de-quench the fluorescence of NBD. To normalize the experiments, the lowest NBD fluorescence signals were set to 0% and the highest signals, after addition of detergent, were set to 100%. Rounds of fusion were determined using a standard curve[16]. **c**, Donor vesicles (5 μl; 3.9 nmol phospholipid) containing the v-SNARE and 45 μl of acceptor vesicles (109 nmol phospholipid) containing t-SNAREs were mixed with 20 μl of either reconstitution buffer or GST–Ykt6 (0.62 mg ml^{-1}), corresponding to a 3.7-fold molar excess over v-SNAREs. Fusion was monitored as in **b**. **d**, Donor vesicles (5 μl; 4.3 nmol phospholipid) containing the v-SNARE Nyv1 were mixed with acceptor vesicles (45 μl; 73 nmole phospholipid) containing either the ternary t-SNARE complex or Vam3 and Vti1, or Vam3 or Vti1. Fusion was monitored as in **b**. **e**, Donor vesicles and acceptor vesicles were prepared with either standard lipid mixture (S) or vacuolar lipid mixture (V). To compare directly the fusion reactions and to correct for the different reconstitution efficiencies, donor liposomes with standard lipid mixture contained half of the amount of GST–Nyv1 used in **a**–**d**. Donor vesicles (5 μl; 2.0 nmol phospholipid) containing the v-SNARE and acceptor vesicles (45 μl 97 nmol phospholipid) containing t-SNAREs were mixed with 15 μl of either reconstitution buffer, or GST–cytoplasmic domain of Nyv1, and fusion was monitored as **b**, except that reactions were incubated at 30 °C.

exocytic/neuronal SNAREs[2], the v-SNAREs reconstitute more efficiently than the t-SNAREs, resulting in a higher number of copies of v-SNAREs per donor liposome than t-SNAREs per acceptor liposome. Assuming a vesicle diameter of 50 nm and 65 Å^2 per phospholipid, we estimate that there are about 400 copies of the v-SNARE Nyv1 protein per donor vesicle and 50 copies of the ternary t-SNARE complex (consisting of Vam3, Vti1 and Vam7) in the acceptor vesicles. About 80% of v-SNAREs and 70% of t-SNAREs are exposed on the outside of the liposomes (data not shown). Fusion was measured by the increase in 7-nitrobenz-2-oxa-1,3-diazole (NBD) fluorescence[15] and converted to rounds of fusion of donor liposome bilayers[16].

Donor and acceptor liposomes formed of the standard lipid mixture (see Methods) fused efficiently at 37 °C (Fig. 3b, open circles). The v-SNARE-containing donor liposomes do not fuse with protein-free acceptor liposomes and vice versa, protein-free donor liposomes do not fuse with t-SNARE-containing acceptor liposomes (Fig. 3b, filled circles and filled squares, respectively). Furthermore, free cytoplasmic domain of Nyv1 completely abolished fusion (Fig. 3b, open squares), indicating that fusion required the formation of SNARE complexes between donor and acceptor liposomes. This 'soluble' v-SNARE titrates the t-SNAREs of acceptor vesicles, preventing them from engaging v-SNAREs on donor vesicles. Addition of Ykt6 in amounts equimolar with the t-SNARE complex (a 3.7-fold molar excess compared with the v-SNARE) also inhibited the fusion reaction by 60% (Fig. 3c). This result is consistent with our protein binding studies (Fig. 2c) and confirms that Ykt6 and Nyv1 compete for binding to the t-SNARE complex. In addition, Ykt6-containing liposomes (in which Ykt6 is anchored by a close mimic of its natural lipid modification) do not fuse with vacuolar t-SNARE-containing liposomes[17].

To confirm that all four SNAREs are needed for fusion, we prepared acceptor liposomes in which one or several components of the t-SNARE were omitted during reconstitution. The different t-SNARE liposomes contain similar protein levels to allow direct comparisons of fusion efficiencies (Fig. 3a). Acceptor liposomes containing only the syntaxin Vam3, or only the light chain Vti1, or both Vam3 and Vti1, did not fuse with the Nyv1 donor liposomes (Fig. 3d). Fusion required the ternary t-SNARE complex.

The kinetics of the above fusion reactions are similar to those observed for the mammalian exocytic SNAREs[2]. The speed of fusion was significantly increased when a mixture of lipids representative of vacuoles (see Methods) was used and when the incubation was carried out at 30 °C, a common temperature for yeast growth (Fig. 3e). Now, the half-time for the first round of fusion was reduced to about 7 min, which is comparable to or faster than the kinetics of fusion of isolated native vacuolar membranes[18].

Our observations support a general model for intracellular t-SNAREs (Fig. 1) in which a syntaxin family member and two associated light chains form a ternary complex, as first suggested from sequence analysis[19], which would be expected to consist of a three-helix bundle[3]. The t-SNARE complex that we report for the yeast vacuole is stable in dilute solution, but this need not always be the case as long as the complex assembles when bound to the v-SNARE in bilayers.

The non-syntaxin SNARE Vti1 forms complexes with most of the yeast syntaxins in intracellular compartments[6]. As for the vacuolar t-SNARE, Vti1 may well be a common light chain of other intracellular t-SNAREs, rather than a promiscious v-SNARE[20]. In general terms, the recognition that non-syntaxin SNAREs can function either as subunits of t-SNAREs or as v-SNAREs provides a straight forward basis for grouping what has become a long and sometimes confusing list of SNARE proteins into a simpler pattern of functional units. Moreover, by no longer equating syntaxins with t-SNAREs and non-synatxin SNAREs with v-SNAREs, several apparent difficulties vanish. For example, the obvious and unresolved question—why would cells need so many v-SNAREs in relation to t-SNAREs—is no longer pertinent. A non-syntaxin SNARE common to the t-SNAREs of different compartments is perfectly consistent with the SNARE hypothesis, but would contradict the hypothesis if the same SNARE could function as a v-SNARE. Finally, combinatorial use of the same syntaxin with different sets of light chains can generate greater diversity in traffic patterns than would be predicted from the number of syntaxins alone, which may be specially important in the Golgi and possibly in endosomes. According to analysis of the yeast genome[6], there are only five syntaxins in the intracellular compartments of yeast (Vam3, Sed5, Ufe1, Pep12 and Tlg2), which, however, could generate a variety of t-SNARE complexes in combination with the 14 yeast v-SNAREs. (On the basis of the new classification[19], Tlg1 is a member of the TSL (t-SNARE-like protein) family and not a direct syntaxin homologue (see also http:// itsa.ucsf.edu/~mostov/supplementary). Indeed, the Golgi-localized syntaxin5 (Sed5 in yeast), which appears to form at least two distinct sets of complexes with non-syntaxin SNAREs (that is, light chains)[6,21], is one example for such a combinatorial code.

Although pairing of SNAREs between vacuoles is important for tight docking and therefore indirectly important for fusion, it has been proposed that SNARE pairing is insufficient for fusion. Instead, a still unknown principle that is somehow activated by SNARE pairing has been invoked to explain the fusion of bilayers[22]. However, our results with isolated vacuolar SNARE proteins directly establish that pairing of these SNARE proteins in itself results in spontaneous and efficient bilayer fusion. Essentially, fusion of vacuoles was largely resistant to excess *N*-ethylmaleimide-sensitive factor (NSF), which is believed to disrupt all SNARE complexes[22]. But it is now known that, whereas *cis*-SNARE complexes are disrupted by NSF, under the same conditions the *trans*-SNARE complexes (SNAREpins) engaged in fusion are resistant to NSF and soluble NSF attachment proteins (SNAPs)[23].

Calcium flux and phosphorylation/dephosphorylation reactions are important for fusion of yeast vacuoles[18,24]. Yet, fusion by the isolated vacuolar SNARE proteins clearly does not depend on additional proteins or enzyme systems, and it is not affected by the addition or absence of calcium ions (data not shown). We propose that the well documented requirements for a phosphatase and calcium flux for fusion of native vacuoles may reflect regulatory mechanisms that are superimposed upon the basic and general core fusion machinery, SNARE proteins. As vacuolar fusion is temporally and spatially regulated to occur during M to G1 phase and within the bud[25], such regulatory mechanisms of one kind or another are obviously expected. Indeed, it is well established that phosphorylation can prevent the assembly of t-SNAREs in other cases[26,27]. Therefore, phosphorylation may also negatively regulate vacuolar SNAREs, and dephosphorylation may relieve such inhibitory effects on membrane fusion. □

Methods

Plasmids and protein purification

The coding sequences of NYV1, VTI1, VAM7 and VAM3 were amplified by PCR from *S. cerevisiae* genomic DNA (Novagen) and cloned into either pET15b, pET28a (Novagen) or pGEX4T-3 (Pharmacia) expression vectors. YKT6 was subcloned from pMS130 (ref. 28) into pGEX4T-3 (Pharmacia). We expressed all SNARE proteins in *E. coli* and purified them by either glutathione- or nickel-affinity chromatography. More details are available from the authors on request.

Protein-binding assay

For preparation of GST–Nyv1, GST–Vam3, GST–Ykt6, or GST affinity matrices, we incubated lysates prepared from cells containing pGEX-Nyv1, pGEX-Vam3, pGEX-Ykt6 or pGEX-4T-3 at 4 °C for 2 h with glutathione agarose equilibrated in binding buffer A (25 mM HEPES-KOH pH 7.4, 500 mM KCl, 10% glycerol, 1 mM DTT and 1% Triton X-100). After centrifugation at 1,000 *g* for 5 min, the beads were washed 6 times with binding buffer A. SNARE proteins and bovine serum albumin (500 μg ml^{-1}) were added and the beads were incubated at 4 °C over night. The beads were then washed three times with 400 μl of binding buffer A, and 30 μl of binding buffer B (25 mM HEPES-KOH pH 7.4, 100 mM KCl, 10% glycerol, 1 mM DTT, 1% Triton X-100) was added. One eighth

of each sample was mixed with 4×Laemmli SDS–PAGE buffer, boiled for 4 min at 98 °C and resolved by SDS–PAGE.

Protein reconstitution into vesicles

For acceptor vesicles containing t-SNAREs, 60 μl of Vam3 (2.9 mg ml^{-1}), 200 μl of His$_6$–Vam7 (8.2 mg ml^{-1}) and/or 20 μl of His$_6$–Vti1 (14 mg ml^{-1}) were mixed and 20 μl of 10% octyl-β-D-glucopyranoside and 250 μl of reconstitution buffer were added. After incubation at 4 °C for 20 h, 500 μl of the reaction was used for reconstitution of acceptor vesicles. For donor vesicles containing the v-SNARE, 100 μl of GST–Nyv1 (3.6 mg ml^{-1}) was used. SNAREs were reconstituted as described[2] except that all the buffers used for reconstitution contained 500 mM KCl. The principal lipid components in the liposomes were 85% DOPC (1,2-dioleoyl phosphatidylcholine) and 15% DOPS (1,2-dioleoyl phosphatidylserine). Typical lipid concentration in the recovered Nycodenz fractions containing the vesicles were 0.7–0.9 mM for donor vesicles and 1.8–2.5 mM for acceptor vesicles. Concentrations of GST–Nyv1 in donor vesicle fractions were 0.7–0.8 mg ml^{-1} and those of t-SNARE complex in acceptor vesicles were 0.45–0.5 mg ml^{-1}, corresponding to a 16% and 9% reconstitution efficiency. For preparation of vesicles containing vacuolar lipids[29,30], we used the following lipid mixture: DOPC (1,2-dioleoyl phosphatidylcholine)/ DOPE (1,2-dioleoyl phosphatidylethanolamine)/ soybean PI (phosphatidylinositol)/ DOPS (1,2-dioleoyl phosphatidylserine)/ DOPA (1,2-dioleoyl phosphatidic acid)/ heart cardiolipin in a 53:20:19:5:2:1 molar ratio. All lipids were obtained from Avanti Polar Lipids. The reconstitution efficiencies for v-SNAREs and t-SNAREs in vacuolar lipid mixtures were 8% and 9%, respectively.

Lipid mixing assay

Lipid mixing assay was carried out as described[2,15], except that *n*-dodecylmaltoside was used to determine the maximum NBD signal instead of Triton X-100. Rounds of fusion were determined by using a calibration curve which was prepared as described[16], except that GST–Nyv1 was used instead of VAMP to reconstitute vesicles and that the mixtures of labelled and unlabelled lipids corresponded to one-, two-, three-, four-, or sixfold dilutions of donor fluorescent lipid. The equation used to convert the percentage dodecylmaltoside signal to rounds of fusion is $y = (0.52247 \times e^{(0.032993x)}) - (0.527 \times e^{(-0.074915x)})$ where y is the rounds of fusion and x is the percentage dodecylmaltoside signal at a given time interval.

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CAP defines a second signalling pathway required for insulin-stimulated glucose transport

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Insulin stimulates the transport of glucose into fat and muscle cells. Although the precise molecular mechanisms involved in this process remain uncertain, insulin initiates its actions by binding to its tyrosine kinase receptor, leading to the phosphorylation of intracellular substrates. One such substrate is the Cbl proto-oncogene product[1]. Cbl is recruited to the insulin receptor by interaction with the adapter protein CAP, through one of three adjacent SH3 domains in the carboxy terminus of CAP[2]. Upon phosphorylation of Cbl, the CAP–Cbl complex dissociates from the insulin receptor and moves to a caveolin-enriched, triton-insoluble membrane fraction[3]. Here, to identify a molecular mechanism underlying this subcellular redistribution, we screened a yeast two-hybrid library using the amino-terminal region of CAP and identified the caveolar protein flotillin. Flotillin forms a ternary complex with CAP and Cbl, directing the localization of the CAP–Cbl complex to a lipid raft subdomain of the plasma membrane. Expression of the N-terminal domain of CAP in 3T3-L1 adipocytes blocks the stimulation of glucose transport by insulin, without affecting signalling events that depend on phosphatidylinositol-3-OH kinase. Thus, localization of the Cbl–CAP complex to lipid rafts generates a pathway that is crucial in the regulation of glucose uptake.