The EMBO Journal Vol.16 No.11 pp.3017–3024, 1997

A novel SNARE complex implicated in vesicle fusion with the endoplasmic reticulum

Michael J.Lewis, Julian C.Rayner and Hugh R.B.Pelham[1]

MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

[1]Corresponding author

Intracellular vesicular traffic is controlled in part by v- and t-SNAREs, integral membrane proteins which allow specific interaction and fusion between vesicles (v-SNAREs) and their target membranes (t-SNAREs). In yeast, retrograde transport from the Golgi complex to the ER is mediated by the ER t-SNARE Ufe1p, and also requires two other ER proteins, Sec20p and Tip20p, which bind each other. Although Sec20p is not a typical SNARE, we show that both it and Tip20p can be co-precipitated with Ufe1p, and that a growth-inhibiting mutation in Ufe1p can be compensated by a mutation in Sec20p. Furthermore, Sec22p, a v-SNARE implicated in forward transport from ER to Golgi, co-precipitates with Ufe1p and Sec20p, and *SEC22* acts as an allele-specific multicopy suppressor of a temperature-sensitive *ufe1* mutation. These results define a new functional SNARE complex, with features distinct from the plasma membrane and *cis*-Golgi complexes previously identified. They also show that a single v-SNARE can be involved in both anterograde and retrograde transport, which suggests that the mere presence of a particular v-SNARE may not be sufficient to determine the preferred target for a transport vesicle.

Keywords: retrograde transport/*SEC20/SEC22*/SNARE/*UFE1*

Introduction

Transport of proteins along the secretory pathway of eukaryotic cells occurs by a process of vesicular budding and fusion. Vesicle formation is aided by the binding of cytosolic coat proteins to the budding membrane. The subsequent docking and fusion of the vesicles requires integral membrane proteins known as v-SNAREs (on vesicles) and t-SNAREs (on the target membranes) (Söllner *et al.*, 1993a,b; Rothman and Wieland, 1996). SNARE proteins were initially identified by yeast genetics, and by biochemical analysis of synaptic components (Ferro-Novick and Jahn, 1994). Further examples were identified by homology searches, and in yeast t-SNAREs are now known for the plasma membrane, the endoplasmic reticulum (ER), the Golgi apparatus and the endosome/vacuole system (Hardwick and Pelham, 1992; Aalto *et al.*, 1993; Becherer *et al.*, 1996; Lewis and Pelham, 1996). Both t- and v-SNAREs have a characteristic structure, typically with a C-terminal transmembrane domain preceded by a stretch of 50–60 residues with the potential to form a coiled-coil structure.

The inter-species conservation of the SNAREs extends to the complexes formed between them. Thus a stable complex can be isolated that contains the synaptic v-SNARE synaptobrevin (also known as VAMP), the plasma membrane t-SNARE syntaxin and a third protein, associated with membranes only via lipids, called SNAP25 (Söllner *et al.*, 1993a; Hayashi *et al.*, 1994). These proteins associate via their coiled-coil motifs, and act as receptors for the soluble proteins α-SNAP and NSF (Chapman *et al.*, 1994; Hayashi *et al.*, 1995; Kee *et al.*, 1995; McMahon and Südhof, 1995); ATP hydrolysis by NSF has the effect of dissociating this complex, and apparently disrupting the SNARE–SNARE interactions. In yeast, all these components have their counterparts: Snc1p and Snc2p (synaptobrevin-like; Protopopov *et al.*, 1993), Sso1p and Sso2p (syntaxin-like; Aalto *et al.*, 1993), Sec9p (SNAP25-like; Brennwald *et al.*, 1994), Sec17p and Sec18p (α-SNAP and NSF homologues, respectively; Eakle *et al.*, 1988; Wilson *et al.*, 1989; Griff *et al.*, 1992). When not associated with the other SNAREs, syntaxin binds to a soluble protein whose yeast homologue is Sec1p (Pevsner *et al.*, 1994).

SNARE complexes from the early Golgi compartment have also been characterized, though in less detail. Immunoprecipitation of the syntaxin-like t-SNARE Sed5p results in the coprecipitation of four putative v-SNAREs implicated in ER–Golgi traffic, namely Bet1p, Bos1p, Sec22p and Ykt6p, together with two other putative v-SNAREs likely involved in retrograde transport from later Golgi compartments, Sft1p and p28 (Søgaard *et al.*, 1994; Banfield *et al.*, 1995). No SNAP25 equivalent has been found. How many distinct complexes are represented is not clear, but all the v-SNARE associations seem to be disruptable by the action of Sec18p. A Sec1p homologue, Sly1p, is also associated with Sed5p (Søgaard *et al.*, 1994). These results suggest that the basic fusion mechanism is similar at different organelles, but whether precisely equivalent complexes form is not clear.

Because v-SNAREs are (for the most part) anchored in the lipid bilayer by a transmembrane domain, their re-use for vesicle targeting can only occur if they are recycled to their membrane of origin. This poses a mechanistic problem: if the v-SNAREs travel both forwards and backwards, how is the direction of movement determined for any given vesicle? This problem is perhaps easiest to pursue in the case of ER–Golgi transport in yeast, since many of the components involved in this step are now known from extensive genetic and biochemical studies. For all cargo molecules so far studied, anterograde transport is mediated by the COPII coat proteins (Barlowe *et al.*, 1994), whereas retrograde transport from the Golgi complex is

```
                          S--(ufe1-1)--P
261.QLKKVETINKTILDIVNIQNELSNHLTVQSQNINLMLNNQDDIEL
        .     .     .     .     .     .
209.LTNNLVRGNQILQSGILQSDLNLDELRAQTNSLTQIDDKYTQFET
           R(sec20-2)        S(sec20-1)

                                  .  .  .  .  K
NIKKGNKELRKAKRAAGR--TAKMTTYGAIIMGVFILFLDYVG* Ufe1p
  .     .                                   |
VFKKTADLVKVLENASHQEKRDVYLSLGFLLCCVSWVLWRRIF. Sec20p
                                          D
```

Fig. 1. Sequences of the membrane-proximal portions of Ufe1p and Sec20p. Residues 261–347 of Ufe1p and 209–297 of Sec20p are shown, with the TMDs underlined and the changes in the ts alleles *sec20-1* and *sec20-2*, and the two changes in this region in *ufe1-1* indicated. Dots between the sequences mark the heptad repeats, and those over the Ufe1p TMD indicate the relatively polar helical face important for function. The compensating changes introduced in the mutants used in Figure 2 are shown in bold. Two spaces have been introduced into the Ufe1p sequence to allow alignment of the TMDs.

mediated by COPI coat proteins (Letourneur *et al.*, 1994). As mentioned above, four putative v-SNAREs have been implicated in forward transport, although it is still not clear whether Bet1p and Ykt6p are necessary components of the transport vesicles, and Sec22p is dispensable at normal growth temperatures (Ossig *et al.*, 1991; Lian and Ferro-Novick, 1993; Barlowe *et al.*, 1994). Recently we described a t-SNARE on the ER, Ufe1p, that is specifically required for retrograde transport (Lewis and Pelham, 1996). Mutation of this protein showed that recycling to the ER is essential for forward transport to be maintained. We also found that a second ER membrane protein, Sec20p, is required for the retrograde step (Lewis and Pelham, 1996). Sec20p was shown to bind to a peripheral membrane protein, Tip20p (formerly designated Tip1p; Sweet and Pelham, 1993), but the precise role of these proteins, and the nature of the v-SNAREs involved in retrograde transport, remained undefined.

In this paper we examine the interactions of Ufe1p with other components of the retrograde transport pathway. We present genetic and biochemical evidence that Sec20p binds to Ufe1p, that this binding involves the transmembrane domains of the two proteins, and that it is required for their function. Tip20p is also associated with Ufe1p, presumably via Sec20p. In addition, this complex contains the v-SNARE Sec22p. These findings define a novel SNARE fusion complex, and indicate that a single v-SNARE can participate in both forward and retrograde steps. The implications for the directionality of traffic between ER and Golgi are discussed.

Results

Genetic interactions between Sec20p and Ufe1p

Since Sec20p and Ufe1p are both membrane proteins located in the ER, and are both required for retrograde transport, we anticipated that they might interact physically. Indeed, Sec20p contains a coiled-coil domain of the appropriate length and position to interact with a SNARE, and ts alleles of *sec20* map to this part of the protein (Figure 1). When temperature-sensitive mutations weaken protein–protein interactions, the defect can often be overcome by overexpression of the normal partner. We therefore attempted to suppress the temperature-sensitive

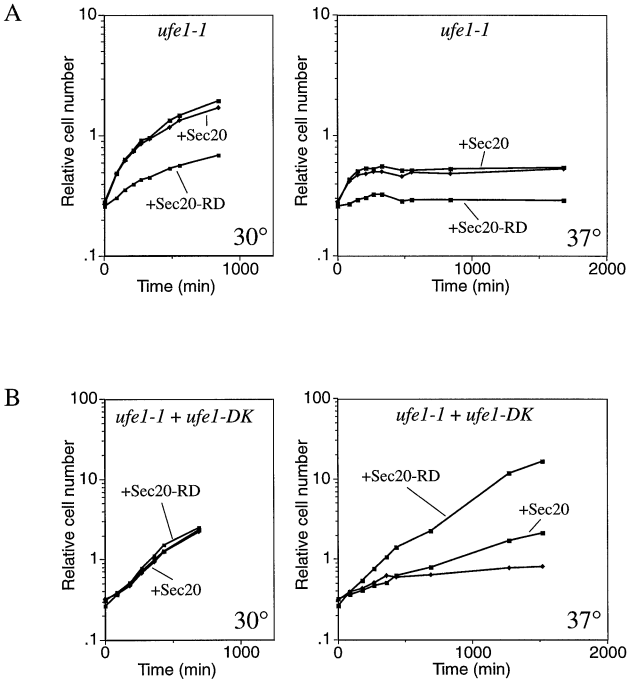

Fig. 2. Suppression of the *ufe1-DK* mutant by the *sec20-RD* mutation. (**A**) Batch growth of *ufe1-1* strains expressing Sec20p or Sec20-RD (as indicated) or with vector alone (unlabelled curves) at 30°C and 37°C. (**B**) As in (A) but strains also expressed the *ufe1-DK* allele. Cultures were diluted to allow continuous growth.

growth defect of *ufe1-1* by overexpressing Sec20p, and that of *sec20-1* by overexpressing Ufe1p. Some suppression was observed in both cases, but the effects were weak and variable.

A more subtle genetic approach was suggested by the observation that the function of Ufe1p, unlike that of other t-SNAREs such as Sed5p, Pep12p and Sso1p, is sensitive to mutations within the transmembrane domain (TMD) (Rayner and Pelham, 1997). A possible explanation is that the Ufe1p TMD interacts with Sec20p, in which case changes in the Sec20p TMD might compensate for mutations in the Ufe1p TMD. Ufe1p function is abolished by conversion of the Asp residue that defines the lumenal end of the TMD to a Lys residue (*ufe1-DK*). The equivalent position in Sec20p is occupied by an Arg residue, so we changed this to Asp (*sec20-RD*) in order to restore a putative positive/negative charge pair. The functions of these proteins were then tested individually and together in a *ufe1-1* strain, which provides Ufe1p activity at low but not high temperature.

The *ufe1-1* strain grew at 30°C, but at 37°C growth ceased (after an initial doubling) as expected (Figure 2A). Moderate overexpression of wild-type Sec20p in this strain had little effect on growth at either temperature. In contrast, expression of *sec20-RD* significantly slowed growth. Our interpretation is that this protein can compete for other components of the fusion machinery (such as Tip20p, see below) but is unable to interact effectively with the normal TMD of Ufe1p.

The experiment was then repeated with a strain expressing both the *ufe1-1* and the *ufe1-DK* alleles (Figure 2B). In the absence of any additional Sec20p this strain grew at 30°C but not at 37°C, confirming that neither allele was capable of functioning at an elevated

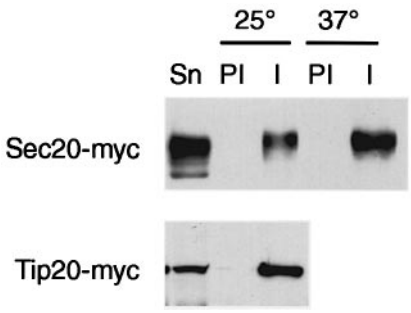

Fig. 3. Sec20p and Tip20p coprecipitate with Ufe1p. Lysates of strains expressing *myc*-tagged versions of Sec20p and Tip20p were incubated with beads containing anti-Ufe1p antibodies (I) or pre-immune antibodies (PI), and the bound *myc*-tagged proteins detected with the 9E10 anti-*myc* mAb. Samples of the supernatants were also analysed (Sn); these correspond to 5% of the precipitated material. Sec20-*myc* was expressed in a *sec18* strain, which was incubated at both 25°C or 37°C, Tip20-*myc* was expressed in a *SEC*$^+$ strain.

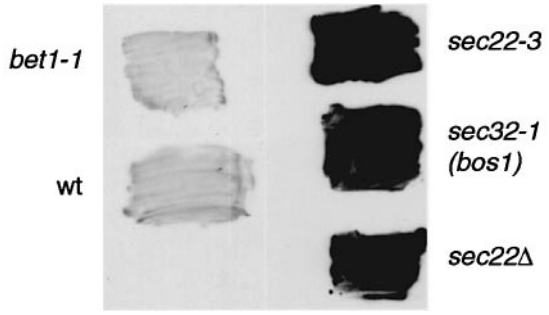

Fig. 4. Secretion of BiP by v-SNARE mutants. The indicated strains were grown in contact with nitrocellulose and the filter probed with anti-BiP antibodies.

temperature. Moderate overexpression of wild-type Sec20p partially rescued growth at 37°C, suggesting that the inactivity of the *ufe1-DK* mutation was indeed due to its poor interaction with Sec20p. Most significantly, however, *sec20-RD* no longer inhibited growth at 30°C, and at 37°C it was a considerably more effective suppressor of the *ufe1-DK* growth defect than was wild-type Sec20p, restoring growth to the same rate as at 30°C. So effective was this suppression that we were able to construct a strain whose only source of Ufe1p was the *ufe1-DK* allele, provided that *sec20-RD* was present; wild-type *sec20* could not substitute (see Materials and methods). Thus, the D→K change in the Ufe1p TMD can be specifically compensated by an R→D change at the corresponding position in Sec20p, providing powerful evidence that the TMDs and flanking regions of these proteins interact in a functionally significant manner *in vivo*.

Physical association of Sec20p and Tip20p with Ufe1p

To confirm the actual binding of Sec20p to Ufe1p, we expressed a *myc*-tagged version of Sec20p in a *sec18* strain, immunoprecipitated Ufe1p with affinity-purified rabbit antiserum, and probed the precipitate with anti-*myc* antibodies. Sec20p was clearly detectable (Figure 3). There was a modest enhancement of the signal after incubation of the cells at 37°C, but binding was clearly evident at a low temperature, and was also observed when the experiment was repeated in a *SEC*$^+$ strain (not shown). A control in which pre-immune serum replaced the anti-Ufe1p serum gave no Sec20p signal (Figure 3). Thus Ufe1p and Sec20p do indeed form a complex. Whether this complex can be dissociated by Sec18p is not clear: we observed only a slight effect of the *sec18* mutation, but we observed a similarly weak effect when we examined the Sed5p-containing SNARE complex as a control. It appears that the effects of Sec18p are very sensitive to minor changes in the conditions used for lysis and precipitation.

We previously identified another protein, originally termed Tip1p but now designated Tip20p, which is not itself an integral membrane protein but which binds to Sec20p. Deletion mutants of *tip20* have the same phenotype as *sec20* mutants (Sweet and Pelham, 1993). To see whether Tip20p is also part of the Ufe1p–Sec20p complex, we expressed a *myc*-tagged version of Tip20p and repeated the Ufe1p immunoprecipitation. As with Sec20p, there was a clear association of Tip20p with Ufe1p even in a *SEC*$^+$ strain (Figure 3). These results strongly suggest that Ufe1p, Sec20p and Tip20p are present in a single complex on the ER membrane.

Assay of v-SNARE mutants for defects in retrograde traffic

We next considered what v-SNAREs might interact with the Ufe1p complex, and sought to determine whether any of the known ER–Golgi v-SNAREs might be involved in retrograde transport in addition to their roles in forward movement. One of the functions of retrograde transport is to retrieve escaped lumenal ER proteins such as BiP/Kar2p, and as a consequence partial defects in components of this pathway, such as those found in temperature-sensitive *ufe1* and *sec20* mutants, tend to increase the secretion of BiP from cells (Semenza *et al.*, 1990; Lewis and Pelham, 1996). This can readily be detected by growing the cells in contact with a nitrocellulose filter and then probing the filter with anti-BiP antibodies. Figure 4 shows that *sec22-3* cells indeed secrete BiP at a growth-permissive temperature; this is not a special feature of this particular allele, since a *sec22* deletion strain had a similar phenotype. BiP was also secreted by a *bos1* temperature-sensitive mutant (*sec32-1*; Wuestehube *et al.*, 1996), though not significantly by a *bet1-1* strain. These results raised the possibility that Sec22p and Bos1p might contribute, directly or indirectly, to the reverse pathway.

Interactions between Sec22p and Ufe1p

As an alternative approach, we looked for genetic interactions between the known v-SNAREs and the ER t-SNARE, Ufe1p. Since the *ufe1-1* mutant has alterations in the conserved coiled-coil domain implicated in v-SNARE binding (Figure 1), we tested the effects of overexpressing each of the ER–Golgi v-SNAREs. Figure 5 shows that elevated levels of Sec22p allowed growth of *ufe1-1* cells at the normally non-permissive temperature of 37°C, whereas Bos1p and Bet1p did not have this effect. Ykt6p, which is most closely related to Sec22p and will suppress a *sec22* mutation (Banfield *et al.*, 1995), showed a very weak effect on *ufe1-1* in some experiments, but did not reproducibly rescue growth at high temperatures. The suppression by Sec22p was allele-specific: though *ufe1-1* was suppressed, another temperature-sensitive *ufe1* allele, with a G to L change at position 336

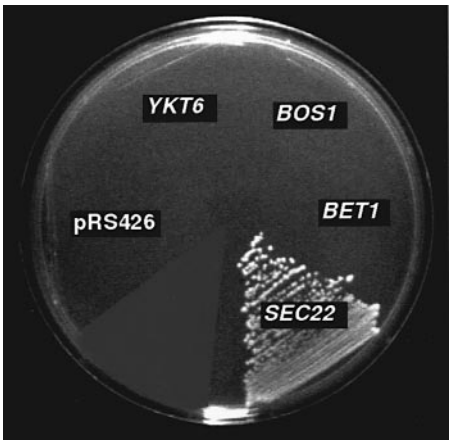

Fig. 5. Suppression of *ufe1-1* by Sec22p. Strains bearing the *ufe1-1* mutation with a multicopy vector alone (pRS426), or one bearing the indicated v-SNARE genes under the control of the *TPI* promoter, were grown at 37°C. Only the *SEC22*-expressing strain grew under these conditions, whereas all the strains grew well at 30°C.

within the TMD, was not (data not shown). This strongly suggests direct binding of Sec22p to Ufe1p.

To see whether a physical interaction between Sec22p and Ufe1p could be demonstrated, we immunoprecipitated Ufe1p from cell extracts and probed the precipitates with anti-Sec22p antibodies. Figure 6A shows that some Sec22p indeed coprecipitated with Ufe1p. In this experiment a *sec18* strain was used and the Sec22p–Ufe1p interaction was enhanced after incubation at 37°C, though as with the Sec20p–Ufe1p interaction, we found the effects of *sec18* to be variable. From the blots, we estimate that ~5–10% of the total Sec22p was associated with Ufe1p. In contrast, no Bet1p could be detected in the immunoprecipitate. Suitable antibodies against Bos1p and Ykt6p were not available, and so we expressed versions of these proteins tagged with a *myc* epitope at the N-terminus. These altered proteins remained functional, as shown by their ability to suppress *sec32-1* (a *bos1* ts allele) and *sec22-3* respectively. However, they could not be detected in the Ufe1p immunoprecipitates. Myc-tagged Sec22p acted as a positive control, and was readily detectable (Figure 6A). We conclude that Sec22p forms a specific association with Ufe1p that is disruptable by Sec18p. The other v-SNAREs, including Bos1p which binds to Sec22p in forward transport vesicles (Lian *et al.*, 1994), evidently do not form stable complexes with Ufe1p under these conditions. Further negative controls were provided by *myc*-tagged versions of the putative Golgi v-SNAREs Sft1p and p28 (the yeast homologue of GS28; Nagahama *et al.*, 1996; Subramaniam *et al.*, 1996), neither of which could be detected in the Ufe1p immunoprecipitate (Figure 6A).

Association between Sec20p, Sec22p and Ufe1p

The data presented above indicate that both Sec20p and Sec22p can associate with Ufe1p, but they do not show whether they can bind simultaneously. To address this question we immunoprecipitated *myc*-tagged Sec20p with the 9E10 anti-*myc* monoclonal antibody and probed the precipitate with anti-Sec22p antibodies. Figure 6B shows that Sec22p was specifically precipitated in this experiment, the efficiency being comparable with that obtained

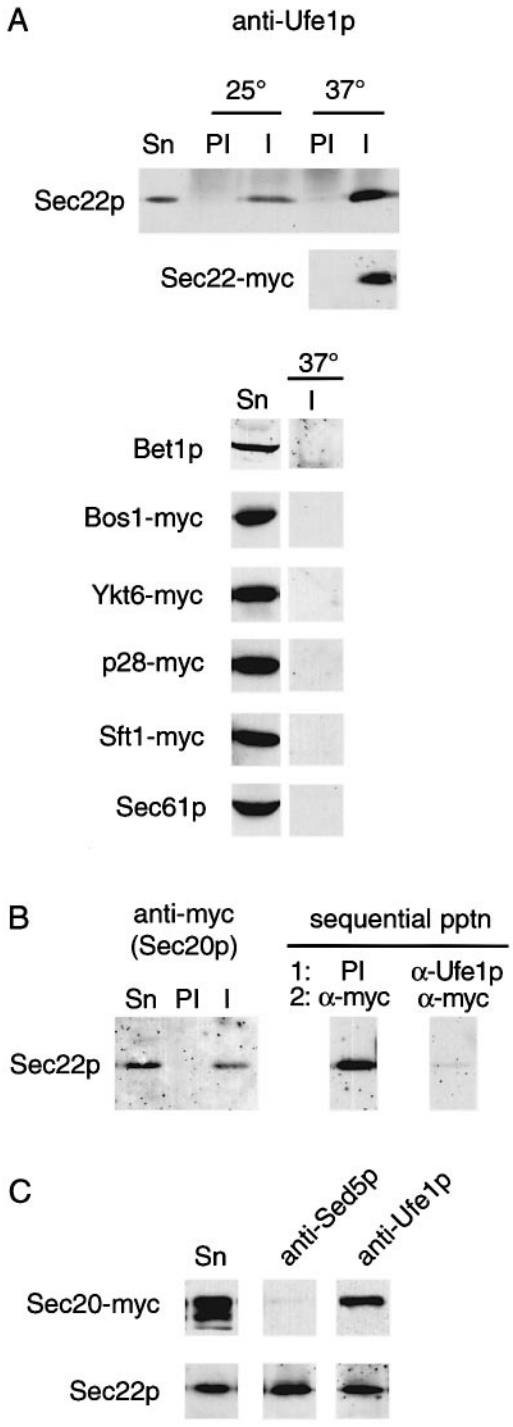

Fig. 6. Coprecipitation of Sec22p with Ufe1p and Sec20p. (**A**) Precipitations of *sec18* strains with anti-Ufe1p (I) or preimmune antibodies (PI) were performed as in Figure 3. Endogenous Sec22p, Bet1p and Sec61p were detected with appropriate antisera, and *myc*-tagged Sec22p, Bos1p, Ykt6p, p28 (the yeast homologue of GS28) and Sft1p with the anti-*myc* mAb. (**B**) Cells expressing *myc*-tagged Sec20p were lysed, immunoprecipitated with the anti-*myc* mAb (I) or non-immune Abs (PI) and the precipitate probed with anti-Sec22p. In a separate experiment, sequential immunoprecipitations were performed: cell extracts were first subjected to immunoprecipitation with pre-immune antibodies (PI) or anti-Ufe1p, and the supernatants then precipitated with anti-*myc*; these second precipitates were then immunoblotted to detect Sec22p. (**C**) As for (B), except that aliquots of the cell extract were immunoprecipitated with either anti-Sed5p or anti-Ufe1p, and the precipitates probed for *myc*-tagged Sec20p and for Sec22p. Supernatant samples (Sn) correspond to 4% of the precipitated material, in all panels.

with anti-Ufe1p antibodies. Since only 5–10% of the total Sec22p coprecipitated with Ufe1p or Sec20p, this result does not rule out the formal possibility that despite their affinity for each other, Sec20p and Ufe1p form quite separate complexes with Sec22p. We therefore performed sequential immunoprecipitations in which first Ufe1p was removed from the cell extracts, together with any associated Sec20p and Sec22p, and then the remaining Sec20p was precipitated from the supernatant and the precipitate probed with anti-Sec22p. Figure 6B shows that although pre-precipitation with control antibodies had little effect, the prior removal of Ufe1p substantially reduced the subsequent recovery of Sec20p–Sec22p complexes. This indicates that the Sec22p molecules that are associated with Sec20p are mostly the same as those that are bound to Ufe1p, and hence that Sec22p, Ufe1p and Sec20p are present in a single complex. Although all of these proteins are found in the ER, their association cannot be explained merely by poor solubilization of the ER membrane. These were shown by the absence not only of Bet1p and Bos1p, but also of the abundant ER membrane protein Sec61p (Figure 6A).

As both Ufe1p and Sed5p appear to be able to bind Sec22p, we performed further controls to establish that these two t-SNARE complexes are distinct. Figure 6C shows the results of precipitating a single extract with anti-Sed5p and anti-Ufe1p. While each antibody precipitated Sec22p in comparable amounts, only anti-Ufe1p precipitated Sec20-*myc*. Additional experiments confirmed that anti-Sed5p precipitated Bet1p but not Tip20-*myc*, and that anti-Ufe1p did not precipitate Sed5p (data not shown). Thus of the components we have tested, only Sec22p could be found associated with both Ufe1p and Sed5p.

Discussion

We have presented evidence in this paper for the existence of a novel SNARE-containing complex on the ER membrane. Biochemical evidence, in the form of co-precipitation, indicates five associations: Ufe1p with Sec20p, Sec20p with Tip20p (Sweet and Pelham 1993), Ufe1p with Tip20p, Sec20p with Sec22p and Ufe1p with Sec22p. The simplest interpretation of these results is that Ufe1p, Sec20p, Tip20p and Sec22p are present in a single complex, and indeed sequential immunoprecipitation provides evidence that Ufe1p, Sec20p and Sec22p are all associated. Preliminary experiments indicate that Tip20p and Sec22p also coprecipitate, implying that they can bind simultaneously to the t-SNARE complex.

We have also presented genetic evidence that supports a functional role for several of these interactions *in vivo*. In particular, the demonstration that growth-inhibiting mutations adjacent to the TMDs of Sec20p and Ufe1p can specifically compensate for each other provides good evidence for the interaction of these proteins. This essential TMD-mediated interaction accounts for our previous finding that mutations that alter the hydrophobicity of the relatively polar helical face of the Ufe1p TMD affect the function of this t-SNARE (Rayner and Pelham, 1997).

The Ufe1p complex shows both similarities to and differences from previously characterized SNARE complexes. Sec20p is in the same membrane as Ufe1p and can be thought of as a partner t-SNARE, analogous to SNAP25 in the synapse. However, unlike SNAP25, Sec20p is a transmembrane protein with a substantial extracytoplasmic domain, and though it has a coiled-coil motif like that of other SNAREs it shows no homology in this region to SNAP25 or to any other known SNARE. This raises the possibility that other SNARE-like proteins exist that have an unusual size and structure and cannot be detected by homology searches alone.

Another unusual feature is the presence of Tip20p, an 81 kDa peripheral membrane protein with no obvious homologue (Sweet and Pelham, 1993). Unlike the Sec1p family of proteins which are thought to associate only with free syntaxins (Pevsner *et al.*, 1994), Tip20p seems to be in a complex containing Sec20p, Ufe1p and Sec22p, and it remains to be determined whether it shares any functional properties with Sec1p. We have not been able to detect by homology searches of the complete yeast genome any candidate Sec1p-like protein that might interact with Ufe1p. It seems that despite the similarity in sequence and structure between different syntaxin family members, the proteins with which they associate can differ substantially.

A retrograde v-SNARE

The interactions that we have identified unite all the ER components known to be involved in retrograde transport and place them in contact with a v-SNARE, Sec22p, whose role in forward transport seems well established. The evidence that Sec22p is involved in retrograde traffic is based not only on its co-precipitation with Ufe1p and Sec20p, but also on its ability to suppress a *ufe1* defect *in vivo* in an allele-specific manner, and on the failure of *sec22* mutants to retain ER proteins efficiently. Given these results, it seems reasonable to ask whether Sec22p is also directly involved in forward transport, or whether its role in this is limited to the indirect one of recycling other v-SNAREs. This is not simple to answer, because Sec22p is not required for viability at 30°C, or for forward transport *in vitro* (Ossig *et al.*, 1991; Jiang *et al.*, 1995). However, it does co-precipitate with Sed5p and, if it is present, antibodies to Sec22p can block the *in vitro* transport reaction (Søgaard *et al.*, 1994; Jiang *et al.*, 1995).

Perhaps the most compelling argument in favour of a dual role for Sec22p comes from electron microscopic studies. When cells are deprived of the activity of *ufe1*, *sec20* or *tip20*, either by temperature-sensitive mutation or by protein depletion, a characteristic phenotype is observed: ER membranes accumulate, but although one might expect retrograde transport vesicles also to accumulate, few vesicles are found. Those that are seen often appear to be trapped between the ER and the cell surface, where they are inaccessible to Golgi membranes (Kaiser and Schekman, 1990; Sweet and Pelham, 1992, 1993; Lewis and Pelham, 1996). In contrast, at 37°C *sec22* mutants accumulate large numbers of vesicles throughout the cell, which are evidently incapable of fusing with any membrane (Kaiser and Scheckman, 1990). This striking difference between the phenotypes of the *ufe1* and *sec22* mutants implies that interaction with Ufe1p is not the only role of Sec22p. We suggest that it participates directly in the fusion of vesicles to both ER and Golgi membranes. The phenotype of the *ufe1*, *sec20* and *tip20* mutants can be explained if retrograde vesicles, thwarted in their

Table I. Yeast strains used in this work

Strain	Genotype	Source
RSY255	MATα *ura3-52 leu2-3, -112*	C.Kaiser
RSY271	MATα *sec18-1 ura3-52 his4-619*	C.Kaiser
RSY275	MATα *sec20-1 ura3-52 his4-619*	C.Kaiser
RSY279	MATα *sec22-3 ura3-52 his4-619*	C.Kaiser
RSY1159	MATα *sec32-1 ura3-52 leu2-3, -112*	R.Schekman
ANY112	MAT**a** *bet1-1 ura3-52*	S.Ferro-Novick
SD1041A	MATα *leu2 his3 sly2::HIS3*	D.Gallwitz
JB811	*ura3 leu2 trp1 prb prc pep4*	P.Sorger
MLY101	MATα Δ*ufe1::TRP1 his3-Δ200 leu2-3, -112 ura3-52* containing pUT1 (*CEN6 LEU2 ufe1-1*)	Lewis and Pelham (1996)
MLY106	*ura3-52 his3-Δ200 trp1-Δ901 sec18-1*	this study
MLY201	MATα *sec18-1 URA3::SEC20-(myc)$_3$ his4-619*	this study
MLY202	MATα *sec18-1 URA3::BOS1-(myc)$_3$ his4-619*	this study

attempts to dock with the ER but containing the same v-SNAREs as forward-moving vesicles, fuse instead with the Golgi apparatus.

If this is so, how does a Sec22p-containing vesicle choose its destination? One possibility is that the v-SNAREs are, upon vesicle formation, put into a special conformation or combinatorial state (Lian *et al.*, 1994) that allows preferential recognition of Sed5p or Ufe1p. Alternatively, vesicles may be distinguished by their coats, forward-moving vesicles being encased in COPII proteins (Barlowe *et al.*, 1994), whereas those returning carry COPI coats (Letourneur *et al.*, 1994). If, for example, removal of COPI were stimulated by Sec20p or Tip20p, fusion of COPI-coated vesicles with the ER would be favoured. This would be consistent with the synthetic lethal interactions that have been observed between mutations in δ-COP and *sec20* (A.Frand and C.Kaiser, personal communication), and between a variety of coatomer mutants and ts alleles of *tip20* (G.Frigerio, personal communication).

Of the known v-SNAREs, it was surprising to find only Sec22p associated with Ufe1p since this protein, unlike Ufe1p itself, is dispensable for growth at 30°C. Perhaps other v-SNAREs do participate in retrograde transport but fail to form a stable complex *in vitro*. There is some suggestion that this may be so, because the *bos1* mutant showed poor retention of BiP and there was some slight suppression of *ufe1-1* by Ykt6p, but the evidence is much weaker than for Sec22p. It is possible that an additional, unidentified v-SNARE is involved, but we have not been able to identify from the genome sequence any candidate that has an appropriate intracellular location. Another explanation might be that low levels of recycling Sec20p (which carries a functional HDEL retrieval signal; Sweet and Pelham, 1992) give retrograde vesicles some affinity for Ufe1p. Indeed, we have recently found that overexpression of Sec20p can suppress the temperature-sensitive growth phenotype of a *sec22* deletion strain, which would be consistent with this. Interestingly, there is a similar puzzle for vesicular transport to the plasma membrane: removal of the t-SNAREs Sso1p and Sso2p is lethal (Aalto *et al.*, 1993), whereas cells lacking the known v-SNAREs Snc1p and Snc2p are still capable of slow growth and secretion (Protopopov *et al.*, 1993).

In conclusion, we have described a SNARE complex with unique features that is likely to mediate vesicle docking with the ER membrane, and have shown that at least one v-SNARE can associate with two different t-SNAREs. It remains to be seen whether the principle of bidirectional targeting can be extended to other vesicular transport steps, and if so whether general mechanisms exist to ensure that vesicles do not simply fuse with their donor membranes.

Materials and methods

Plasmids

Multicopy suppression analysis was performed using plasmids based on the vector JS 209 (2 μ, *URA3*, *TPI* promoter) (Semenza *et al.*, 1990). Constructs containing the open reading frames of *YKT6*, *BOS1*, *BET1* and *SEC22* were generated by PCR using either Vent (New England Biolabs) or *Pfu* (Stratagene) polymerases. PCR-generated constructs were checked by dideoxy sequencing or, where appropriate, by their ability to complement the relevant temperature-sensitive alleles.

N-terminal triple c-*myc* tagged versions of Sec20p and Bos1p were generated by PCR using *Pfu* polymerase and cloned into an integration vector based on YIP56X (Hardwick and Pelham, 1992). The *ufe1* Asp to Lys mutant was generated by site directed mutagenesis (Kunkel *et al.*, 1987) and transferred to a JS209-based plasmid. The complementary Arg to Asp mutation in the Sec20p TMD region was also made by site-directed mutagenesis. The *SEC20* coding region was transferred from the plasmid STM20 (Sweet and Pelham, 1992) into a pRS413-based plasmid (a *CEN* vector carrying the *HIS3* gene and the *TPI* promoter; Sikorski and Hieter, 1989) and used unaltered in this background as the control. All mutagenesis was checked by DNA sequencing. N-terminal c-*myc* tagged versions of p28 (orf YHL031c), Sft1p, Sec22p and Ykt6p were expressed from derivatives of the JS209 vector. C-terminal c-*myc*-tagged Tip20p was expressed from the JS209 derivative pTM2 (Sweet and Pelham, 1993).

Yeast strains

The main strains used for this work are listed in Table I. Other strains were generated by transforming these with appropriate replicating plasmids. The data in Figure 2 are based on MLY101. For Figure 3 the Sec20-*myc* immunoprecipitation was performed using MLY201, while the Tip20-*myc* were expressed in the protease-deficient strain JB811. Figure 4 shows assays of RSY255 (wt), ANY112, RSY279, RSY1159 and SD1041A. Figure 5 shows transformants of MLY101. For Figure 6, versions of RSY271 were used, with appropriate plasmids or integrated genes (MLY201, MLY202), except that p28-*myc* and Sft1-*myc* were expressed in the related strain MLY106.

To test the suppressibility of the *ufe1-DK* allele, a derivative of strain MLY101 that carried *UFE1* on a 2 μ *URA3* vector was transformed with a plasmid identical to pU315 (*CEN, UFE1, LEU2*; Lewis and Pelham, 1996) except that it carried the *ufe1-DK* mutation, together with the *SEC20* or *sec20-RD* expression plasmids described above. The transformants were plated at 30°C in the presence of 5-fluoro-orotic acid to counter-select for the *URA3* vector. Colonies lacking this vector and hence the wild-type *UFE1* gene grew readily from the strain expressing *sec20-RD*, but not from the strain expressing wild-type *SEC20*.

Antibodies

An antibody to Ufe1p was raised against a bacterially expressed version of the protein lacking the transmembrane domain (truncated at residue 326). A cDNA encoding this was expressed from the vector pMW172 (Way *et al.*, 1990) and protein from purified inclusion bodies (Harlow and Lane, 1988) was further SDS–PAGE-purified and used as antigen to immunize rabbits. The resulting serum was purified on an affinity column containing the antigen. The antibody recognized only a band of appropriate size (sometimes appearing as a doublet) on Western blots of total yeast extracts, and control experiments confirmed that it did not cross-react with Sed5p, the t-SNARE most closely related to Ufe1p.

Antibodies to Bet1p and Sec22p were generously provided by Randy Schekman. The human c-*myc* epitope was detected with the monoclonal antibody 9E10 (Munro and Pelham, 1987). The anti-BiP antibody was a gift from Mark Rose (Rose *et al.*, 1989). Horseradish peroxidase-coupled secondary antibodies were obtained from Sigma.

Immunoprecipitations

Affinity-purified anti-Ufe1p and 9E10 were coupled to protein A–Sepharose under appropriate conditions and cross-linked with dimethyl pimelimidate (Harlow and Lane, 1988). Detergent extracts were prepared from the indicated strains essentially as described by Söllner *et al.*, (1994) using cells which were spheroplasted using 0.1 mg of oxalyticase (Enzogenetics, Corvallis, OR)/300 OD units of yeast cells. Washed spheroplasts were regenerated at either 25°C or 37°C for 1 h, lysed and extracts prepared. Detergent extracts were diluted to 2 mg/ml protein and typically 1 ml of extract was precipitated overnight at 4°C with 25 μl of covalently coupled anti-Ufe1p beads. Immunoprecipitates were washed four times with buffer E (Söllner *et al.*, 1994) followed by buffer E containing increasing concentrations of KCl up to 1 M, and complexes were eluted from the beads with pH 2.6 glycine–HCl. Similar results were obtained by washing precipitates with buffer E alone. TCA-precipitated eluates were analysed by SDS–PAGE, blotted onto nitrocellulose and probed with the indicated antibodies, followed by ECL detection (Amersham International).

BiP secretion blots

The secretion of lumenal ER proteins was analysed by colony blotting. Cells were streaked out thinly onto YPD plates and overlaid with wetted nitrocellulose discs (Schleicher and Schuell 0.45 μm). Following overnight growth at 25°C, filter discs were extensively washed with distilled water and subsequently probed in the same manner as Western blots with an antibody to BiP (Kar2p).

Acknowledgements

We are grateful to Susan Ferro-Novick, Randy Schekman and Dieter Gallwitz for yeast strains and antibodies, and to Ben Nichols for plasmids. We would also like to thank Gabriella Frigerio for communicating her unpublished results on the genetic interactions of *tip20*, *ufe1* and *sec20*. J.R. was supported by the Commonwealth Scholarship Commission in the UK.

References

Aalto,M.K., Ronne,H. and Keränen,S. (1993) Yeast syntaxins Sso1p and Sso2p belong to a family of related membrane proteins that function in vesicular transport. *EMBO J.*, **12**, 4095–4104.

Banfield,D.K., Lewis,M.J. and Pelham,H.R.B. (1995) A SNARE-like protein required for traffic through the Golgi complex. *Nature*, **375**, 806–809.

Barlowe,C., Orci,L., Yeung,T., Hosobuchi,M., Hamamoto,S., Salama,N., Rexach,M.F., Ravazzola,M., Amherdt,M. and Scheckman,R. (1994) COPII: A membrane coat formed by Sec proteins that drive vesicle budding from the endoplasmic reticulum. *Cell*, **77**, 895–907.

Becherer,K.A., Rieder,S.E., Emr,S.E. and Jones,E.W. (1996) Novel syntaxin homologue, Pep12p, required for the sorting of lumenal hydrolases to the lysosome-like vacuole in yeast. *Mol. Biol. Cell*, **7**, 579–594.

Brennwald,P., Kearns,B., Champion,K., Keränen,S., Bankiatis,V. and Novick,P. (1994) Sec9 is a SNAP-25-like component of a yeast SNARE complex that may be the effector of Sec4 function in exocytosis. *Cell*, **79**, 245–258.

Chapman,E.R., An,S., Barton,N. and Jahn,R. (1994) SNAP-25, a t-SNARE which binds to both syntaxin and syaptobrevin via domains that may form coiled coils. *J. Biol. Chem.*, **269**, 27427–27432.

Eakle,K.A., Bernstein,M. and Emr,S.D. (1988) Characterization of a component of the yeast secretion machinery: identification of the *SEC18* gene. *Mol. Cell. Biol.*, **8**, 4098–4109.

Ferro-Novick,S. and Jahn,R. (1994) Vesicle fusion from yeast to man. *Nature*, **370**, 191–193.

Griff,I.C., Schekman,R., Rothman,J.E. and Kaiser,C.A. (1992) The yeast *SEC17* gene product is functionally equivalent to mammalian alpha-SNAP protein. *J. Biol. Chem.*, **267**, 12106–12115.

Harlow,E. and Lane,D. (1988) *Antibodies: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Hardwick,K.G. and Pelham,H.R.B. (1992) SED5 encodes a 39-kD integral membrane protein required for vesicular transport between the ER and the Golgi complex. *J. Cell Biol.*, **119**, 513–521.

Hayashi,T., McMahon,H., Yamasaki,S., Binz,T., Hata,Y., Südhof,T.C. and Niemann,H. (1994) Synaptic vesicle membrane-fusion complex—action of clostridial neurotoxins on assembly. *EMBO J.*, **13**, 5051–5061.

Hayashi,T., Yamasaki,S., Nauenburg,S., Binz,T. and Niemann,H. (1995) Disassembly of the reconstituted synaptic vesicle membrane fusion complex *in vitro*. *EMBO J.*, **14**, 2317–2325.

Jiang,Y., Sacher,M., Singer-Krüger,B., Lian,J.P., Stone,S. and Ferro-Novick,S. (1995) Factors mediating the late stages of ER-to-Golgi transport in yeast. *Cold Spring Harbor Symp. Quant. Biol.*, **60**, 119–126.

Kaiser,C.A. and Schekman,R. (1990). Distinct sets of *SEC* genes govern transport vesicle formation and fusion early in the secretory pathway. *Cell*, **61**, 723–733.

Kee,Y., Lin,R.C., Hsu,S. and Scheller,R.H. (1995) Distinct domains of syntaxin are required for synaptic vesicle fusion complex formation and dissociation. *Neuron*, **14**, 991–998.

Kunkel,T.A., Roberts,J.D. and Zakour,R.A. (1987) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.*, **154**, 367–382.

Letourneur,F., Gaynor,E. C., Hennecke,S., Démollière,C., Duden,R., Emr,S., Riezman,H. and Cosson,P. (1994) Coatomer is essential for retrieval of dilysine-tagged proteins to the endoplasmic reticulum. *Cell*, **79**, 1199–1207.

Lewis,M. and Pelham,H.R.B. (1996) SNARE-mediated retrograde traffic from the Golgi complex to the endoplasmic reticulum. *Cell*, **85**, 205–215.

Lian,J.P. and Ferro-Novick,S. (1993) Bos1p, an integral membrane protein of the endoplasmic reticulum to Golgi tranport vesicles, is required for their fusion competence. *Cell*, **73**, 735–745.

Lian,J.P., Stone,S., Jiang,Y., Lyons,P. and Ferro-Novick,S. (1994) Ypt1p implicated in v-SNARE activation. *Nature*, **372**, 698–701.

McMahon,H.T. and Südhof,T.C. (1995) Synaptic core complex of synaptobrevin, syntaxin, and SNAP25 forms high-affinity alpha-snap binding site. *J. Biol. Chem.*, **270**, 2213–2217.

Munro,S. and Pelham,H.R.B. (1987) A C-terminal signal prevents the secretion of lumenal ER proteins. *Cell*, **48**, 899–907.

Nagahama,M., Orci,L., Ravazzola,M., Amherdt,M., Lacomis,L., Tempst,P., Rothman,J.E. and Söllner,T.H. (1996) A v-SNARE implicated in intra-Golgi transport. *J. Cell Biol.*, **133**, 507–516.

Ossig,R., Dascher,C., Trepte,H., Schmitt,H. and Gallwitz,D. (1991) The yeast SLY gene products, suppressors of defects in the essential GTP-binding Ypt1p protein, may act in endoplasmic reticulum-to-Golgi transport. *Mol. Cell. Biol.*, **11**, 2980–2993.

Pevsner,J., Hsu,S., Braun,J.E.A., Calakos,N., Ting,A.E., Bennett,M.K. and Scheller,R.H. (1994) Specificity and regulation of a synaptic vesicle docking complex. *Neuron*, **13**, 353–361.

Protopopov,V., Govindan,B., Novick.,P. and Gerst,J.E. (1993) Homologs of the synaptobrevin/VAMP family of synaptic vesicle proteins function on the late secretory pathway in *S. cerevisiae*. *Cell*, **74**, 855–861.

Rayner,J. and Pelham,H.R.B. (1997) Transmembrane domain dependent sorting of proteins to the ER and plasma membrane in yeast. *EMBO J.*, **16**, 1832–1841.

Rose,M., Misra,L.M. and Vogel,J.P. (1989) *KAR2*, a karyogamy gene, is the yeast homologue of the mammalian BiP/GRP78 gene. *Cell*, **57**, 1211–1221.

Rothman,J.E. and Wieland,F.T. (1996) Protein sorting by transport vesicles. *Science*, **272**, 227–234.

Semenza,J.C., Hardwick,K.G., Dean,N. and Pelham,H.R.B. (1990) *ERD2*, a yeast gene required for the receptor-mediated retrieval of luminal ER proteins from the secretory pathway. *Cell*, **61**, 1349–1357.

Sikorski,R.S. and Hieter,P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics*, **122**, 19–27.

Søgaard,M., Tani,K., Ye,R.R., Geromanos,S., Tempst,P., Kirchhausen,T., Rothman,J.E. and Söllner,T. (1994) A Rab protein is required for the assembly of SNARE complexes in the docking of transport vesicles. *Cell*, **78**, 937–948.

Söllner,T., Bennett,M.K., Whiteheart,S.W., Scheller,R.H. and Rothman,J.E. (1993a) A protein assembly-disassembly pathway *in vitro* that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell*, **75**, 409–418.

Söllner,T., Whiteheart,S.W., Brunner,M., Erdjument-Bromage,H., Geromanos,S., Tempst,P. and Rothman,J.E. (1993b) SNAP receptors implicated in vesicle targeting and fusion. *Nature*, **362**, 318–324.

Subramaniam,V.N., Peter,F., Philp,R., Wong,S.H. and Hong,W.J. (1996) GS28, a 28-kilodalton Golgi SNARE that participates in ER-Golgi transport. *Science*, **272**, 1161–1163.

Sweet,D.J. and Pelham,H.R.B. (1992) The *Saccharomyces cerevisiae SEC20* gene encodes a membrane glycoprotein which is sorted by the HDEL retrieval system. *EMBO J.*, **11**, 423–432.

Sweet,D.J. and Pelham,H.R.B. (1993) The *TIP1* gene of *Saccharomyces cerevisiae* encodes an 80 kDa cytoplasmic protein that interacts with the cytoplasmic domain of Sec20p. *EMBO J.*, **12**, 2831–2840.

Way,M., Pope,B., Gooch,J., Hawkins,M. and Weeds,A.G. (1990) Identification of a region in segment 1 of gelsolin critical for actin binding. *EMBO J.*, **9**, 4103–4109.

Wuestehube,L.J., Duden,R., Eun,A., Hamamoto,S., Korn,P., Ram,R. and Schekman,R. (1996) New mutants of *Saccharomyces cerevisiae* affected in the transport of proteins from the endoplasmic reticulum to the Golgi complex. *Genetics*, **142**, 393–406.

Wilson,D.W., Wilcox,C.A., Flynn,G.C., Chen,E., Kuang,W.J., Henzel,W. J., Block,M.R., Ullrich,A. and Rothman,J.E. (1989) A fusion protein required for vesicle-mediated transport in both mammalian cells and yeast. *Nature*, **339**, 355–359.

Received on January 6, 1997; revised on February 17, 1997