The EMBO Journal vol.12 no.7 pp.2831 - 2840, 1993

The *TIP1* gene of *Saccharomyces cerevisiae* encodes an 80 kDa cytoplasmic protein that interacts with the cytoplasmic domain of Sec20p

Deborah J.Sweet and Hugh R.B.Pelham[1]

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

[1]Corresponding author

Communicated by H.R.B.Pelham

The *SEC20* gene of *Saccharomyces cerevisiae* encodes a 50 kDa type II integral membrane glycoprotein that is required for endoplasmic reticulum (ER) to Golgi transport. Here, we have used a genetic screen, based on the lethal effect of overexpressing the cytoplasmic domain of Sec20p, to identify a novel cytosolic factor that interacts with *SEC20*. This factor is an 80 kDa cytoplasmic protein encoded by the *TIP1* (*SEC* twenty *i*nteracting *p*rotein) gene. Coimmunoprecipitation and immunofluorescence using Tip1p and Sec20p or its cytoplasmic domain showed that the two proteins physically interact to form a stable complex. Like *SEC20*, *TIP1* is required for ER to Golgi transport and depletion of Tip1p results in accumulation of an extensive network of ER plus small transport vesicles. We therefore propose that Sec20p and Tip1p act together as a functional unit in the ER to Golgi transport step.

Key words: ER to Golgi transport/*Saccharomyces cerevisiae*/*SEC20*/secretion/*TIP1*

Introduction

The secretory pathway of eukaryotic cells is divided into a number of distinct compartments, enabling efficient synthesis and modification of the proteins that pass along it. Traffic between these organelles is thought to be mediated by small transport vesicles (Palade, 1975). Genetic and biochemical analyses have identified a number of the components that are involved in the budding, transfer and fusion of transport vesicles (Rothman and Orci, 1992) and shown that several of them interact to form complex molecular machines.

Transport from the endoplasmic reticulum (ER) to the Golgi apparatus is the first vesicle-mediated step in secretion and in many ways can be considered a paradigm for later parts of the pathway. In *Saccharomyces cerevisiae*, genetic analyses have shown that at least 17 genes are involved in ER to Golgi transport: *SEC12*, *13*, *16*, *17*, *18*, *20*, *21*, *22*, *23*, *SAR1*, *YPT1*, *SLY1*, *BET1*, *BET2*, *BOS1*, *USO1*, *SED5* (Novick *et al.*, 1980; Newman and Ferro-Novick, 1987; Segev *et al.*, 1988; Nakano and Muramatsu, 1989; Nakajima *et al.*, 1991; Ossig *et al.*, 1991; Shim *et al.*, 1991; Hardwick and Pelham, 1992). Analysis of mutant phenotypes has allowed further subdivision of some of these genes into interacting groups that are required either for the formation of transport vesicles (*SEC12*, *13*, *16*, *21*, *23*) or their targeting and fusion with the next compartment (*SEC17*, *18*, *22*, *BOS1*) (Kaiser and Schekman, 1990; Newman *et al.*, 1990; Rexach and Schekman, 1991). Recently, the convergence of genetic and biochemical analyses has led to demonstrations of functional homology between yeast and mammalian proteins and has shown that several of them act in multicomponent complexes. For example, purification of the yeast *SEC21* protein showed that it is found in a 700–800 kDa complex that seems to be the yeast homologue of a mammalian vesicle coat precursor (Hosobuchi *et al.*, 1992), and yeast Sec18p can functionally replace NSF, a mammalian vesicle fusion factor, in an *in vitro* transport assay (Wilson *et al.*, 1989).

We originally became interested in *SEC20* because the *sec20*-1 mutant has a somewhat unusual phenotype, suggesting that Sec20p may have a slightly different role in this part of the pathway from the other *SEC* genes (for discussion see Sweet and Pelham, 1992). *SEC20* encodes a 50 kDa integral membrane glycoprotein that is required for ER to Golgi transport. Sec20p has a type II orientation and its relatively small (91 amino acids) luminal domain terminates in the ER retention signal HDEL. This signal, which is found on a number of soluble ER proteins and one other membrane protein (*SED4*; Hardwick *et al.*, 1992), is used for receptor-mediated retrieval of resident ER proteins if they escape to post-ER compartments (for review see Pelham, 1989, 1990). Although the HDEL sequence is not essential for *SEC20* function, Sec20p does interact with the HDEL retention system, suggesting that it can recycle between ER and Golgi during secretion. However, the precise role of *SEC20* protein in ER to Golgi transport is not yet clear, and genetic and biochemical analyses have so far led to relatively few insights. It seems likely that the N-terminal cytoplasmic domain of Sec20p is important for its

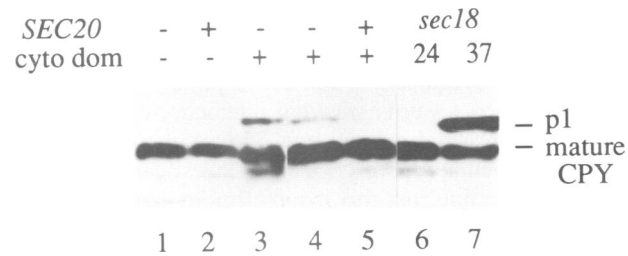

Fig. 1. The *SEC20* cytoplasmic domain causes accumulation of p1 CPY. Total protein extracts from wild type cells (SEY6210) plus cells expressing the *SEC20* cytoplasmic domain and/or intact *SEC20* were immunoblotted and probed with anti-CPY antiserum. Lane 1, protein from wild type cells; lane 2, protein from cells expressing intact *SEC20* from an integrated construct (pSTI20); lane 3, protein from cells expressing the *SEC20* cytoplasmic domain from an equivalent integrated construct (pSTI20T); lanes 4 and 5, the effect of coexpressing additional wild type *SEC20* (pSLM20; lane 5) in comparison with a control plasmid (pZUC13; lane 4) with the *SEC20* cytoplasmic domain (pSTI20T); lanes 6 and 7 show protein extracts from a *sec18* ts mutant strain at the permissive (lane 6) and non-permissive (lane 7) temperature. The *sec18* mutant causes a block in ER to Golgi transport and accumulation of the p1 form of CPY. Each track contains protein from ~0.5 OD_{600} equivalents of cells.

Table I. Lethal effect of the *SEC20* cytoplasmic domain

Cotransformation	Growth of colonies
Control L/control U	+++
Control L/STM20T	−
SLM20/STM20T	+
STLM20/STM20T	++

Plasmids

STM20T	*TPI*-driven *SEC20* cytoplasmic domain, 2μ, *URA3*
SLM20	Genomic *SEC20* insert, 2μ, *LEU2*
STLM20	*TPI*-driven *SEC20*, 2μ, *LEU2*
Control L	pZUC13, *LEU2* vector
Control U	pYCP86, *URA3* vector

Wild type cells (SEY6210) were cotransformed with plasmids expressing the *SEC20* cytoplasmic domain or wild type *SEC20*, as indicated in Table I. Growth was measured by examining colony size 2 and 3 days after transformation.

secretory function, as the amino acid changes in all three temperature-sensitive (ts) alleles map to this part of the protein (Sweet and Pelham, 1992). Also, high level expression of a truncated form of *SEC20* in which the luminal domain has been deleted can rescue the ts growth of a *sec20*-1 mutant strain (D.Sweet and H.R.B.Pelham, unpublished observations). Therefore, in an attempt to learn more about the role of Sec20p in ER to Golgi transport, we decided to look for additional components of the secretion machinery that interact with this cytoplasmic domain.

In this paper, we describe the cloning and characterization of the *TIP1* gene, which encodes an 80 kDa cytoplasmic protein that interacts with Sec20p. *TIP1* (*SEC twenty interacting protein*) was isolated using an unusual genetic screen based on rescue of the lethal effect of overexpressing the *SEC20* cytoplasmic domain in wild type cells. We show that *TIP1* protein interacts with both intact Sec20p and the truncated cytoplasmic domain and that, like Sec20p, it is required for ER to Golgi transport. We discuss our findings in relation to the possible role of these two proteins in vesicular traffic.

Results

The SEC20 cytoplasmic domain is toxic

As a first step towards studying interactions between the *SEC20* cytoplasmic domain and other components of the secretion machinery, we decided to investigate the effects of overexpressing just this cytoplasmic domain without a membrane anchor. We constructed a truncated form of *SEC20* by inserting a stop codon immediately before the predicted transmembrane region (at amino acid 275). This gene was expressed under the control of the constitutive *TPI* (triose phosphate isomerase) promoter either by integration at the *URA3* locus or from a multicopy (2μ-based) plasmid.

In wild type cells (SEY6210), expression of the *SEC20* cytoplasmic domain from an integrated construct (pSTI20T) resulted in accumulation of the ER (p1) form of the vacuolar protease carboxypeptidase Y (CPY) (see Figure 1 lane 3). This suggests that when present at relatively low levels, the cytoplasmic domain causes a reduction in the rate of ER to Golgi transport. Expression from a 2μ plasmid (pSTM20T) appeared to be extremely toxic as no viable transformants were obtained (see Table I). Effects such as these could

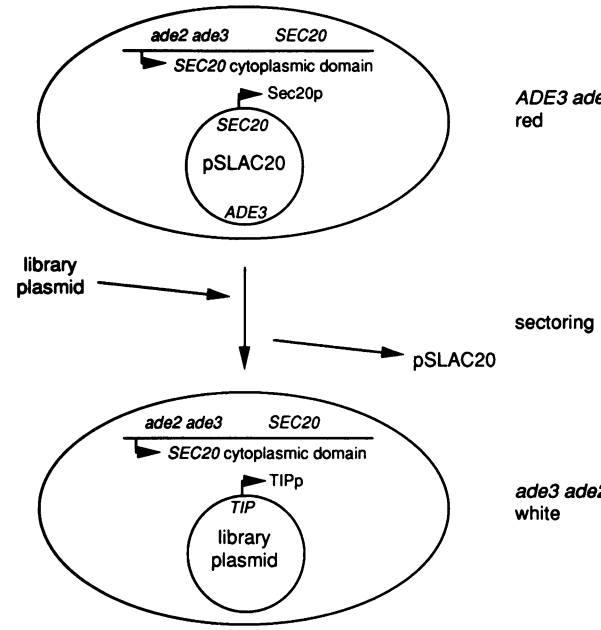

Fig. 2. Schematic diagram of the sectoring assay screen. The parent strain, DSYSC1, is red in colour due to maintenance of the plasmid pSLAC20. Transformation with a library plasmid containing a *TIP* gene capable of rescuing cytoplasmic domain lethality permits loss of pSLAC20 and therefore results in sectoring.

either be the result of a specific block in secretion by the cytoplasmic domain or simply an indirect consequence of overexpressing a mutant protein. However, all the observed effects, i.e. the inhibition of ER to Golgi transport and the lethality, could be rescued by cotransformation with additional copies of the wild type *SEC20* gene (see Figure 1, lanes 4 and 5, and Table I), and the efficiency of rescue depended on its level of expression (Table I). From this, it seems likely that the cytoplasmic domain specifically inhibits wild type Sec20p function. The cytoplasmic domain constructs were not capable of suppressing the ts growth of a *sec20*-1 mutant and in fact the toxic effects were even more pronounced in this strain: neither the integration construct nor the multicopy plasmid gave viable transformants (at 24°C) in the absence of additional wild type *SEC20*. This ts mutant therefore appears to be more sensitive than wild type to interference by the cytoplasmic domain.

The most obvious interpretation of these results is that the cytoplasmic domain competes with intact *SEC20* protein for a factor (or factors) that interacts with it during ER to Golgi transport. This factor could in principle be cytoplasmic or associated with the ER, Golgi or transport vesicle membrane. We therefore chose to use a genetic screen to look for such *SEC20*-interacting proteins.

Screen for interacting proteins

As described above, the *SEC20* cytoplasmic domain is lethal to wild type cells when expressed at a sufficiently high level, but this can be rescued by the presence of additional wild type *SEC20* protein. If the cytoplasmic domain is competing with Sec20p for an interacting factor, overexpression of that factor should also be capable of rescuing the lethal effect. We therefore decided to design a genetic screen aiming to identify the interacting factor using such rescue. Initial attempts to generate a conditionally lethal strain by expressing the cytoplasmic domain or intact *SEC20* under

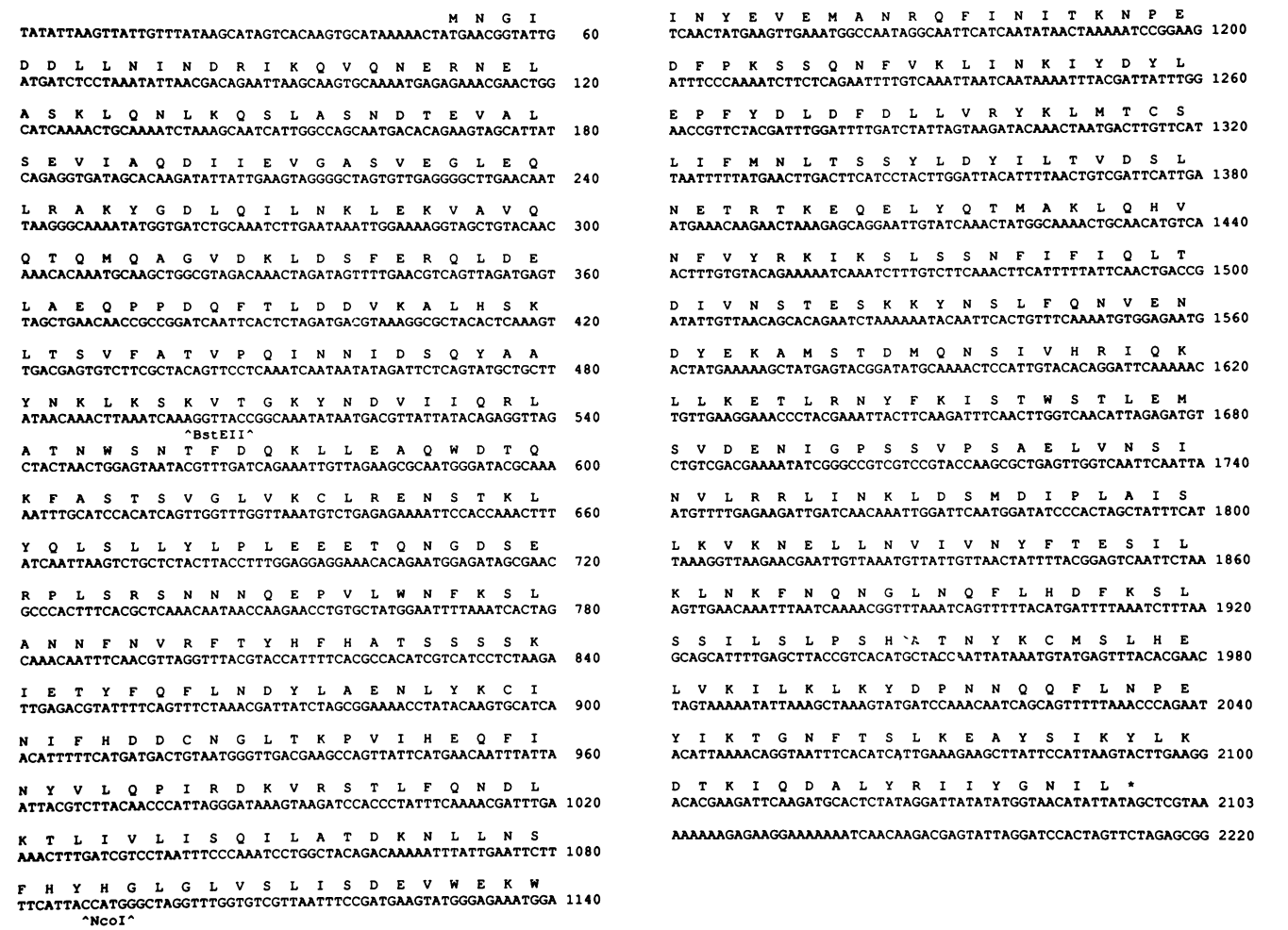

Fig. 3. Nucleotide and predicted protein sequence of the *TIP1* gene. Disruption of the marked *Bst*EII or *Nco*I site abolishes *TIP1* activity. This sequence has been submitted to the EMBL database (accession number X72699).

the control of the inducible *GAL1* promoter, or using cytoplasmic domain constructs derived from ts *sec20* alleles, were unsuccessful, so we changed to a slightly less direct strategy involving plasmid shuffling.

The method eventually used to search for *TIP* genes was based on an *ADE2/ADE3* sectoring assay (Koshland *et al.*, 1985). We used this sectoring assay to screen for genes that could functionally replace *SEC20* in rescuing lethality due to the cytoplasmic domain. The strain constructed for use in the screen (DSYSC1) contained a *TPI*-driven *SEC20* cytoplasmic domain construct (pSTI20T), integrated at the *URA3* locus, plus additional wild type *SEC20* on a centromere plasmid (pSLAC20), which also contained the *ADE3* gene. This strain was generated by cotransformation, in an *ade2 ade3* background (strain CH1304) and from the resulting transformants individual colonies were selected that could not lose the wild type *SEC20* plasmid, presumably because they had integrated sufficient copies of the cytoplasmic domain construct to otherwise be lethal. Maintenance of the plasmid resulted in an *ade2 ADE3* genotype for the strain, so the cells accumulated a red pigment (the product of Ade3p) and colonies appeared red on plates. If this strain was transformed with another *SEC20*-containing plasmid, which lacked the *ADE3* gene, the resulting transformants were able to lose the *ADE3–SEC20* plasmid, producing white sectors in the otherwise red colonies. This colour change formed the basis of the genetic screen, as transformation with any gene capable of rescuing cytoplasmic domain lethality should give a similar sectoring effect (see Figure 2). Potentially rescued transformants could therefore be identified by visual examination of colonies.

The strain DSYSC1 was transformed with a yeast genomic library on a *HIS3* multicopy plasmid (pHUC13; Hardwick *et al.*, 1992) and transformants were streaked onto rich plates to test for sectoring. Ten thousand transformants were screened, which with this library should represent at least three genomes, and from these 224 apparently sectoring colonies were selected for further analysis. These were tested on rich plates again to verify their sectoring phenotype and then white sectored colonies were tested on appropriate selective plates to ensure that they retained the cytoplasmic domain construct (*URA3*) and lacked the *ADE3–SEC20* plasmid (*LEU2*). Seventy-nine of the selected transformants fitted these criteria and were analysed in more detail. Immunoblotting of total protein extracts, using anti-Sec20p antibodies, showed that six of these transformants were expressing far lower levels of the cytoplasmic domain than the parent strain, so they were discarded. Plasmid DNA was prepared from the remaining candidates and used to transform *E.coli*. At least five bacterial colonies were analysed from all but one of the 73 yeast transformants. We

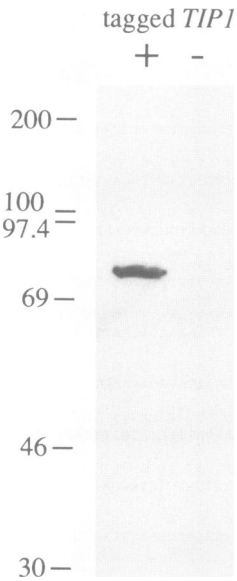

Fig. 4. Detection of tagged Tip1p by immunoblotting. Total protein extracts from wild type (SEY6210) cells and cells transformed with a plasmid containing c-myc-tagged *TIP1* (SEY6210 + pPTM2) were immunoblotted with the monoclonal antibody 9E10, which specifically recognizes the epitope tag. Each track contains protein from ~0.75 OD_{600} equivalents of cells. The gel mobilities of molecular weight markers are indicated at the side.

recovered plasmids from 14 of the yeast transformants that, by restriction mapping, seemed to originate from the yeast genomic library, and these fell into four main categories. Class 1 plasmids (isolated from eight yeast transformants) contained an ~7.1 kb insert and were capable of producing sectoring in the parent strain (DSYSC1). Class 2 plasmids (from two yeast transformants) contained a 4.5 kb insert related to the class 1 insert by restriction mapping but these were not capable of producing sectoring. Class 3 plasmids (from two yeast transformants) contained inserts that were related to each other, but not to class 1 or 2, and class 4 (from two yeast transformants) did not give yeast colonies on retransformation. PCR analysis showed that the class 3 plasmids both contain the *SEC20* gene itself but class 1 and 2 plasmids do not. From the design of the screen we expected to isolate the *SEC20* gene, which acts as an internal control for the method. Having isolated what appeared to be a very similar (by restriction mapping) plasmid from eight separate yeast transformants, and the *SEC20* gene twice, we considered that we had covered the library adequately and did not continue to screen plasmids from the other yeast transformants. The novel rescuing gene, found in the class 1 plasmids, was named *TIP1* and characterized as described below.

Sequencing and genetic analysis of TIP1

Subcloning of the 7.1 kb insert of one of the class 1 plasmids (pIVd2) resulted in identification of a 3.9 kb *Bam*HI–*Sac*I fragment that encoded the rescuing activity. Sequence analysis of this fragment revealed a 2103 bp open reading frame whose DNA and predicted protein sequences are shown in Figure 3. Disruption of either the *Nco*I or the *Bst*EII site within this ORF abolished the rescuing activity of the fragment. The sequence predicts an 81 kDa protein and database searches revealed no significant homology to other known genes. There are no potential membrane spanning regions and no obvious signal peptide, so on the basis of sequence data Tip1p is predicted to be a cytoplasmic protein. The codon bias index (CBI) of the *TIP1* gene is 0.09, which is the same value as obtained for *SEC20*. Codon bias is quite a reliable indicator of protein abundance in yeast (Bennetzen and Hall, 1982; Sharp and Cowe, 1991): scores range from 0 to 1 with 1 indicating exclusive use of preferred codons, so a score of 0.09 suggests that the protein is relatively rare. For comparison, scores for transcription factors are normally in the 0–0.05 range while the BiP gene has a CBI of 0.58. Sec20p does indeed appear to be a rare protein (Sweet and Pelham, 1992) and it is likely that the same is also true for Tip1p.

Disruption of *TIP1* was performed using a two-step strategy (see Materials and methods) to generate a diploid strain with one wild type and one deleted copy of the *TIP1* gene. The *TIP1*/Δ*tip1* diploid was identified by Southern blotting. Upon sporulation of this strain, nine out of 10 analysed tetrads gave a 2:2 viable:non-viable pattern, suggesting that *TIP1* is an essential gene. To verify this, sporulation was also carried out on the diploid strain transformed with a *TIP1* plasmid containing a *URA3* marker (pPTM1). Haploid spores were isolated that could not grow on *URA3* counterselective plates containing 5-fluoorotic acid (Boeke *et al.*, 1984) unless they had previously been transformed with another *TIP1* plasmid (*HIS3* marker, pHSB). From this, we concluded that *TIP1* is an essential gene.

Tip1p interacts with Sec20p

To allow detection of Tip1p, we attached a c-myc epitope tag at the C-terminus of the protein, which is recognized by the monoclonal antibody 9E10 (Evan *et al.*, 1985; Munro and Pelham, 1986). Addition of this tag did not appear to interfere with Tip1p function as the tagged protein was still able to rescue lethality due to the *SEC20* cytoplasmic domain. Western blotting of total protein extracts from cells expressing the tagged construct (Figure 4) revealed a single 9E10-reactive band of ~80 kDa that was not present in wild type cells. This confirms the predicted size of Tip1p and shows that epitope tagging allows specific recognition of the protein.

Initially, the subcellular location of Sec20p was examined using double label immunofluorescence on a wild type strain expressing c-myc-tagged *SEC20*. The cells were probed with 9E10 to detect the Sec20p and anti-BiP to stain the ER (see Figure 5A). In these cells, the Sec20p colocalized with BiP, indicating that it is mainly found in the ER. To compare the localization of Sec20p and Tip1p, we performed similar double label immunofluorescence on cells containing wild type Sec20p and tagged Tip1p. Sec20p was stained using affinity purified rabbit anti-Sec20p antiserum and Tip1p with 9E10. To facilitate detection, both genes were expressed under *TPI* promoter control on multicopy (2μ-based) plasmids. The copy number of such plasmids varies between different cells within the population, so individual cells can contain high levels of one or the other or both of the overexpressed proteins. This strain therefore allowed us to examine the localization of each protein individually and to look at any interactions that occur when they are both present at high levels in the same cell. In cells where Tip1p is

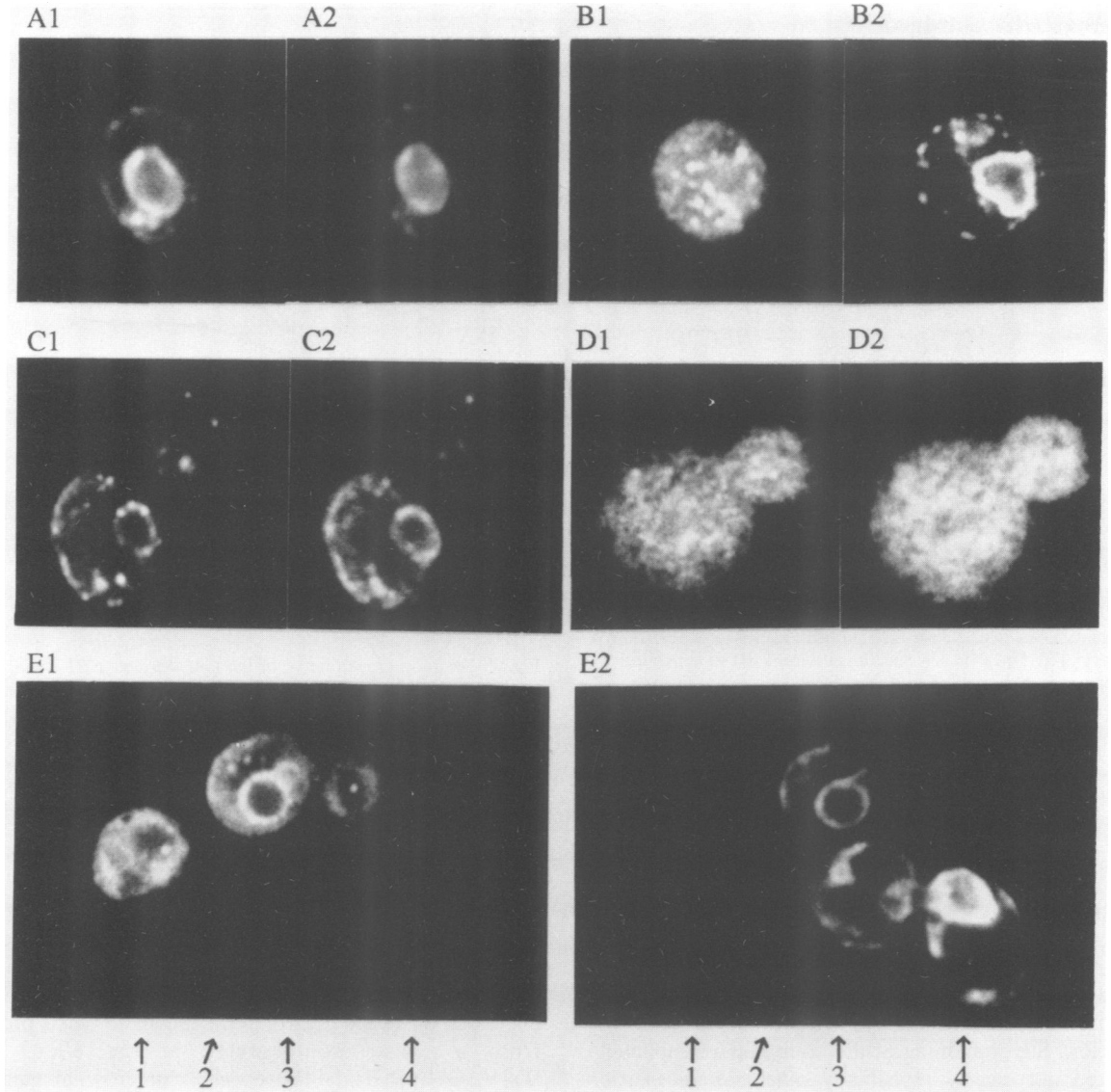

Fig. 5. Immunolocalization of Sec20p and Tip1p. This shows wild type cells (SEY6210) expressing tagged Sec20p, tagged Tip1p, untagged Sec20p and the *SEC20* cytoplasmic domain from 2μ plasmids, either singly or in combination. The cells were double labelled using 9E10 (mouse monoclonal) to detect the tagged proteins plus either rabbit anti-Sec20p antiserum to detect Sec20p and its cytoplasmic domain or rabbit anti-BiP antiserum to look at the endogenous ER marker BiP. (**A**) Cell expressing tagged Sec20p (pSTM22) stained with (1) 9E10 and (2) anti-BiP; (**B**) cell expressing tagged Tip1p (pPTM2) stained with (1) 9E10 and (2) anti-BiP; (**C**) cell expressing tagged Tip1p (pPTM2) and wild type Sec20p (pSTLM20) stained with (1) 9E10 and (2) anti-Sec20p; (**D**) cell expressing tagged Tip1p and the *SEC20* cytoplasmic domain stained with (1) 9E10 and (2) anti-Sec20p; (**E**) field of cells stained with (1) 9E10 and (2) anti-Sec20p expressing tagged Tip1p (cell 1), Sec20p (cells 3 and 4) and both proteins together (cell 2).

abundant but Sec20p is rare, the Tip1p (Figure 5B) shows diffuse cytoplasmic staining and does not colocalize with BiP, consistent with the prediction that Tip1p is a cytoplasmic protein. However, in cells where both Tip1p and Sec20p are present at high levels, the Tip1p is no longer cytoplasmic but instead becomes colocalized with the Sec20p to the ER (Figure 5C). Figure 5E shows a field of cells from the cotransformed strain where all three types of expression pattern are visible i.e. high levels of Sec20p (cells 3 and 4), Tip1p (cell 1) and both (cell 2). ER localization of overexpressed Tip1p was entirely dependent on the presence of high levels of Sec20p and incomplete ER localization was observed in cells with less *SEC20* protein. Therefore, it seems that the two proteins do interact *in vivo* and Tip1p can be attached to the ER by association with Sec20p.

Bearing in mind the genetic screen used to isolate *TIP1*, it seemed likely that *TIP1* protein would also interact with the *SEC20* cytoplasmic domain. Immunofluorescence on cells overexpressing Tip1p and the cytoplasmic domain (Figure 5D) showed that both proteins are cytoplasmic and therefore could form a soluble complex. To study the interaction more directly, we used coimmunoprecipitation of *TIP1* protein and the *SEC20* cytoplasmic domain. Tip1p was immunoprecipitated, using the 9E10 antibody, from cytoplasmic extracts of cells overexpressing c-myc-tagged *TIP1* protein and the *SEC20* cytoplasmic domain. The resulting immunoprecipitate and supernatant fractions were tested by immunoblotting to look at the distribution of the two proteins. To control for non-specific binding to the 9E10−Sepharose beads, the experiment was performed in parallel on a corresponding strain in which the overexpressed *TIP1* protein was not myc-tagged. The results of these

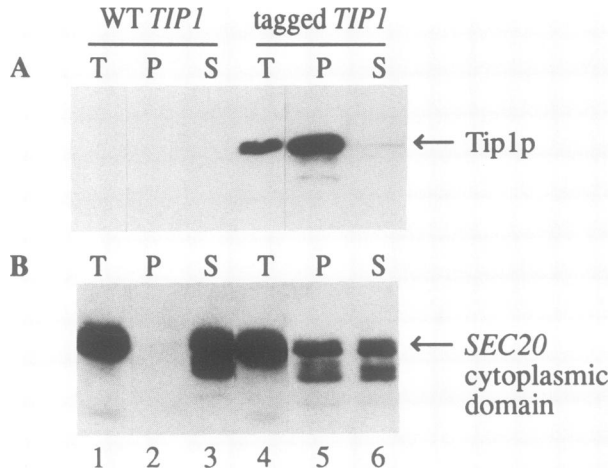

Fig. 6. Coimmunoprecipitation of Tip1p and the *SEC20* cytoplasmic domain. This immunoblot shows total protein samples (T), and the antibody bound (P) and unbound (S) fractions from 9E10 immunoprecipitation of extracts from wild type cells (SEY6210) expressing the *SEC20* cytoplasmic domain (pSTLM20T) and tagged or wild type *TIP1* (pPTM2/pPTM1). After transfer, the blot was cut into top and bottom halves which were then probed either with 9E10 (**A**) to detect the tagged Tip1p or anti-Sec20p antiserum (**B**) to detect the *SEC20* cytoplasmic domain. Tracks 1 and 4 contain protein from ~0.75 OD_{600} equivalents of cells, and track 2, 3, 5 and 6 contain immunoprecipitate and supernatant from 2 OD_{600} equivalents of cell lysate.

immunoprecipitations are shown in Figure 6. The myc-tagged Tip1p was efficiently immunoprecipitated (lane 5) and very little of the protein remained in the supernatant (lane 6). In these samples, ~50% of the *SEC20* cytoplasmic domain was also immunoprecipitated (lanes 5 and 6), whereas in control samples with untagged Tip1p it remained in the supernatant (lanes 2 and 3). This specific immunoprecipitation of the *SEC20* cytoplasmic domain with tagged Tip1p provides further evidence that the two proteins are present in a complex. Silver staining of the coimmunoprecipitated material did not clearly reveal any other stoichiometric components of the overexpressed Sec20p−Tip1p complex (data not shown), but this does not rule out the possibility that less abundant proteins can interact with it. It is interesting that the *SEC20* cytoplasmic domain and its major proteolytic product appear to run as a closely spaced doublet (see lanes 3, 5 and 6), which is not apparent from the heavier loaded total protein sample (lanes 1 and 4). This could be a result of partial proteolysis, but may reflect a post-translational modification, such as phosphorylation, of Sec20p on its cytoplasmic domain which could be involved in regulating its activity.

Both the immunofluorescence and immunoprecipitation data indicate that *TIP1* protein interacts with the cytoplasmic domain of Sec20p, whether it is soluble in the cytoplasm or attached to the ER membrane. These experiments therefore provide a biochemical confirmation of the genetic interaction originally used to identify *TIP1* and suggest that Sec20p and Tip1p are closely associated in wild type cells.

TIP1 is required for ER to Golgi transport

Previous experiments have shown that *SEC20* is required for ER to Golgi transport (Novick *et al.*, 1980; Sweet and Pelham, 1992) and lack of *SEC20* function causes build up of ER membranes and clusters of vesicles (Kaiser and Schekman, 1990; Sweet and Pelham, 1992). If *TIP1* has a

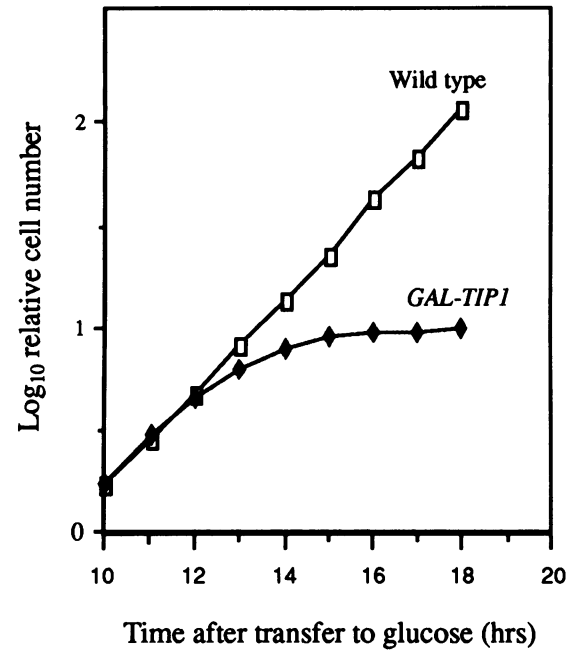

Fig. 7. Semi-log growth curve of the *GAL1*-driven *TIP1* strain. Cells [strains SEY6210 and DSYΔ*tip1*(pPGI1)] were grown to log phase in galactose-containing medium then diluted to an OD_{660} of 0.001 in glucose-containing medium and grown for 10 h, by which time they had reached an OD_{660} of ~0.15. The cultures were adjusted to ensure their densities were equal, then growth monitored by measuring the OD_{660} at hourly intervals. This reading was used to calculate the relative cell number, which was then plotted on a semi-log scale. 1:2 dilutions were made as necessary to maintain log phase growth.

close functional interaction with *SEC20*, one would predict that lack of Tip1p would have similar effects on the secretory pathway. To investigate this, we constructed a strain in which the chromosomal copy of *TIP1* was deleted and *TIP1* instead expressed under the control of the inducible *GAL1* promoter from a construct integrated at the *URA3* locus [DSYΔ*tip1*(pPGI1)]. These cells can grow in galactose-containing medium, but in glucose the *GAL1* promoter is repressed and they cease to grow due to a lack of *TIP1* protein. Figure 7 shows a growth curve of this strain after transfer to glucose medium, compared with a wild type control strain grown under the same conditions. Growth of the *GAL−TIP1* strain slowed down after a lag of ~11 h following medium transfer and had completely stopped after 15 h. This profile is similar to that observed for an equivalent *GAL−SEC20* strain (Sweet and Pelham, 1992), where the cells first showed slower growth 8 h after transfer and ceased to grow 4 h later. The 3 h time difference between the growth arrest of these strains represents ~1−2 additional divisions for the *GAL−TIP1* cells.

To look at the effects of depletion of Tip1p on the secretory pathway, protein samples were taken over this time course and immunoblotted with antibodies to the vacuolar protease carboxypeptidase Y (CPY) (see Figure 8). As the growth of the cells slowed, they showed progressive accumulation of the p1 (ER) form of CPY, suggesting that lack of *TIP1* protein causes a block in ER to Golgi transport. To allow more detailed investigation of the effects on intracellular membrane organization, cells were also prepared for thin section TEM, using potassium permanganate fixation to accentuate membrane structures (Riemersma, 1970). Figure 9 shows cells from the *GAL−TIP1* strain at 5 (A)

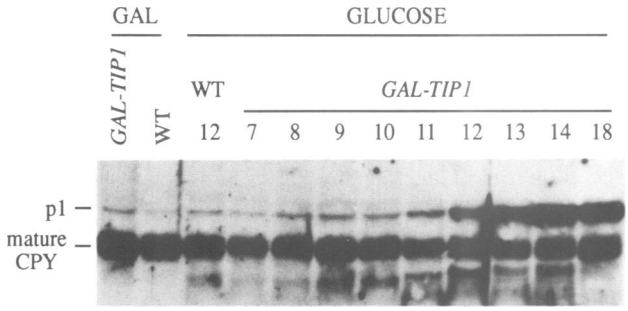

Fig. 8. Depletion of Tip1p results in accumulation of p1 CPY. Total protein extracts from wild type (SEY6210) and *GAL−TIP1* [DSYΔ*tip1*(pPGI1)] yeast strains grown in galactose medium or after transfer to glucose medium were immunoblotted with anti-CPY antiserum. The numbers above the glucose medium samples indicate the time (in h) after medium transfer at which the sample was taken. Each track contains protein from ~0.5 OD_{600} equivalents of cells.

and 13 (B) h after switching to glucose medium, compared with a wild type control (C). At 13 h after transfer, when the growth of the culture had slowed significantly (see Figure 7), the *GAL−TIP1* cells showed extensive accumulation of ER membranes and some small poorly staining vesicles (see Figure 9, panel Bi), which were commonly found between the ER and the plasma membrane. Their phenotype was strikingly similar to that observed for cells lacking *SEC20* function, either from inactivation of a ts allele (Kaiser and Schekman, 1990) or in an equivalent glucose repression experiment (Sweet and Pelham, 1992). This close correspondence between the effects of lack of Tip1p and Sec20p provides further evidence that the two proteins are involved in the same step of ER to Golgi transport.

Discussion

In this paper, we have shown that *S.cerevisiae SEC20* protein physically interacts with the product of the *TIP1* gene. *TIP1* is a novel gene encoding an 80 kDa cytoplasmic protein, which like Sec20p is required for ER to Golgi transport.

Interaction with *TIP1* protein is mediated by the cytoplasmic domain of Sec20p and can occur both when it is membrane-associated and when the cytoplasmic domain is expressed in a soluble form. Several lines of evidence suggest that the interaction between Tip1p and Sec20p is a stable one and the two proteins normally exist as a complex. Perhaps the strongest of these is that the association between Tip1p and the soluble *SEC20* cytoplasmic domain is stable during immunoprecipitation, which would be expected to dissociate any loosely interacting components. The clear shift in steady state localization of overexpressed Tip1p from the cytoplasm to the ER when the expression level of Sec20p is increased, demonstrates that the interaction between Tip1p and intact Sec20p is also stably maintained *in vivo*. In addition, the toxic effects of the *SEC20* cytoplasmic domain and success of the screen suggest that the interaction is stable, as the strong dependence on expression level and recessive nature of the inhibition are most consistent with sequestration of an essential factor by the overexpressed cytoplasmic domain. Sec20p and Tip1p therefore appear to be an example of two components of the secretion machinery that physically interact to form a stable complex. Biochemical experiments on other proteins involved in secretion have shown that this

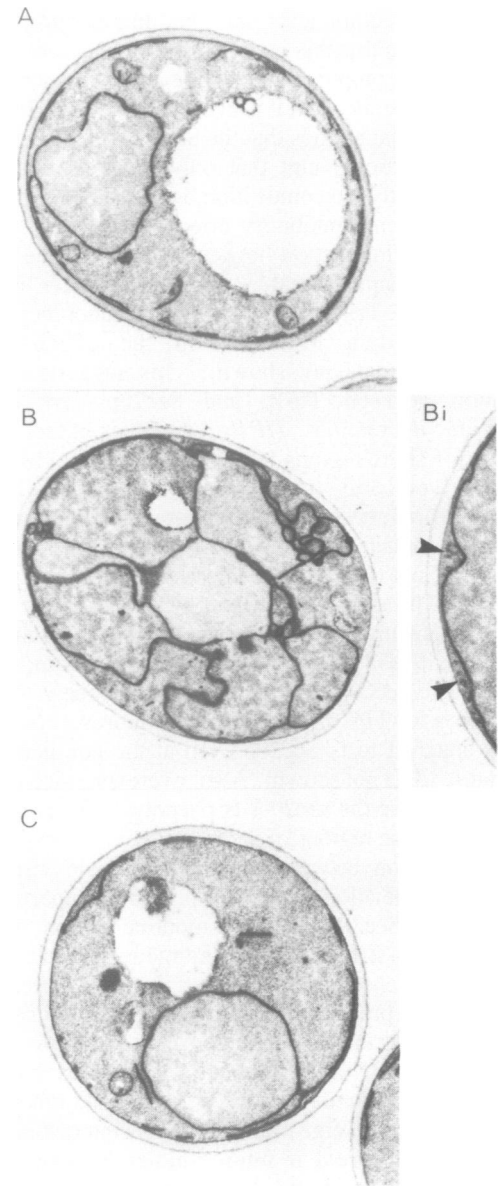

Fig. 9. Thin section electron micrographs of Tip1p-depleted cells. (**A**) *GAL−TIP1* [strain DSYΔ*tip1*(pPGI1)] at 5 h after transfer to glucose medium; (**B**) *GAL−TIP1* at 13 h after transfer to glucose; (**C**) wild type (strain SEY6210) grown in glucose medium. The total width of the main panels is 4.3 μm (A,B) or 3.3μm (C) and the inset in panel B (Bi) is at 1.6× the magnification of the main picture.

is not uncommon, for example Sec23p is an 84 kDa peripheral ER membrane protein found in association with a 105 kDa protein (Hicke *et al.*, 1992) and Sec8p and Sec15p form part of a 19.5S particle that is required for fusion of secretory vesicles with the plasma membrane (Bowser *et al.*, 1992).

In contrast to the toxicity of the *SEC20* cytoplasmic domain, high level expression of the Tip1p−cytoplasmic domain complex, which clearly cannot perform a wild type secretory function, does not seem to have detrimental effects. This soluble complex does not therefore appear to titrate out another limiting component of the secretion machinery. It is possible that the Sec20p−Tip1p complex does contain additional proteins, but these are present in excess in wild

type cells so their sequestration does not become growth limiting. However, the apparent lack of additional stoichiometric components in the immunoprecipitated material suggests that this is not the case. Alternatively, the Sec20p−Tip1p complex may only be able to associate with its other components when it is membrane bound. However, the simplest explanation is that the Sec20p−Tip1p complex is a stable functional unit that only interacts with other proteins transiently, so competition by the soluble form does not have significant inhibitory effects.

Although the interaction between Tip1p and the *SEC20* cytoplasmic domain was stable in phosphate buffer, addition of detergent (1% Triton X-100) to the immunoprecipitation reaction caused a reduction in the efficiency of coprecipitation (data not shown). This suggests that the association between Tip1p and Sec20p may involve hydrophobic interactions. *TIP1* protein does in fact contain a number of short regions that could potentially assume a coiled coil type configuration (e.g. amino acids 141−169) and thus form such a hydrophobic interaction. The cytoplasmic domain of Sec20p also contains a potential coiled coil region, close to the transmembrane domain (amino acids 231−266). The amino acid change in the *sec20*-1 mutant (L234 to S) is within this region and the *sec20*-2/3 mutation is close to it (G216 to R) (Sweet and Pelham, 1992). However, the *sec20*-1 mutation does not appear to affect interaction with Tip1p as, by immunofluorescence, Tip1p remains attached to ts Sec20p even at the non-permissive temperature (data not shown). Also, overexpression of *TIP1* does not suppress the *sec20*-1 ts phenotype. Thus it seems unlikely that these regions of the two proteins form the main site of interaction between them. They could instead be involved in association with other proteins or, particularly in the case of Sec20p, allow homodimerization. Further studies using site-directed mutagenesis and either immunofluorescence or immunoprecipitation, could be used to identify more precisely the domains of Sec20p and Tip1p that are involved in the interaction between them.

Previous genetic and biochemical analyses have not identified any interactions between *SEC20* and other genes involved in ER to Golgi transport. Such interactions may become more apparent if future studies involve treating *SEC20* and *TIP1* proteins as a functional unit and, for example, examine the effect of overexpressing both simultaneously. Clearly interaction between Tip1p and membrane bound Sec20p is essential for transport, and cytoplasmic sequestration of Tip1p can block secretion. However, it seems unlikely that the sole function of Sec20p is to anchor Tip1p to the membrane, as the *sec20*-1 ts mutation, which renders the protein non-functional in ER to Golgi transport, does not seem to strongly affect interaction between the two proteins (see above). The ts mutation does nevertheless appear to have a subtle effect, as a *sec20*-1 mutant strain is more sensitive to transport inhibition by the cytoplasmic domain than wild type cells. This is probably due to the ts Sec20p−Tip1p complex acting in a subfunctional way and therefore being required at a higher level than one containing the wild type protein. Further studies on Sec20p and Tip1p will undoubtedly reveal interactions with other components of the secretion machinery, although many of the relevant proteins may not have been identified yet.

The codon bias indices of *SEC20* and *TIP1* predict that they both encode relatively rare proteins of approximately equal abundance. Bearing in mind the stability of their interaction and assuming that their expression levels are indeed comparable, it is likely that the majority of cellular Tip1p and Sec20p exists in the form of a heteromeric complex. The presence of an HDEL signal on Sec20p suggests that it can recycle between ER and Golgi during secretion (Sweet and Pelham, 1992). Immunolocalization of overexpressed protein showed that, like many soluble HDEL proteins, it is mainly found in the ER. Unfortunately, the wild type levels of Sec20p are too low to detect by immunofluorescence, but cells expressing intermediate levels of Sec20p showed no evidence of a shift in its distribution. It therefore seems likely that the normal location of the Tip1p is as a peripheral ER membrane protein associated with Sec20p.

If the usual location of Sec20p and Tip1p is indeed in the ER, one might predict that the role of these proteins in transport would be in the budding of vesicles that are destined for the Golgi complex. However, the phenotypic effects of depletion of Sec20p or Tip1p and of the *sec20*-1 ts mutation are not entirely consistent with such a role. The *sec20*-1 mutant seems to have a slightly incomplete block in transport and accumulates ER and some vesicles, which are in a characteristic location, at the non-permissive temperature (for discussion see Sweet and Pelham, 1992). Similar membrane organization is observed in cells depleted of Tip1p or Sec20p (Sweet and Pelham, 1992; this study). The phenotypic effects of lack of *SEC20* or *TIP1* function are in fact more consistent with a partial defect in vesicle targeting or fusion. If so, how might this function be carried out? The presence of a functional HDEL signal on Sec20p suggests that during secretion it leaves the ER at a sufficient rate to merit retrieval and thus recycles between ER and Golgi. Sec20p and Tip1p may therefore be examples of cycling vesicle components required for fusion whose steady state localization is nevertheless in the ER. This is not unprecedented as Bos1p, a 27 kDa membrane protein that is also required for ER to Golgi transport (Newman *et al.*, 1990), has been shown by fractionation to be mainly associated with the ER, yet is also present in the transport vesicles (Newman *et al.*, 1992) and is required for vesicle consumption (Shim *et al.*, 1991). Alternatively, Sec20p and Tip1p may perform their function in the ER and, for example, be required for recruitment of additional factors to vesicles as they bud to make them competent for fusion. It remains to be seen whether Sec20p and/or Tip1p are actually present at significant levels in ER to Golgi vesicles or, more importantly, whether their activity in these vesicles is necessary for fusion to take place. Recent advances in biochemical assays for transport in yeast (Baker *et al.*, 1990; Rexach and Schekman, 1991) mean that, using anti-Sec20p or anti-Tip1p antibodies, it may be possible to investigate these questions and test the various models directly.

Materials and methods

Yeast strains

The wild type strains used for assays and disruption were SEY6210 (*MATα ura3*-52 *his3*Δ200 *leu2*-3,112 *trp*-Δ901 *lys2*-801 *suc2*-Δ9) and SEY6211 (*MATa ura3*-52 *his3*Δ200 *leu2*-3,112 *trp*-Δ901 *ade2*-101 *suc2*-Δ9) (both gifts from S.D.Emr); the strain used for making the parent strain in the screen, DSYSC1, was CH1304 (*MATα ade2 ade3 his3 leu2 ura3*−52 *gal2*) (a gift from C.Holm); the *sec20* strain was RSY275 (*MATα ura3*-52 *his4*-619 *sec20*-1) and the *sec18* strain was RSY271 (*MATα ura3*-52 *his4*-619 *sec18*-1) (gifts from C.Kaiser). The *tip1* chromosomal deletion strains were

collectively known as DSY*Δtip1*(p) where p is a *TIP1* plasmid - this strain was derived from a SEY6210/6211 diploid by sporulation.

Plasmids

The *SEC20* cytoplasmic domain construct was generated by oligonucleotide-directed mutagenesis of the *SEC20* gene in Bluescript KS− (Bluescript 1, Stratagene). The truncated *SEC20* (0.8 kb) was transferred to the expression vectors pJS209 (2μ, *URA3*, *TPI* promoter; Semenza *et al.*, 1990) and pHP209 (integration, *URA3*, *TPI* promoter) to create plasmids pSTM20T (2μ) and pSTI20T (integration) respectively. Plasmid pSTI20 contains the wild type *SEC20* gene in an equivalent construct to pSTI20T. Plasmids pSLM20 and pSTLM20 contain the *SEC20* gene under the control of its endogenous promoter (pSLM20) or the *TPI* promoter (pSTLM20) in the vector pZUC13 (*LEU2*, 2μ). The *SEC20* plasmid pSTM22 has been described previously (Sweet and Pelham, 1992). The *ADE3* plasmid used in the genetic screen, pSLAC20, was constructed by inserting a 3.7 kb fragment containing the *ADE3* gene (a gift from K.Hardwick) into plasmid pRSL201 (*SEC20* genomic fragment, *LEU2*, *CEN6*; Sweet and Pelham, 1992). pIVd2 is a yeast genomic library plasmid in the vector pHUC13 (*HIS3*, 2μ; Hardwick *et al.*, 1992). It contains a 7.1 kb insert that includes the *TIP1* gene. A 3.9 kb fragment spanning the *TIP1* gene was subcloned from pIVd2 into Bluescript KS− and pHUC13 to create pKSSB and pHSB respectively. *TIP1* was subcloned for insertion into expression vectors using PCR amplification of the gene, either with or without addition of a C-terminal myc tag sequence. The PCR products were first cloned into Bluescript KS− and sequenced to ensure they did not contain PCR errors, then transferred to pJS209 (2μ, *URA3*, *TPI* promoter) for expression. pPTM2 contains tagged *TIP1*, which has the C-terminal amino acid sequence (added residues are in italics, epitope tag in bold type) ...LYRIIYGNIL***SMEQKLISEEDLN***, and pPTM1 contains untagged *TIP1*. The untagged *TIP1* gene was also cloned into pHP208 (integration, *URA3*, *GAL1* promoter) to form pPGI1.

Gene disruption

This was carried out using a two-step strategy. A disrupted form of the *TIP1* gene was generated using a 1.2 kb *Sna*BI−*Spe*I internal deletion, which renders the gene non-functional in rescuing *SEC20* cytoplasmic domain lethality. The resulting 2.7 kb *Δtip1* fragment was cloned into the *URA3*-marker vector pRS306 (Sikorski and Hieter, 1989) then linearized with *Xba*I and integrated at the *TIP1* locus of the wild type strain SEY6210. The resulting strain was crossed with SEY6211 then the integrated *URA3* was looped out by contraselection with 5-fluoorotic acid (FOA) (Boeke *et al.*, 1984). FOA-resistant colonies were tested by Southern blotting to identify those that retained the deleted copy of *tip1*. A *TIP1*/*Δtip1* diploid was then sporulated and tetrads analysed for spore viability. This diploid strain was also transformed with the plasmid pPTM1 and sporulated, then haploids with deleted chromosomal *tip1* were selected by their inability to grow in the presence of FOA unless transformed with another *TIP1* plasmid (pHSB).

DNA sequence analysis

The 3.9 kb DNA fragment described was transferred to Bluescript KS− to form pKSSB, and sequencing performed on this and smaller fragments derived from it. Nested deletion series were created using the *Exo*III−*Exo*VII method (Yanisch-Perron *et al.*, 1985). Dideoxy sequencing (Sequenase kit, United States Biochemical Corp.) was used to create 3.2 kb of continuous sequence containing the 2103 bp open reading frame. The sequence was confirmed on the complementary strand using oligonucleotide primers.

Immunoblotting

Total yeast protein samples were prepared using either lyticase lysis (see Sweet and Pelham, 1992) or, for detection of CPY, by resuspending cells at 5 OD_{600} equivalents/100 μl in SDS sample buffer, vortexing with Ballotini No. 8 glass beads for 5 min at 4°C then boiling for 2 min. Proteins were transferred to nitrocellulose (0.45 μm, Schleicher and Schuell) using a TE70 Semi-Phor semi-dry blotting unit (Hoefer) according to the manufacturer's instructions. Antibody incubations were carried out in PBS + 2% dried milk, with 2 mM NaN_3 for overnight incubations and 0.2% Tween-20 for the initial blocking. 9E10 (2.68 mg/ml) was diluted to 1/2000, anti-CPY antiserum to 1/7000 and anti-Sec20p antiserum (this study) to 1/750 and incubated overnight. Immunoreactive bands were detected using peroxidase conjugated anti-rabbit (Sigma, 1/3000) and anti-mouse (Dakopatts, 1/1000) secondary antibodies, incubated for 2 h, followed by chemiluminescence (ECL kit, Amersham) and autoradiography.

Anti-Sec20p antibodies

Rabbits were immunized with bacterially expressed *SEC20* cytoplasmic domain. The truncated *SEC20* gene was inserted into the T7 expression vector pSKPT7 (a gift from M.Wehrli) and expression was induced with 0.6 mM IPTG (isopropyl β-D-thiogalactopyranoside). The protein was eluted from an acrylamide gel by rolling fragments overnight at 4°C in elution buffer (50 mM Tris−HCl pH 7.9, 0.1 mM EDTA, 1 mM DTT, 0.1 M NaCl and 0.1% SDS) and concentrated by precipitation with 4 vol of acetone, then resuspended in a small volume of elution buffer. Typical yields were ~1 mg of purified protein from a 500 ml culture, which was resuspended in 500 μl elution buffer. For each immunization, 300 μg of this protein were emulsified with Freund's adjuvant (complete adjuvant was used for the initial immunization then incomplete adjuvant for subsequent boosts) in a total volume of 1 ml. Boosts were carried out at 6 week intervals after the primary immunization.

Affinity purification of anti-Sec20p antiserum was carried out on a 1 ml Affi-Gel (Bio-Rad) column coupled to bacterially expressed glutathione-S-transferase−*SEC20* fusion protein. This construct was generated by cloning the *SEC20* cytoplasmic domain into the expression vector pGEX-3X (Pharmacia) as an in-frame fusion to glutathione-S-transferase. Expression of this protein was also induced using IPTG (1 mM) then it was extracted by digesting the bacterial cell wall with lysozyme (10 mg/ml lysozyme in 100 mM Tris−HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol and 1 mM DTT) and lysing the cells by sonication in PBS + 1% Triton X-100 + 1 mM PMSF + 2 mM benzamidine. These relatively stringent conditions were necessary as expression of the fusion protein made the bacterial cells resistant to lysis by sonication. The protein was purified by incubation with glutathione−agarose (Sigma) and then eluted from these beads in 5 mM reduced glutathione, 50 mM Tris pH 8, 1 mM PMSF and 2 mM benzamidine. Typical yields were ~1 mg protein per litre of cells. The purified protein was dialysed against PBS at 4°C for 10 h then a total of 2.4 mg of protein was coupled to the Affi-Gel according to the manufacturer's instructions. Antibody purification on this column was carried out as described by Harlow and Lane (1988).

Immunofluorescence

Cells were fixed and mounted on slides as described by Hardwick and Pelham (1992). Antibody incubations were carried out in PBS + 2% dried milk, with primary antibodies incubated overnight at 4°C and secondary antibodies for 2 h at room temperature. Anti-BiP antiserum was used at 1/30000, 9E10 antibody (2.68 mg/ml) at 1/200 and affinity purified anti-Sec20p antiserum (this study) at 1/10. Secondary antibodies (Texas Red and Fluorescein conjugated, Amersham) were used at 1:50. Images were obtained using an MRC-600 confocal laser scanning microscope (Bio-Rad). When images were obtained with simultaneous excitation of both fluorophores some bleed through of signal from the FITC channel was observed in the Texas Red channel with brightly stained cells, so for all cells shown here the dual images were obtained by separate excitation at the two appropriate wavelengths.

Immunoprecipitation

Log phase cells were incubated at 20 OD_{600} equivalents/ml in DTT solution (10 mM DTT, 100 mM Tris pH 9.4, 10 mM NaN_3) for 10 min at 30°C then resuspended at 20 OD_{600} equivalents/ml in spheroplasting buffer (1.4 M sorbitol, 60 mM β-mercaptoethanol, 2 mM $MgCl_2$, 25 mM Tris pH 7.4 and 10 mM NaN_3) plus lyticase at 25 U/OD equivalent of cells and incubated at 30°C for 30 min to digest the cell wall. The resulting spheroplasts were then osmotically lysed by resuspension (by vortexing) at 10 OD_{600} equivalents/400 μl in PBS + 0.1% BSA + protease inhibitors (1 mM PMSF, 2 mM benzamidine, 3 μg/ml pepstatin and 3 μg/ml leupeptin). The cell debris was removed by centrifugation (10 min in a microfuge at 4°C) and 380 μl of the supernatant were removed to a fresh tube. 40 μl of a 1:1 suspension of 9E10-coupled protein A−Sepharose beads in PBS [antibody was attached to the beads at high salt (3 M NaCl) then crosslinked to them using 20 mM dimethylpimelimidate (Sigma) at pH 9] were added to this supernatant and the mixture was rolled for 60 min at 4°C. After this incubation, proteins remaining in the supernatant were precipitated by adding TCA to 10%, incubating on ice for 30 min, washing the resulting pellet in acetone, resuspending in SDS sample buffer and boiling for 2 min. The beads were washed three times in PBS + protease inhibitors then the bound proteins eluted by boiling for 2 min in SDS sample buffer. Samples were analysed by SDS gel electrophoresis and immunoblotting (see above).

Electron microscopy

Samples were prepared by potassium permanganate fixation as described previously (Sweet and Pelham, 1992).

Acknowledgements

We are very grateful to Douglas Kershaw for his assistance with sectioning and staining samples for electron microscopy and to Theresa Langford and Gareth King of the LMB animal facility for injection and bleeding of rabbits. The anti-CPY antiserum was a gift from Neta Dean, the 9E10 from Sean

Munro, the anti-BiP antiserum from Joe Vogel and Mark Rose and the 9E10-coupled protein A–Sepharose beads from Rowan Chapman. We also thank Mark Bretscher, Mike Lewis and Duncan Wilson for comments on the manuscript.

References

Baker,D., Wuestehube,L., Schekman,R., Botstein,D. and Segev,N. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 355–359.
Bennetzen,J.L. and Hall,B.D. (1982) *J. Biol. Chem.*, **257**, 3026–3031.
Boeke,J.D., Lacroute,F. and Fink,G.R. (1984) *Mol. Gen. Genet.*, **197**, 345–346.
Bowser,R., Muller,H., Govindan,B. and Novick,P. (1992) *J. Cell Biol.*, **118**, 1041–1056.
Evan,G.I., Lewis,G.K., Ramsay,G. and Bishop,J.M. (1985) *Mol. Cell. Biol.*, **5**, 3610–3616.
Hardwick,K.G. and Pelham,H.R.B. (1992) *J. Cell Biol.*, **119**, 513–521.
Hardwick,K.G., Boothroyd,J.C., Rudner,A.D. and Pelham,H.R.B. (1992) *EMBO J.*, **11**, 4187–4195.
Harlow,E. and Lane,D. (1988) *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Hicke,L., Yoshihisa,T. and Schekman,R. (1992) *Mol. Biol. Cell*, **3**, 667–676.
Hosobuchi,M., Kreis,T. and Schekman,R. (1992) *Nature*, **360**, 603–605.
Kaiser,C. and Schekman,R. (1990) *Cell*, **61**, 723–733.
Koshland,D., Kent,J.C. and Hartwell,L.H. (1985) *Cell*, **40**, 393–403.
Munro,S. and Pelham,H.R.B. (1986) *Cell*, **46**, 291–300.
Nakajima,H., Hirata,A., Ogawa,Y., Yonehara,T., Yoda,K., and Yamasaki, M. (1991) *J. Cell Biol.*, **113**, 245–260.
Nakano,A. and Muramatsu,M. (1989) *J. Cell Biol.*, **109**, 2677–2691.
Newman,A.P. and Ferro-Novick,S. (1987) *J. Cell Biol.*, **105**, 1587–1594.
Newman,A.P., Shim,J. and Ferro-Novick,S. (1990) *Mol. Cell. Biol.*, **10**, 3405–3414.
Newman,A.P, Groesch,M.E. and Ferro-Novick,S. (1992) *EMBO J.*, **11**, 3609–3617.
Novick,P., Field,C. and Schekman,R. (1980) *Cell*, **21**, 205–317.
Ossig,R., Dascher,C., Trepte,H.-H., Schmitt,H.D. and Gallwitz,D. (1991) *Mol. Cell. Biol.*, **11**, 2980–2993.
Palade,G. (1975) *Science*, **189**, 347–358.
Pelham,H.R.B. (1989) *Annu. Rev. Cell Biol.*, **5**, 1–23.
Pelham,H.R.B. (1990) *Trends Biochem. Sci.*, **15**, 483–486.
Rexach,M. and Schekman,R. (1991) *J. Cell Biol.*,**114**, 219–229.
Riemersma,J.C. (1970) In Parson,D.F. (ed.), *Some Biological Techniques in Electron Microscopy*. Academic Press, New York, pp. 69–100.
Rothman,J.E. and Orci,L. (1992) *Nature*, **355**, 409–415.
Segev,N., Mulholland,J. and Botstein,D. (1988) *Cell*, **52**, 915–924.
Semenza,J.C., Hardwick,K.G., Dean,N. and Pelham,H.R.B. (1990) *Cell*, **61**, 1349–1357.
Sharp,P.M. and Cowe,E. (1991) *Yeast*, **7**, 657–678.
Shim,J., Newman,A.P. and Ferro-Novick,S. (1991) *J. Cell Biol.*, **113**, 55–64.
Sikorski,R.S. and Hieter,P. (1989) *Genetics*, **122**, 19–27.
Sweet,D.J. and Pelham,H.R.B. (1992) *EMBO J.*, **11**, 423–432.
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) *Nature*, **339**, 355–359.
Yanisch-Perron,C., Vieira,J. and Messing,J. (1985) *Gene*, **33**, 103–119.

Received on February 11, 1993; revised on March 26, 1993