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Combinatorial SNARE complexes with VAMP7 or VAMP8 define different late endocytic fusion events

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Both heterotypic and homotypic fusion events are required to deliver endocytosed macromolecules to lysosomes and remodel late endocytic organelles. A *trans*-SNARE complex consisting of Q-SNAREs syntaxin 7, Vti1b and syntaxin 8 and the R-SNARE VAMP8 has been shown by others to be responsible for homotypic fusion of late endosomes. Using antibody inhibition experiments in rat liver cell-free systems, we confirmed this result, but found that the same Q-SNAREs can combine with an alternative R-SNARE, namely VAMP7, for heterotypic fusion between late endosomes and lysosomes. Co-immunoprecipitation demonstrated separate syntaxin 7 complexes with either VAMP7 or VAMP8 in solubilized rat liver membranes. Additionally, overexpression of the N-terminal domain of VAMP7, in cultured fibroblastic cells, inhibited the mixing of a preloaded lysosomal content marker with a marker delivered to late endosomes. These data show that combinatorial interactions of SNAREs determine whether late endosomes undergo homotypic or heterotypic fusion events.

Keywords: lysosome; endosome; endocytosis

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INTRODUCTION

The delivery of endocytosed macromolecules from late endosomes to lysosomes probably involves 'kiss and run' and complete fusion events, the latter resulting in formation of hybrid organelles from which lysosomes may be re-formed (reviewed in Mullins & Bonifacino, 2001; Luzio *et al*, 2003). Late endosomal fusion events conform to the tenets of the SNARE hypothesis (reviewed in Chen & Scheller, 2001). At the core of each fusion event is the formation of a specific tetrameric *trans*-SNARE complex consisting of interacting coiled coils from a t- (target membrane) SNARE made up of a heavy chain and two light chains (Fukuda *et al*, 2000) plus a v- (vesicle membrane) SNARE or, using an alternative nomenclature (defined in Bock *et al*, 2001), Qa-, Qb- and Qc-SNARE plus an R-SNARE. There is good evidence that the Qa-SNARE syntaxin 7 is required for both homotypic late endosome fusion (Antonin *et al*, 2000) and heterotypic fusion with lysosomes (Mullock *et al*, 2000; Ward *et al*, 2000). In the former case, the other SNAREs required have been identified, using antibody inhibition of cell-free assays, as Vti1b, syntaxin 8 and VAMP8. The structure of the four-helix SNARE protein bundle (syntaxin 7, Vti1b, syntaxin 8 and VAMP8) has been solved (Antonin *et al*, 2002). Uncertainty remains over the SNAREs involved in heterotypic fusion of late endosomes with lysosomes. Immunoprecipitated syntaxin 7 complexes have been found to contain a number of different SNAREs: Vti1b, syntaxin 6, VAMP7 and VAMP8 (Wade *et al*, 2001). Antibodies against VAMP7 have been shown to inhibit both ligand delivery to lysosomes in permeabilized cells (Advani *et al*, 1999) and homotypic lysosome fusion *in vitro* (Ward *et al*, 2000). In contrast, we have previously observed no inhibition of cell-free late endosome–lysosome fusion with antibodies against VAMP8 (Mullock *et al*, 2000).

RESULTS AND DISCUSSION

Candidate SNAREs for a specific fusion event must be present in the membranes of vesicles or organelles that fuse. We found that syntaxin 7, Vti1b, syntaxin 8, VAMP7 and VAMP8 could all be detected by immunoblotting, in late endosome- and lysosome-enriched rat liver membrane fractions prepared for cell-free fusion assays (Fig 1A). Moreover, immunogold labelling of the SNAREs in specific organelles in these fractions showed the presence of Vti1b, syntaxin 8 and VAMP7, in addition to syntaxin 7 and VAMP8, on the limiting membrane of late endosomes and lysosomes (Fig 1B,C). All except VAMP8 were more enriched on the limiting membrane of lysosomes than on late endosomes.

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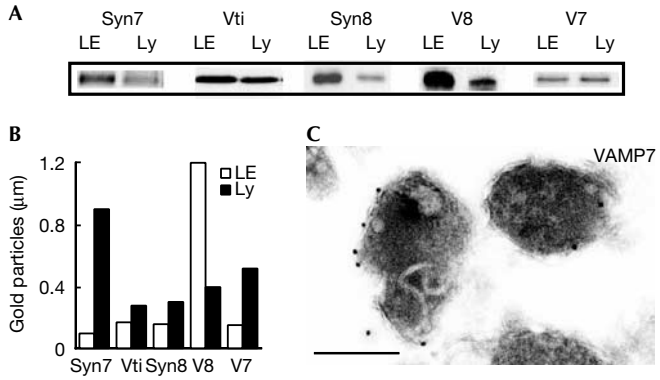

Fig 1 | SNAREs in the late endocytic pathway. (**A**) Immunoblots of SDS–PAGE-separated SNAREs from late endosome- and lysosome-enriched membrane fractions from the rat liver. 20 μg protein/track. (**B**) Quantification of gold particles per micrometre limiting membrane of late endosomes and lysosomes after immunogold labelling with anti-SNARE antibodies. Data for Syn7 and V8 from Mullock *et al* (2000) and the same EM blocks were used to localize the other SNAREs. (**C**) Immunoelectron micrograph showing labelling of VAMP7 on lysosomes. Gold particles, 10 nm; scale bar, 0.5 μm. Syn7, syntaxin 7; Vti, Vti1b; Syn8, syntaxin 8; V8, VAMP8; V7, VAMP7; LE, late endosome; Ly, lysosome.

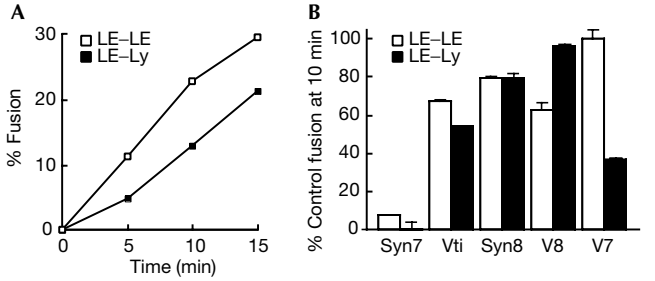

Fig 2 | Cell-free fusion assays. (**A**) Time courses of content mixing assays for late endosome–lysosome fusion and homotypic late endosome fusion using membrane fractions from the rat liver. (**B**) Inhibition of fusion by affinity-purified antibodies against SNAREs. Standard fusion assays were run for 10 min at 37 °C in the presence of 100 μg/ml affinity-purified anti-SNARE antibodies. Representative experiments (means ± range of pairs) are shown in which the same affinity-purified antibody preparations were used in each of the two assays on the same day with the same late endosome preparations containing avidin–ASF.

We next established a cell-free homotypic late endosome fusion assay using late endosome-enriched fractions from rat liver to compare with our previously established heterotypic lysosome–late endosome fusion assays (Mullock *et al*, 1998; Pryor *et al*, 2000). The assays showed similar initial time courses and extent of fusion (Fig 2A). We then added affinity-purified anti-SNARE antibodies to fusion reactions, which were run for 10 min to ensure that we were in the linear range of the fusion assays shown in Fig 2A. All antibodies, when added at 100 μg/ml, showed inhibition of fusion in one or both of the assays, with some antibodies being more effective than others (Fig 2B). We have previously shown that different antibodies against syntaxin 7 inhibit the heterotypic fusion assay to different extents (Mullock *et al*, 2000). In the present experiments, we found that antibodies against the cytosolic domains of syntaxin 7, Vti1b and syntaxin 8 inhibited to a similar extent both late endosome homotypic fusion and late endosome–lysosome fusion (Fig 2B). In contrast, antibodies against VAMP8 inhibited only the homotypic fusion, and antibodies against VAMP7 only the heterotypic fusion (Fig 2B). Another syntaxin 7-interacting SNARE is syntaxin 6 (Wade *et al*, 2001). However, antibodies against syntaxin 6 that inhibit immature secretory granule fusion (Wendler *et al*, 2001) had no effect on our assays. The inhibition data on homotypic late endosome fusion are entirely consistent with those reported by Antonin *et al* (2000), using organelles from PC12 cells. The results of the antibody inhibition experiments on heterotypic fusion of late endosomes and lysosomes suggest a requirement for a *trans*-SNARE complex consisting of syntaxin 7, Vti1b, syntaxin 8 and VAMP7, consistent with the Qa-, Qb-, Qc- and R-SNARE core complex model proposed by Bock *et al* (2001).

As modelled in Fig 3A, our data suggest that two combinatorial SNARE complexes are required for homotypic and heterotypic fusion events involving late endosomes such that a different v-(R-)SNARE interacts with the same t-(Qa,b,c)SNARE. Combinatorial SNARE complexes have been demonstrated previously, based on *in vitro* binding studies and genetic/biochemical studies of yeast SNAREs (Tsui *et al*, 2001; Parlati *et al*, 2002). Additional data to support our model were obtained by immunoprecipitation of detergent-solubilized rat liver membranes. Both VAMP7 and VAMP8 co-immunoprecipitated with syntaxin 7, and syntaxin 7 co-immunoprecipitated with antibodies against either R-SNARE (Fig 3B). However, immunoprecipitation of VAMP7 did not co-precipitate VAMP8 or vice versa. Although our model for heterotypic fusion (Fig 3A) shows the functional v-(R-)SNARE VAMP7 on lysosomes and the functional t-(Qa,b,c)SNARE on late endosomes, we have no data to exclude the reverse model, as all of the relevant SNAREs were found on both organelles. Preliminary experiments in which late endosome and lysosome fractions were pretreated separately with anti-VAMP7 antibodies before re-isolation, washing and addition to the cell-free fusion assay were consistent with functional VAMP7 being present on both organelles because partial inhibition was observed in either case (data not shown). Nevertheless, we have not observed inhibition of late endosome homotypic fusion with antibodies against VAMP7.

VAMP7 is the first component of the late endocytic fusion machinery shown to be differentially required for heterotypic fusion of late endosomes and lysosomes rather than homotypic fusion events. VAMP7 (also known as toxin-insensitive VAMP, TI-VAMP or SYBL1) is, along with yeast and mammalian orthologues of Sec22p (Gonzalez *et al*, 2001) and Ykt6p (Tochio *et al*, 2001), a member of the longin subfamily of R-SNAREs (Filippini *et al*, 2001). All longins are distinguished from brevins by the presence of an additional ~110–140 amino-acid N-terminal domain. The structure of this longin domain has been solved for both mammalian Sec22b (Gonzalez *et al*, 2001) and Ykt6p (Tochio *et al*, 2001), showing it to resemble a circular permutation of the overall fold of the actin regulatory protein, profilin, the σ-subunit of the clathrin adaptor AP2, the N-terminal domain of the μ-subunit of AP2 and the N-terminal domain of the SRα subunit of the signal recognition particle receptor (Schwartz & Blobel, 2003).

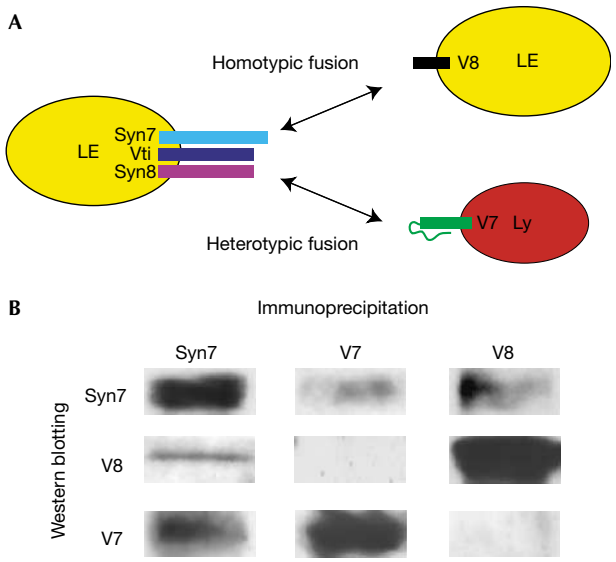

Fig 3 | Combinatorial SNARE complexes. (**A**) Schematic representation of combinatorial SNARE interactions late in the endocytic pathway with the same t-(Q-)SNARE complex interacting with different v-(R-)SNAREs (V8 and V7) to allow, respectively, homotypic fusion of late endosomes or heterotypic fusion with lysosomes. Although not shown, individual SNAREs are present, and may be functional, on either membrane. (**B**) Immunoprecipitation of SNAREs from solubilized liver membrane fractions followed by SDS–PAGE and immunoblotting.

The N-terminal longin domains have been suggested to regulate *trans*-SNARE complex formation either by folding over the R-SNARE motif and/or by an unknown mechanism possibly involving interaction with non-SNARE proteins (Gonzalez *et al*, 2001; Tochio *et al*, 2001; Martinez-Arca *et al*, 2000, 2003).

To demonstrate that VAMP7 is required for fusions of late endosomes with lysosomes in living cells, we took advantage of the observation that expression of a chimaera consisting of green fluorescent protein (GFP) fused to the longin domain of VAMP7 could inhibit neurite outgrowth in PC12 cells (Martinez-Arca *et al*, 2000). This process requires VAMP7 in the formation of 'apical' SNARE complexes, which mediate fusion of intracellular vesicles with the plasma membrane. We tested whether expression of the same GFP–longin domain chimaera could inhibit mixing between endocytosed markers in lysosomes and late endosomes of cultured fibroblastic cells. The markers were taken up by fluid-phase endocytosis and examined either by fluorescence or EM. When fluorescent-labelled dextran was taken up into cultured normal rat kidney (NRK) cells for 4 h followed by a 20 h chase, it was, as expected, all delivered to compartments, which could be stained with antibodies against the lysosomal membrane protein lgp120 (Fig 4A–C). When cells were preloaded with Texas Red–dextran (4 h pulse, 20 h chase), then transfected with the plasmid encoding GFP–VAMP7 longin domain (24 h), there was reduced delivery of subsequently endocytosed biotinylated dextran (4 h pulse, 20 h chase) to lgp120-positive compartments (Fig 4G–L). Consistent with this, there was incomplete mixing of the biotinylated dextran with the Texas Red–dextran (Fig 4G,J,K). By the end of this experiment (72 h), fewer of the lgp120-containing organelles were positive for Texas Red–dextran (Fig 4G–I) than after 24 h (Fig 4A–C), possibly as a result of inactivation of the Texas Red fluor. The endocytosed biotinylated dextran had travelled beyond early endosomes in the transfected cells because it did not colocalize with EEA1 (Fig 4D–F). To confirm the effect of the VAMP7 longin domain on inhibiting delivery to lysosomes, we monitored the traffic of endocytosed gold particles to lysosomes previously marked with a different size colloidal gold. We had earlier shown that when bovine serum albumin (BSA)–gold is taken up into NRK cells for 4 h and chased for 20 h, >85% is delivered to dense core lysosomes with all of the gold having aggregated as a result of passing through compartments in which BSA is hydrolysed (Bright *et al*, 1997). In the cells expressing the GFP–VAMP7 longin domain, identified by green fluorescence before fixation and embedding, there was significant inhibition of mixing of the two sizes of gold, and inhibition of delivery of the second pulse of gold to dense core lysosomes, when compared with the surrounding nontransfected cells or cells expressing GFP-tagged cytosolic domain of VAMP8 (Fig 5A–D). In agreement with earlier experiments on NRK cells (Bright *et al*, 1997), almost all of the second pulse of gold was delivered to dense core lysosomes containing the first pulse of gold in the nontransfected and GFP–VAMP8-expressing cells (Fig 5A–C). Taken together, our microscopy experiments show that overexpression of the longin domain of VAMP7 has no effect on the passage of fluid-phase markers through early endosomes but prevents mixing of markers that are in late endosomes and dense core lysosomes. This is consistent with the ability of bacterially expressed, purified VAMP7 longin domain to inhibit cell-free lysosome–late endosome content mixing (Fig 5E) and with the proposed function of VAMP7 in heterotypic fusion of late endosomes and lysosomes.

In our present study, we have defined the SNAREs required for fusion of late endosomes with lysosomes in mammalian cells and shown that the R-SNARE VAMP7 determines heterotypic fusion in contrast to the R-SNARE VAMP8 required for homotypic fusion of late endosomes. Recent work on SNARE complexes required for fusion events in the macropinocytic pathway of the slime mould *Dictyostelium discoideum* has shown the formation and function of a SNARE complex made up of syntaxin 7, Vti1, syntaxin 8 and VAMP7 (Bogdanovic *et al*, 2002), consistent with our present observations being relevant beyond mammals. As VAMP7 is such a key player in heterotypic fusion events involving lysosomes, there is now a need to understand its biology more fully. Little is known about how late endocytic SNAREs are localized or recycle. Recently, it has been shown that VAMP7 is targeted to late endocytic compartments by interaction of its longin domain with the adaptor protein AP-3 (Martinez-Arca *et al*, 2003). VAMP7 has also been localized to small vesicles leaving cholesterol-containing organelles late in the endocytic pathway (Ko *et al*, 2001), which may reflect its recycling route. Further work is clearly required to understand how the longin domain of VAMP7 regulates heterotypic fusion and whether any interacting proteins are important to this regulation.

METHODS

Antibodies against SNAREs. Polyclonal antibodies against syntaxin 6 were a gift from Dr Sharon Tooze (CRUK, London) and polyclonal antibodies against syntaxin 7 and VAMP8 were as described (Mullock *et al*, 2000). Polyclonal antibodies against

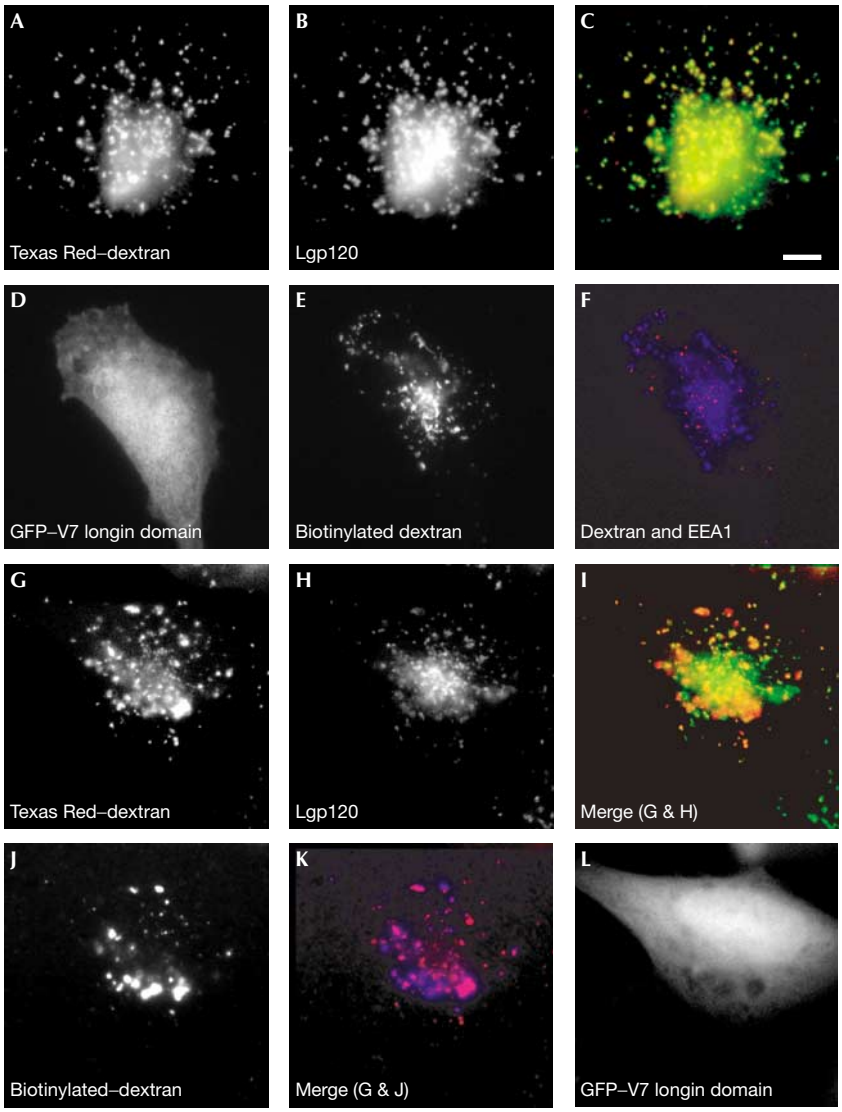

Fig 4 | Effect of overexpressing the longin domain of VAMP7 on traffic through the endocytic pathway of NRK cells. (**A**) Distribution of Texas Red–dextran after uptake for 4 h followed by 20 h chase. (**B**) Immunofluorescence localization of lgp120 in the same cell as in (**A**). (**C**) Colocalization (yellow) of Texas red–dextran (**A**, red) and lgp120 (**B**, green). (**D**) Expression of GFP–V7 longin domain (24 h) in a cell subsequently pulse-chased (4 h pulse, 20 h chase) with biotinylated dextran (**E**). (**F**) Immunofluorescence localization of EEA1 (red) in the same cell shown in (**E**) containing biotinylated dextran (blue). (**G–L**) Distribution of Texas Red–dextran (**G**), lgp120 (**H**) and biotinylated dextran (**J**) in a cell preloaded with Texas Red–dextran (4 h pulse, 20 h chase), transfected with a plasmid encoding GFP–longin domain of VAMP7 (**L**), and after 24 h, incubated with biotinylated dextran (4 h uptake, 20 h chase). (**I**) Colocalization (yellow) of Texas Red–dextran (**G**, red) and lgp120 (**H**, green). (**K**) Colocalization (magenta) between biotinylated dextran (**J**, blue) and Texas Red–dextran (**G**, red). Scale bar, 10 μm.

cytosolic domains of other SNAREs were raised against GST fusion proteins and affinity-purified as described previously (Mullock *et al*, 2000), using mouse syntaxin 8 (amino acids (aa) 1–215, GenBank™ accession number AB040054), VAMP7 (aa 1–188, XP135737) and Vti1b (aa 1–207, O88384; cDNA a gift from Dr Gabriele Fischer von Mollard).

Immunoblotting and immunoprecipitation. Preparation of fractions, immunoprecipitations of solubilized rat liver membranes, SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting were as described previously (Mullock *et al*, 2000; Wade *et al*, 2001; Poupon *et al*, 2003).

Cell-free fusion assays. The late endosome–lysosome content mixing assay was carried out as described previously using rat liver organelle fractions loaded respectively with avidin–asialofetuin (ASF) and iodinated, biotinylated polymeric IgA (Mullock *et al*, 1998; Pryor *et al*, 2000).

The late endosome–late endosome homotypic fusion assay was established as follows. ASF was biotinylated and iodinated as described for polymeric IgA (Mullock *et al*, 1994). A late endosome-enriched fraction (dense endosomes) was prepared from the liver of a rat that had received an intravenous injection of ~0.03 nmol biotinylated, iodinated ASF, 6 min before killing. The

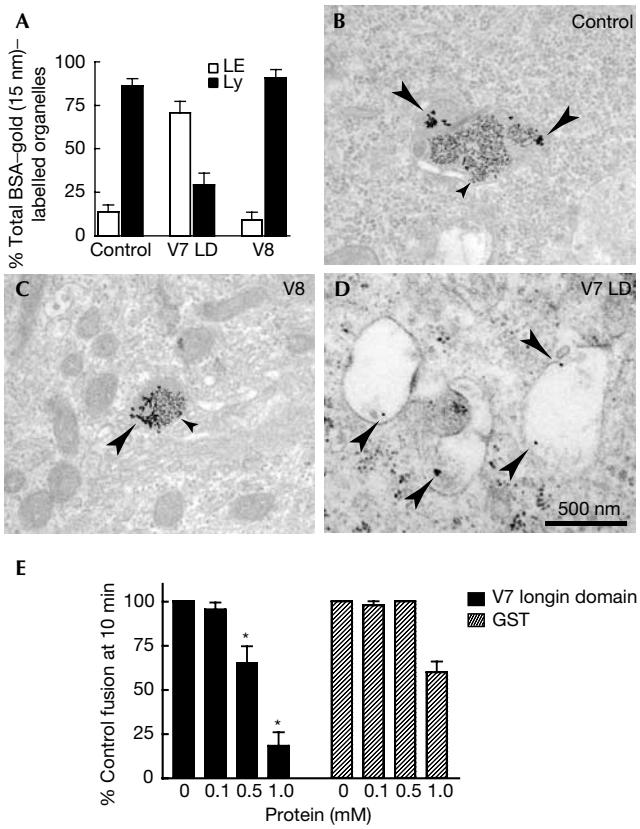

Fig 5 | Inhibition of delivery to lysosomes by the VAMP7 longin domain. NRK cells were preloaded with BSA–5 nm gold (4 h pulse, 20 h chase). They were then transiently transfected with a plasmid encoding GFP–longin domain of VAMP7 (V7 LD), or GFP–VAMP8 (V8), and after 24 h allowed to take up BSA–15 nm gold (4 h pulse, 20 h chase). (**A**) After EM, the percentage of organelles containing BSA–15 nm gold that were electron dense/contained (closed bars), or electron lucent/did not contain (open bars), BSA–5 nm gold measured in cells expressing V7 LD or V8, or surrounding cells not expressing GFP (control). The data shown are mean ± s.e.m. from single sections of six control and six V7 LD-transfected cells from two separate experiments and four V8-transfected cells from a different experiment (surrounding nontransfected cells had a distribution of gold particles not significantly different from the control shown). (**B–D**) Representative images of main organelles containing 15 nm gold in transfected and control cells (large arrows, 15 nm gold; small arrows, 5 nm gold). (**E**) Cell-free late endosome–lysosome fusion after addition of bacterially expressed his6-tagged V7 longin domain or GST as a control (*$P < 0.03$ versus GST control). Data are means ± s.e.m., $n = 4$.

dense endosome peak from a Ficoll gradient was diluted and sedimented, then resuspended in pig brain cytosol and mixed with dense endosomes that had been loaded with avidin–ASF and stored in liquid N_2 (Mullock *et al*, 1998). Content mixing between the two sets of endosomes was assayed exactly as for endosome–lysosome content mixing.

The effect of anti-SNARE antibodies and bacterially expressed proteins on fusion was determined as described by Mullock *et al* (2000). The VAMP7 longin domain (aa 1–120) was cloned into the plasmid pMWH6, expressed as His_6-tagged protein in BL21(DE3) pLysS cells and purified using nickel-nitrilotriacetic acid–agarose. Glutathione *S*-transferase (GST) was expressed in BL21(DE3) pLysS cells, using plasmid pGEX-4T1, and purified using glutathione–Sepharose 4B.

Microscopy. NRK cells were incubated with labelled dextrans (Molecular Probes Inc., Eugene, OR) at 10 mg/ml, fixed and stained with antibodies against EEA1 (BD Biosciences, Lexington, KY) and lgp120 as described previously (Poupon *et al*, 2003). Biotinylated dextran was observed by staining fixed cells with 100 μg/ml NeutrAvidin-Alexa Fluor 350 (Molecular Probes Inc., Eugene, OR, USA). Fluorescence was observed with a Zeiss Axioplan microscope equipped with a CCD camera. Multiple fluors were visualized using a single stationary (multiband) beam splitter and appropriate filters fitted to filter wheels. We used the 86000: Sedat Quad filter set with single-band excitation and emission filters, details of which can be found at http://www.chroma.com/products/products/byfilterset_seriesdetail.cfm?ID = 86000. Uptake of BSA–gold, ultrastructural analysis using transmission EM with 70 nm sections observed in a Philips CM100 EM and quantification of gold mixing were as described previously (Bright *et al*, 1997). In experiments using transiently transfected cells, transfection was as described by Poupon *et al* (2003) using a plasmid encoding GFP–VAMP7 longin (N-terminal) domain (aa 1–120) constructed as described by Martinez-Arca *et al* (2000), or the same plasmid encoding GFP–VAMP8 (aa 1–215). ImmunoEM and quantification of SNAREs on organelle membranes were as described by Mullock *et al* (2000).

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