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# Homotypic vacuole fusion requires Sec17p (yeast α-SNAP) and Sec18p (yeast NSF)

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**In *Saccharomyces cerevisiae*, vacuoles are inherited by the formation of tubular and vesicular structures from the mother vacuole, the directed projection of these structures into the bud and the homotypic fusion of these vesicles. We have previously exploited a cell-free inheritance assay to show that the fusion step of vacuole inheritance requires cytosol, ATP and the GTPase Ypt7p. Here we demonstrate, using affinity-purified antibodies and purified recombinant proteins, a requirement for Sec17p (yeast α-SNAP) and Sec18p (yeast NSF) in homotypic vacuole fusion *in vitro*. Thus, Sec17p and Sec18p, which are typically involved in heterotypic transport steps, can also be involved in homotypic organelle fusion. We further show that vacuole-to-vacuole fusion is stimulated by certain fatty acyl-coenzyme A compounds in a Sec18p-dependent fashion. Finally, our data suggest the presence of a cytosolic factor which activates vacuole membrane-bound Sec18p.**

*Keywords*: membrane fusion/NSF/organelle inheritance/SNAP/vacuole

## Introduction

Homotypic (vacuole-to-) vacuole fusion allows *Saccharomyces cerevisiae* (hereafter referred to as yeast) to maintain vacuoles as low copy-number organelles. Cytological and genetic studies (Weisman *et al.*, 1987; Weisman and Wickner, 1988, 1990; Klionsky *et al.*, 1990; Gomes de Mesquita *et al.*, 1991; Raymond *et al.*, 1992; Shaw and Wickner, 1992; Wada *et al.*, 1992; Jones *et al.*, 1993) have shown that the bud inherits vacuole material from the mother cell via tubular and/or vesicular 'segregation' structures. Segregation structure formation is coordinated by the cell cycle (occurring between S phase and $G_2$/M phase transition) and is spatially restricted to a narrow axis between mother cell and bud. The last discernible step of vacuole inheritance is the fusion of vacuoles in the daughter cells, giving rise to one or a few new vacuoles per cell.

Recently, we have reconstituted segregation structure formation and vacuole fusion *in vitro*. These reactions require cytosol, ATP, a moderate salt concentration (Conradt *et al.*, 1992, 1994; Haas *et al.*, 1994) and the *YPT7* gene product (a *ras*-like GTPase; Haas *et al.*, 1995). *Ras*-like GTPases regulate intracellular fusion events (Nuoffer and Balch, 1994) but most likely are not fusion proteins *per se*. It has been hypothesized that the *N*-ethylmaleimide-sensitive fusion protein NSF (Block *et al.*, 1988; Beckers *et al.*, 1989; Whiteheart and Kubalek, 1995) couples ATP consumption to lipid-bilayer fusion in mammals (Malhotra *et al.*, 1988; Wilson *et al.*, 1992; Söllner *et al.*, 1993). NSF is involved in several fusion events such as ER-to-Golgi transport (Beckers *et al.*, 1989), transport of vesicles between successive Golgi compartments (Balch *et al.*, 1984), endosome-to-endosome fusion (Diaz *et al.*, 1989), neurotransmission (Söllner *et al.*, 1993) and transcytosis (Sztul *et al.*, 1993). NSF is a homotrimeric ATPase (Sumida *et al.*, 1994; Whiteheart *et al.*, 1994) whose ATPase activity is required for membrane fusion *in vitro* (Rothman, 1994; Whiteheart *et al.*, 1994). Model studies have shown that ATP hydrolysis dissociates detergent-solubilized 'fusion complexes' comprised of NSF, SNAP (soluble NSF attachment protein) and membrane-bound SNAP receptors (SNAREs; Söllner *et al.*, 1993). Recent data, however, question the role of NSF as a component of the actual fusion machinery (reviewed in Morgan and Burgoyne, 1995; Mayer *et al.*, 1996).

Yeast Sec18p and NSF have 48% sequence identity and perform the same function (Wilson *et al.*, 1989), as Sec18p is also involved in steps along the biosynthetic transport pathway (Kaiser and Schekman, 1990; Graham and Emr, 1991). Loss of Sec18p function is lethal (Eakle *et al.*, 1988; Kaiser and Schekman, 1990). Cytosol from Sec18p-overexpressing yeast cells can substitute for NSF in a mammalian cell-free assay reconstituting transport from the ER to the Golgi complex (Wilson *et al.*, 1989), establishing a close functional relationship. However, it has not yet been shown that purified Sec18p can support membrane fusion *in vitro*.

α-SNAP, a 35 kDa mammalian protein, or its homologues, β-SNAP or γ-SNAP, are also absolutely required in several fusion events, probably to provide a membrane anchor for NSF (Weidman *et al.*, 1989; Clary *et al.*, 1990; Whiteheart *et al.*, 1992) and to stimulate ATP hydrolysis at one of the two ATPase sites of NSF (Morgan *et al.*, 1994). As for NSF, a yeast homolog of α-SNAP was described, the *SEC17* gene product (Clary *et al.*, 1990). Sec17p, the 34 kDa yeast homolog of α-SNAP, is 34% identical to α-SNAP. Sec17p can substitute with low efficiency for α-SNAP in mammalian *in vitro* transport assays (Clary *et al.*, 1990; Griff *et al.*, 1992). Sec17p is required for membrane binding of Sec18p *in vitro* (Griff *et al.*, 1992) and loss of Sec17p function is lethal to the cell (Novick *et al.*, 1980; Kaiser and Schekman, 1990). Both *sec17* and *sec18* mutant cells accumulate small (transport) vesicles at the restrictive temperature (Novick *et al.*, 1980; Rexach and Schekman, 1991), suggesting that they may be required for a step which precedes vesicle docking.

Despite the general role of NSF and α-SNAP in heterotypic fusion events of the secretory pathway, recent

studies (Latterich and Schekman, 1994; Acharya *et al.*, 1995a,b; Latterich *et al.*, 1995; Rabouille *et al.*, 1995) have suggested that intracellular homotypic fusion events may require neither NSF, SNAP nor Rab/Ypt proteins. Even some heterotypic fusion events may require factors different from NSF and SNAP (Goda and Pfeffer, 1991; Ikonen *et al.*, 1995). Our data, obtained *in vitro* with affinity-purified antibodies and purified recombinant proteins, clearly establish that homotypic vacuole fusion requires Sec17p and Sec18p and is stimulated by acyl-coenzyme A (acyl-CoA), a factor which also supports heterotypic intra-Golgi fusion (Glick and Rothman, 1987). We have previously shown that fusion is dependent on Ypt7p (Haas *et al.*, 1995) and on Vac1p and Vac2p (Weisman and Wickner, 1991; Shaw and Wickner, 1992). Homotypic fusion can therefore, in principle, involve Sec17p and Sec18p as well as a Rab/Ypt-GTPase. The Sec17p and Sec18p in our reactions are vesicle-bound, and Sec18p can be activated by a novel cytosolic factor.

## Results

### *The in vitro fusion reaction*

Vacuole-to-vacuole fusion *in vitro* is measured by mixing vacuoles isolated from two different yeast strains, one with a deletion in the *PHO8* gene, encoding the vacuolar membrane alkaline phosphatase, and the other with a deletion in the *PEP4* gene, coding for the vacuolar lumenal proteinase A. Neither vacuole population has alkaline phosphatase activity, as Pho8p is transported into the vacuole as an enzymatically inactive precursor, proPho8p, which only becomes enzymatically active upon specific cleavage by proteinase A. Alkaline phosphatase activity can only be generated by the mixing of the lumenal contents of both vacuole types and hence is a measure of frequency of vacuole fusion *in vitro* (Haas *et al.*, 1994). As fusion reactions are defective when components of the *in vitro* assay are derived from vacuole inheritance mutants (Conradt *et al.*, 1992; Haas *et al.*, 1994, 1995; T.Nicolson, B.Conradt and W.Wickner, unpublished), they represent physiologically authentic fusion reactions.

### *Affinity-purified antibodies against Sec17p or Sec18p inhibit homotypic vacuole fusion in vitro*

All secretion mutants, including *sec17-1* and *sec18-1*, show immediate arrest of bud growth and of cell cycle progression upon shift to a non-permissive temperature, while expression of the '*vac*' phenotype of defective vacuole inheritance requires bud growth and progression in the cell cycle. Thus the potential role of Sec17p and Sec18p in vacuole inheritance cannot be tested *in vivo* by simply shifting thermosensitive strains to a non-permissive temperature. We therefore tested the requirement for Sec17p and Sec18p in vacuole fusion using our *in vitro* reaction. Polyclonal rabbit antibodies were raised against recombinant, oligohistidine-tagged Sec18p or against $His_6$-tagged Sec17p. In immunoblot analysis, our affinity-purified antibodies to Sec17p detected a protein of $M_r$ 34 000 in either vacuole or cytosol preparations (Figure 1C) whereas affinity-purified anti-Sec18p detected a protein of $M_r$ 84 000 in vacuoles and cytosol as well as a few other cytosolic proteins (Figure 1C). Both Sec17p and Sec18p are enriched in our vacuole fractions (100 μg cytosolic protein or 50 μg vacuolar protein per lane was used; Figure 1C). Both affinity-purified Sec17p and Sec18p antibodies inhibited fusion (Figure 1A and B). When low concentrations of recombinant antigens (Figure 2B) were preincubated with the antibodies, inhibition of fusion was reduced or abolished (Figure 1A and B). We have also raised antibodies to a Sec17p–protein A hybrid, and have affinity purified these on a nitrocellulose strip containing a denatured β-galactosidase–Sec17p hybrid protein (Griff *et al.*, 1992). These purified antibodies also inhibited vacuole fusion (unpublished data). In addition, a prepara-

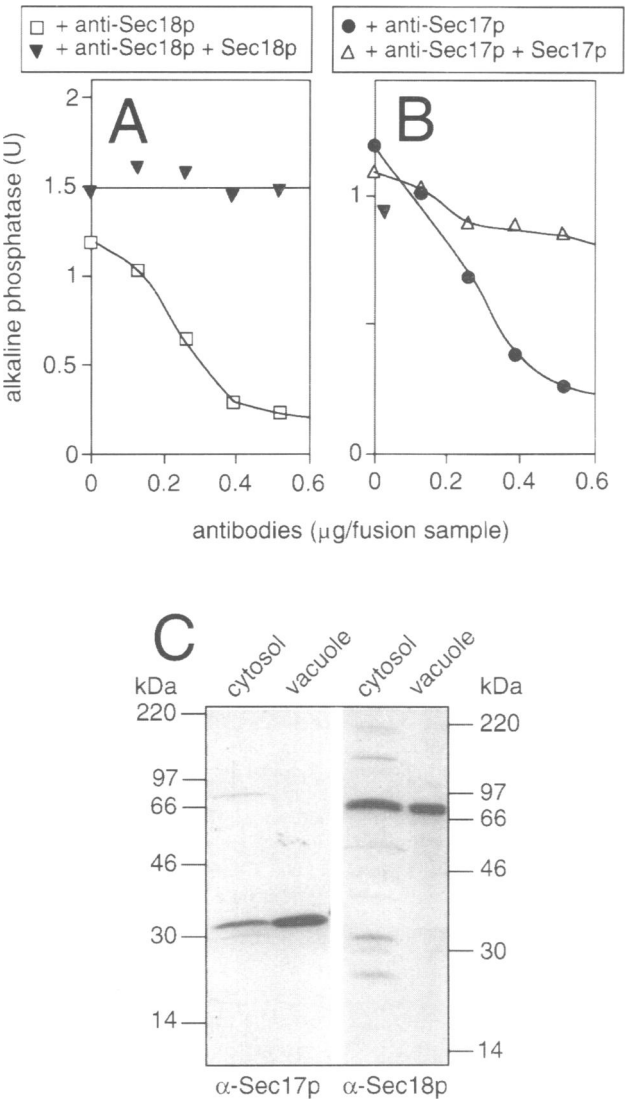


**Fig. 1.** Affinity-purified antibodies to Sec17p or Sec18p inhibit vacuole fusion. (**A**) Antibodies to $His_6$-Sec18p were affinity purified and mixed at the indicated concentrations with vacuoles. In parallel, the same amounts of antibody were preincubated on ice for 10 min with 0.14 μg purified $His_6$-Sec18p, fusion reaction buffer and cytosol (35 μg/reaction), followed by addition of vacuoles and a second incubation on ice for 5 min. Fusion reactions were started by adding the ATP-regenerating system and transferring the samples to 25°C. (**B**) As in (A), except that the affinity-purified antibodies were to $His_6$-Sec17p. $His_6$-Sec17p was added at 0.04 μg/fusion sample. (**C**) Immunoblot analysis of affinity-purified antibodies to Sec17p and Sec18p. Purified vacuoles (50 μg protein) or cytosol from strain K91-1A (100 μg protein) were immunodecorated with affinity purified antibodies to Sec18p or Sec17p. The positions of molecular mass markers (×1000; rainbow markers, Bio-Rad) are indicated.

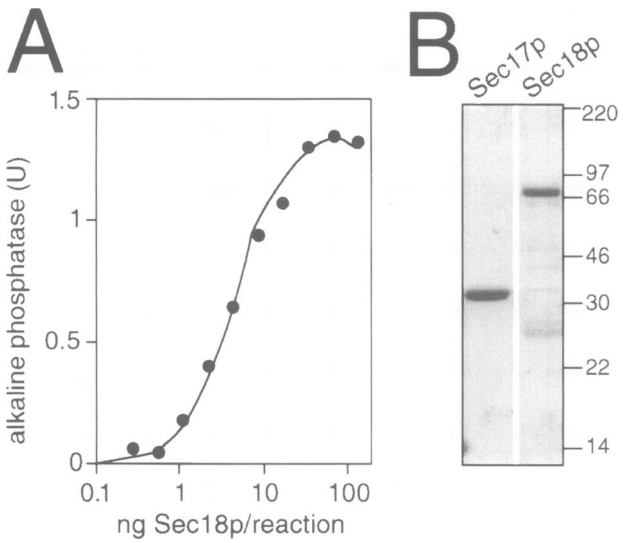


**Fig. 2.** Vacuole fusion is supported by $His_6$-Sec18p. (**A**) Vacuoles were preincubated with affinity-purified anti-Sec18p IgG (0.7 μg/5 μg vacuole protein) in reaction buffer for 10 min at 30°C, reisolated by centrifugation, and resuspended in fresh reaction buffer without antibodies. Serial dilutions of $His_6$-Sec18p were prepared in reaction buffer containing 1 mM DTT and 1 mM ATP and added to standard fusion reactions without cytosol (containing the preincubated vacuoles). After 90 min at 25°C, alkaline phosphatase activities were determined. Fusion-independent background alkaline phosphatase activity (0.111 U) was subtracted from all samples. (**B**) Coomassie-stained SDS–15% polyacrylamide gel showing $His_6$-Sec17p (5 μg) and $His_6$-Sec18p (2.5 μg), purified on nickel-NTA-agarose columns and desalted by gel chromatography. The preparations shown are identical to those used in the fusion experiments. The minor proteins seen in the Sec18p preparation are degradation products of Sec18p, as demonstrated by immunoblotting (not shown).

tion of Sec17p antibodies from C.Kaiser (MIT) that had been shown to inhibit ER-to-Golgi transport (Griff *et al.*, 1992), but not homotypic ER-fusion *in vitro* (Latterich and Schekman, 1994), completely inhibited homotypic vacuole fusion at approximately the same concentrations needed for ER-to-Golgi transport inhibition (not shown). Monovalent Fab fragments produced from total anti-Sec18p IgG inhibited the fusion reaction by 90% at a concentration of 0.3 μg per standard fusion reaction (Figure 4B and not shown), demonstrating that cross-linking of vacuolar Sec18p by antibodies does not play a major role in fusion inhibition.

Another homotypic fusion event in yeast, ER-to-ER fusion, is not dependent on Sec18p, but on its relative Cdc48p (Latterich *et al.*, 1995). Therefore, we have tested an IgG fraction prepared from a polyclonal Cdc48p antiserum (Fröhlich *et al.*, 1991) in the vacuole fusion assay. No effect was observed at a concentration of 2.5 μg IgG per reaction (not shown), a concentration which was sufficient for maximal inhibition of homotypic ER fusion (Latterich *et al.*, 1995; the same antiserum was used in both studies). This finding suggests that homotypic vacuole fusion is dependent on Sec17p/Sec18p but not on Cdc48p.

### *Purified recombinant Sec18p stimulates homotypic vacuole fusion in vitro*

As Sec18p antibodies inhibited vacuole fusion, we asked whether purified recombinant hexahistidine-tagged Sec18p can promote vacuole fusion. Freshly harvested vacuoles may often fuse in the absence of cytosol (see Figure 6C, black bar, 'ATP') since they have bound Sec17p and Sec18p and presumably also possess other necessary factors. As vacuolar ATPases are inhibited by very low concentrations of *N*-ethylmaleimide (NEM; Forgac, 1989; Klionsky *et al.*, 1990) and as VATPase activity is required in our reactions (Haas *et al.*, 1994), we could not use this reagent to inactivate membrane-bound Sec18p, an approach which has been used successfully in mammalian cell-free systems (Glick and Rothman, 1987). Instead, vacuoles were preincubated with anti-Sec18p, reisolated by centrifugation, resuspended in fresh reaction buffer (without antibodies) and used in the fusion assay. This pretreatment renders the vacuoles completely dependent upon the addition of soluble components. When $His_6$-Sec18p was used in fusion experiments with anti-Sec18p-preincubated vacuoles, fusion was Sec18p dependent (Figure 2A) and occurred as efficiently as with added cytosol (Figure 6A). It would be formally possible that this 'activation' of fusion was in fact a 're-activation' of vacuoles via the removal of bound Sec18p antibodies by added antigen. We can exclude this possibility, as (i) Sec18p also stimulates fusion of vacuoles that have not been pretreated with anti-Sec18p (Figure 6C), (ii) low concentrations of NEM-inactivated Sec18p did not support fusion of vacuoles preincubated with anti-Sec18p (Figure 3A), and (iii) Sec18p heated at 95°C did not support fusion (not shown).

To investigate whether the Sec18p-driven fusion follows the same pathway as fusion supported by cytosol, we have tested several pharmacological reagents which had been demonstrated to effectively inhibit cytosol-driven vacuole fusion (Figure 3B; Conradt *et al.*, 1994; Haas *et al.*, 1994, 1995). Neomycin (which binds to polyphosphoinositides), mastoparan (activates heterotrimeric G proteins), Gdi1p (complexes membrane bound Ypt-GTPases), anti-Ypt7p (inhibits Ypt7p) or apyrase (hydrolyses ATP) completely inhibited fusion driven by Sec18p or cytosol (Figure 3B). Therefore, fusion via purified Sec18p needs ATP and is biochemically indistinguishable from the fusion reaction driven by cytosol.

### *Acyl-coenzyme A and CoA stimulate vacuole fusion*

Glick and Rothman (1987) found that coenzyme A and some of its acyl-derivatives stimulate NSF-mediated fusion of Golgi transport vesicles with their acceptor compartment *in vitro*. Later studies demonstrated that the addition of acyl-CoA stimulated both fusion (Pfanner *et al.*, 1990) and budding of transport vesicles (Pfanner *et al.*, 1989; Ostermann *et al.*, 1993). We find that long-chain acyl-coenzyme A compounds (myristoyl-CoA and palmitoyl-CoA) as well as coenzyme A itself stimulate vacuole *in vitro* fusion with limited amounts of Sec18p, whereas short-chain and very long-chain acyl-CoA do not (Figure 4A). The CoA-sulfhydryl group which participates in acyl-group transfer is essential, since the desulfo-analog of CoA did not stimulate the reaction (Figure 4A). Furthermore, the fusion stimulation by these reagents is dependent on the presence of functional Sec18p, as the stimulation was completely inhibited by preincubation of vacuoles with affinity-purified anti-Sec18p or Sec17p antibodies (Figure 6C), addition to the fusion reactions of Sec18p antibodies

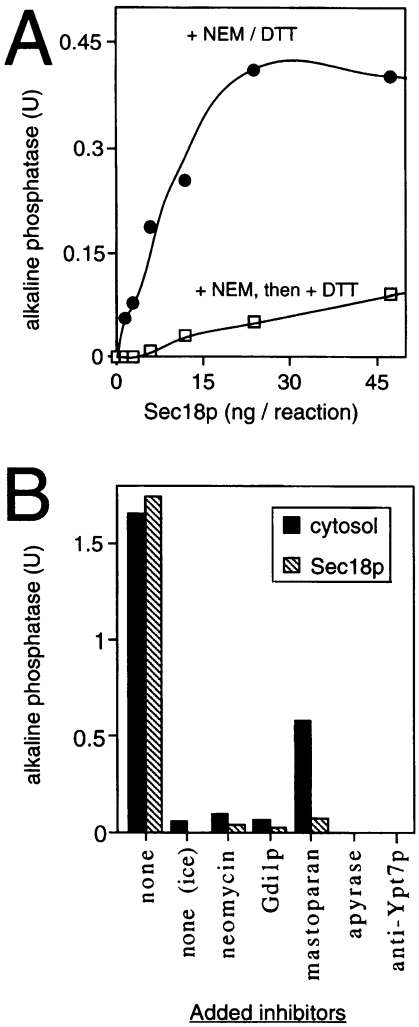


**Fig. 3.** Fusion-promoting activity of $His_6$-Sec18p is NEM-sensitive. (**A**) Recombinant Sec18p in reaction buffer (containing 1 mM DTT and 2 mM ATP) was incubated on ice for 15 min either in the presence of 6 mM NEM (open squares) or in the presence of 6 mM NEM and 6 mM DTT (closed circles). DTT (6 mM) was then added to the first sample (open squares). Incubation of both samples was continued for 15 min on ice. Serial dilutions (in reaction buffer with 1 mM DTT and 2 mM ATP) were assayed for their ability to support the fusion of vacuoles that had been preincubated with anti-Sec18p (as in Figure 2A). Fusion reactions were for 90 min at 25°C. A fusion-independent background of 0.38 U was subtracted for each sample. (**B**) Fusion reactions supported by either cytosol or recombinant Sec18p are inhibited by the same reagents. Vacuoles were preincubated with reaction buffer for 10 min at 25°C, reisolated and used in standard fusion reactions (containing 0.1 mM DTT and 0.04× PIC) with either cytosol (black bars; 35 μg/reaction) or with Sec18p (hatched bars; 140 ng per reaction). Either neomycin (1.5 mM), Gdi1p (1.8 μg per reaction), mastoparan (30 μM) or affinity-purified anti-Ypt7p (2 μg per reaction) was added with the ATP-regenerating system. Apyrase (1.25 U per reaction) was added without the ATP-regenerating system. Fusion reactions were performed at 25°C (or on ice, where indicated) for 90 min. Data were corrected for background alkaline phosphatase activity (0.12 U); fusion samples that were incubated at 25°C without cytosol or added Sec18p, but with ATP, had an activity of 0.63 U.

(Figure 4A) or Fab fragments (Figure 4B) or of anti-Sec17p or Ypt7p antibodies (not shown).

The stimulation of vacuole fusion by palmitoyl-CoA is maximal at ~1 μM palmitoyl-CoA (Figure 4B), similar to the 10 μM needed for maximal stimulation in the Golgi

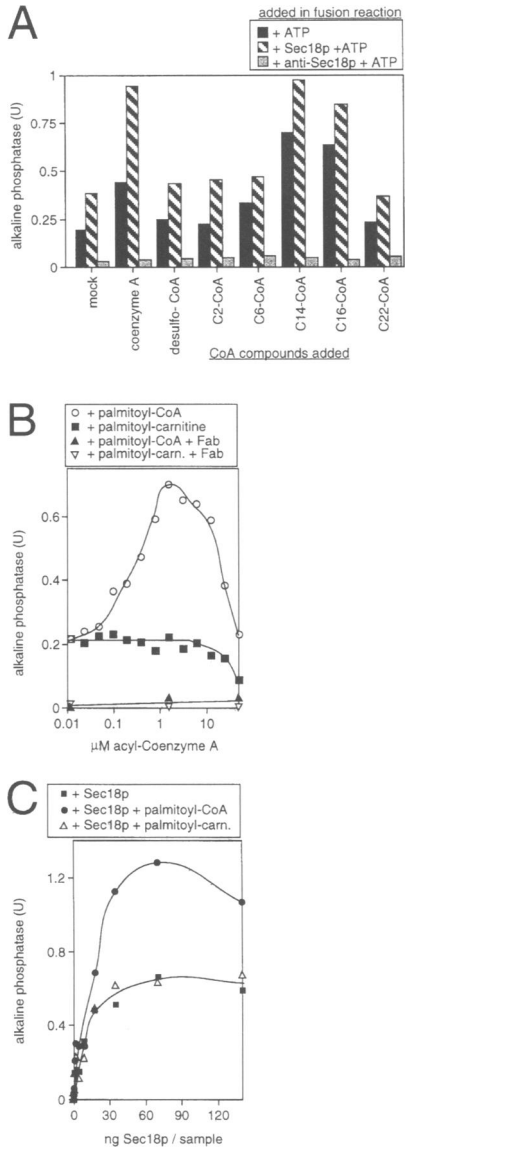


**Fig. 4.** Vacuole fusion is stimulated by certain coenzyme A compounds. (**A**) Vacuoles were preincubated in fusion reaction buffer for 15 min at 30°C, reisolated and used in standard fusion reactions (with 0.1 mM DTT and 0.04× PIC) without cytosol and containing ATP (filled bars), Sec18p (10 ng per reaction; hatched bars) or affinity purified anti-Sec18p IgG (0.65 μg per reaction; gray bars). To each of these samples, buffer only was added (mock) or 3 μM (final) of: coenzyme A, desulfo-coenzyme A (desulfo-CoA), acetyl-coenzyme A ($C_2$-CoA), hexanoyl-CoA ($C_6$-CoA), myristoyl-CoA ($C_{14}$-CoA), palmitoyl-CoA ($C_{16}$-CoA), or behenoyl-CoA ($C_{22}$-CoA). Fusion reactions were performed at 25°C for 90 min. Data were corrected for background activity (0.195 U). (**B**) Dependence of fusion stimulation on palmitoyl-CoA concentration. Vacuoles were preincubated in fusion reaction buffer for 10 min at 30°C, reisolated and used in standard fusion reactions containing either palmitoyl-CoA or palmitoyl-carnitine. Fab fragments of anti-Sec18p IgG (0.68 μg per reaction) were added to the indicated samples. Data were corrected for background activity (0.116 U). (**C**) Palmitoyl-CoA and Sec18p stimulate vacuole fusion synergistically. Vacuoles were preincubated with anti-Sec18p IgG (3.5 μg/standard reaction equivalent) for 10 min at 30°C, reisolated and used in 30 μl fusion reactions containing increasing amounts of $His_6$-Sec18p as indicated. Either buffer alone, palmitoyl-CoA (2 μM) or palmitoyl-carnitine (2 μM) was added to these reactions. Data shown were corrected for background activity (0.162 U).

transport system (Glick and Rothman, 1987). The structurally related palmitoyl-carnitine was used as a control for possible non-specific, detergent-like effects of palmitoyl-CoA. Palmitoyl-carnitine did not stimulate fusion (Figure 4B) whereas non-specific, inhibitory effects may occur with either reagent at higher concentrations (Figure 4B). The fusion stimulated by palmitoyl-CoA was again inhibited by Sec18p antibodies (Figure 4B, closed triangles).

To address the question of whether fusion stimulation by palmitoyl-CoA and purified Sec18p (Figure 2B) is synergistic or additive, we preincubated the vacuoles with Sec18p antibodies (see above) and then added recombinant Sec18p and palmitoyl-CoA, but no cytosol. Under these reaction conditions, fusion requires Sec18p (Figure 4C) and was stimulated ~2-fold by palmitoyl-CoA, suggesting that these reagents act synergistically as in intra-Golgi transport (Glick and Rothman, 1987).

### *Cytosol contains a 'Sec18p-recruiting factor'*

To test whether added Sec18p is absolutely required to activate vacuoles that had been preincubated with anti-Sec18p for fusion, cytosol was treated with NEM to inactivate Sec18p (Wilson *et al.*, 1989) and used in standard fusion reactions. Surprisingly, treated and control cytosols stimulated fusion equally (Figure 5A), although NEM treatment has previously been shown to inactivate Sec18p under these conditions (Wilson *et al.*, 1989). As an alternative approach, cytosol was prepared from either a wild-type or from an isogenic *sec18-1* strain and tested in standard fusion reactions with vacuoles that had been preincubated with anti-Sec18p. Again, both cytosols supported fusion, although the *SEC18* cytosol was somewhat more active than the *sec18-1* cytosol (Figure 5B). However, cytosol from *sec18-1* mutant yeast could not drive fusion at all in another *in vitro* system (Wilson *et al.*, 1990). We therefore hypothesized that a distinct cytosolic factor was recruiting vacuole-bound Sec18p (Figure 1C) to drive the fusion reaction, even though vacuoles had been pretreated with anti-Sec18p. To test this hypothesis rigorously, we have immunodepleted cytosol using either preimmune IgG, anti-Sec17p IgG or anti-Sec18p IgG. As Sec17p is not complexed with Sec18p in the soluble, cytosolic phase (Griff *et al.*, 1992), incubation of cytosol with either preimmune IgG or anti-Sec17p IgG should not reduce the level of Sec18p in the cytosol, whereas preincubation of cytosol with Sec18p IgG should efficiently deplete the cytosol of Sec18p. Cytosol treated with Sec18p IgG was completely depleted of Sec18p which could be recovered in the IgG fraction (Figure 6B). The other IgGs did not deplete the cytosol of Sec18p, as expected (Figure 6B). Anti-Sec18p-treated vacuoles fused almost as efficiently with Sec18p-depleted cytosol as with cytosol pretreated with either preimmune IgG (Figure 6A; compare lanes 3 and 5, black bars) or with anti-Sec17p IgG (not shown). Similar fusion activities were also obtained with both cytosols when vacuoles were pretreated with preimmune IgG (Figure 6A; compare lanes 3 and 5, hatched bars), and this was true at all cytosol concentrations between 0.05 and 1.3 mg protein/ml reaction (1.0 mg/ml being the standard concentration used; data not shown). This demonstrated that the fusion reaction did not require the addition of soluble Sec18p, even when the vacuoles had been pretreated with Sec18p antibodies. Fusion reactions driven by anti-Sec18p-depleted cytosol were still sensitive to anti-Sec18p, indicating that they were Sec18p dependent and not following an alternative pathway (Figure 6A; compare lanes 3 and 4). In agreement with this observation, purified recombinant $His_6$-Sec18p supported more fusion than was observed with either of the cytosols tested (Figure 6A; lane 7). As expected, purified Sec18p did not support fusion of vacuoles that had been pretreated with Sec17 antibodies (Figure 6C) or reactions which contained anti-Sec17p (not shown). We interpret these data as indicating that two pools of Sec18p exist on the vacuoles, one which can be inhibited by anti-Sec18p antibodies and one which is resistant to them. This second pool can only be recruited for the reaction if cytosolic factors (different from Sec18p) are present. Activated Sec18p then becomes sensitive to inhibition by Sec18p antibodies. Therefore, fusion is always suppressed when an excess of Sec18p antibodies is present in the test tube. A heat-stable, complex form of thioredoxin which has recently been demonstrated to support vacuole fusion *in vitro* (Xu and Wickner, 1996), could not activate anti-Sec18p-pretreated vacuoles for fusion (data not shown), nor could palmitoyl-CoA (Figure 6C). These data strongly suggest the presence of a novel factor. This factor seems to be a heat-insensitive macromolecule, as we could recover re-activating activity after incubation at 95°C from a heated cytosol supernatant (HCS; Figure 7A) and as most of the re-activating activity was not passing through a membrane with a 10 000

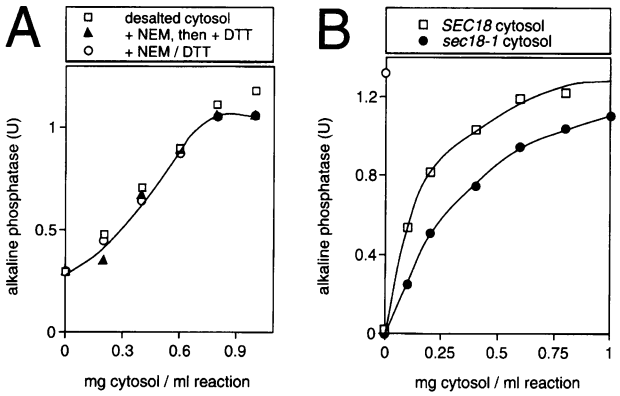


**Fig. 5.** Vacuole fusion can occur in the absence of active cytosolic Sec18p. (**A**) Pretreatment of yeast cytosol with NEM does not reduce its fusion activity. Desalted cytosol (containing 2 mM DTT and 4 mM ATP) from a *SEC18* strain (K91-1A) was split into three aliquots. One aliquot was used directly in the fusion reactions (open squares), a second aliquot received 10 mM NEM (closed triangles) and a third received a mixture of 10 mM NEM and 10 mM DTT (open circles). The samples were incubated on ice for 30 min, then NEM was quenched by adding DTT (closed triangles) and the samples were desalted and used in standard fusion reactions. Alkaline phosphatase activities are shown after correction for the background (0.232 U). (**B**) Cytosol preparations from *SEC18* or *sec18-1* cells support fusion to a similar extent. Standard fusion reactions contained cytosol preparations isolated from either a *SEC18* strain (RSY248) or an isogenic *sec18-1* strain (RSY271) at the final concentrations indicated. The vacuoles were first incubated with anti-Sec18p IgG for 10 min at 30°C, reisolated and used in standard fusion reactions. After 90 min at 25°C, vacuoles were reisolated and cytosol (containing alkaline phosphatase activity) was removed as described in Materials and methods. The open circle at '0 mg cytosol' represents a sample that has received 140 ng of $His_6$-Sec18p without cytosol. Alkaline phosphatase activities were determined and the background (0.135 U) was subtracted from all data.

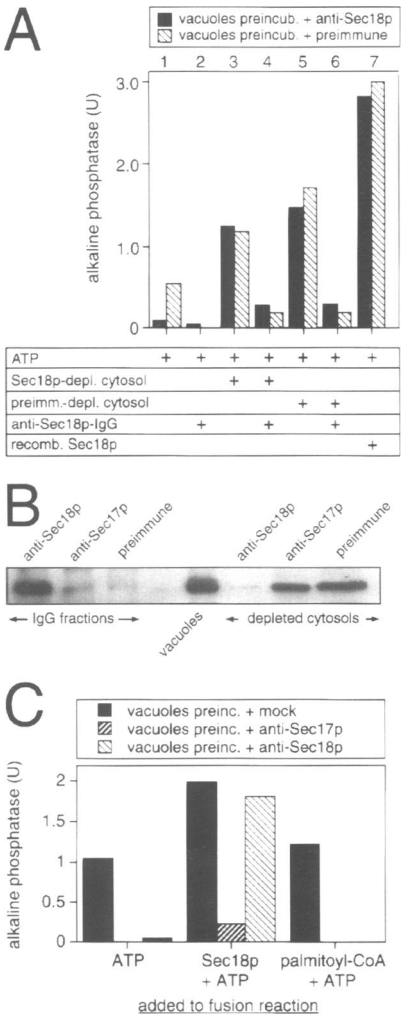


**Fig. 6.** Yeast cytosol contains an 'activating' factor (different from Sec18p itself) for vacuoles pretreated with anti-Sec18p. (**A**) Vacuoles were preincubated at 30°C for 10 min with 3.5 μg (per sample) of either anti-Sec18p complete IgG or preimmune IgG (as in Figure 2A), reisolated and used in fusion reactions (90 min, 25°C) containing cytosol (35 μg/reaction), affinity-purified Sec18p antibodies (anti-Sec18p, at 1.1 μg/reaction), or $His_6$-Sec18p (140 ng/reaction). 'Preimmune cytosol' was cytosol pretreated with preimmune IgG and 'Sec18p-depleted cytosol' was pretreated with anti-Sec18p IgG. Alkaline phosphatase activities were determined and the fusion-independent background was subtracted (0.158 U for vacuoles preincubated with anti-Sec18p, 0.209 U for vacuoles preincubated with preimmune IgG). (**B**) Sec18p is completely removed from the cytosol by immunodepletion. Protein A–Sepharose resins were preincubated with either preimmune serum, Sec18p immune serum or Sec17p immune serum, followed by incubation with cytosol ('depleted cytosols' were used at 100 μg protein per lane). Untreated vacuoles (50 μg protein/lane) were added as a marker fraction. 'IgG fractions' with bound antigens (IgG fraction; 5 μl) were separated in SDS–10% polyacrylamide gels, transferred to PVDF membranes (Bio-Rad), and immunodecorated with affinity-purified Sec18p antibodies. (**C**) Purified Sec18p does not re-activate vacuoles for fusion that have been pretreated with anti-Sec17p. Vacuoles were incubated with either 5.0 μg Sec17p IgG or 3.5 μg Sec18 IgG /5.0 μg vacuole protein, then reisolated and resuspended as in Figure 2A. Fusion reactions contained pretreated vacuoles, an ATP-regenerating system and, where indicated, purified $His_6$-Sec18p (140 ng/sample) or palmitoyl-CoA (3 μM). After 90 min at 25°C, alkaline phosphatase activities were determined and the fusion-independent background activity (0.047 U) was subtracted from all data.

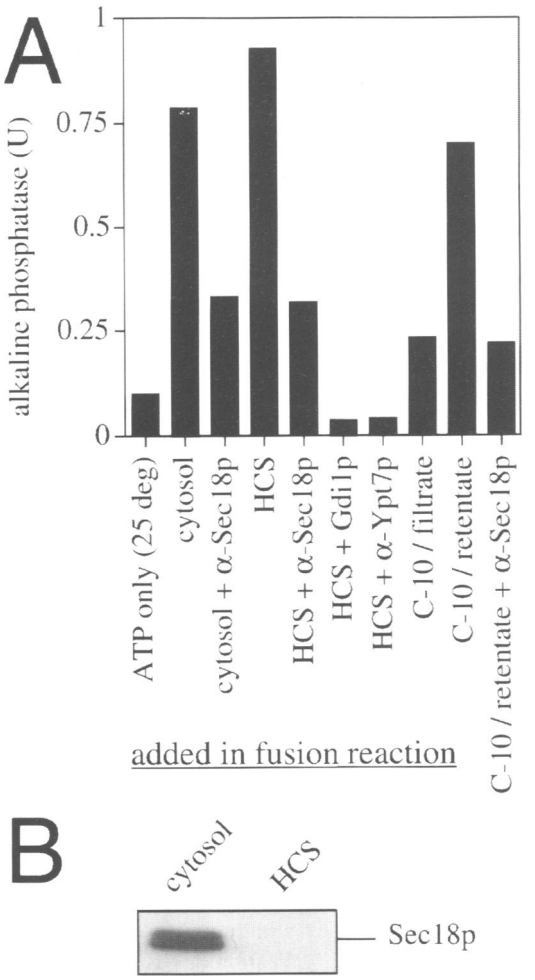


**Fig. 7.** (**A**) The 'sec18p-activating factor' is a macromolecule and can be recovered in heat-treated cytosol. Cytosol was heated at 95°C for 210 s and the insoluble fractions was separated by centrifugation in a minifuge (14 000 r.p.m., 4°C, 10 min). The supernatant ('heated cytosol supernatant'; HCS) was withdrawn and used in fusion reactions. Fusion reactions were performed with vacuoles that had been pretreated with Sec18p IgG as in Figure 2A (the IgG used in this experiment was prepared from the immune serum and was not affinity-purified on its antigen). Cytosol was added at 9.0 μg protein per standard reaction either in the presence or absence of 6.0 μg Sec18p IgG. Heated cytosol supernatant ('HCS', 1.8 μg protein per reaction) was added either alone or together with anti-Sec18p IgG (6.0 μg), Gdi1p (1.8 μg), or affinity-purified anti-Ypt7p (1.8 μg). HCS was further filtered through a microcon-10 unit (Amicon; 'C-10') at 10 000 *g* and 3 μl of either the retentate (reconstituted to the original volume with fusion reaction buffer) or flow-through were added in the presence or absence of anti-Sec18p IgG (6.0 μg). Fusion was for 90 min at 25°C. All alkaline phosphatase activities were corrected for background (0.072 U). (**B**) Heat treatment and subsequent centrifugation remove Sec18p from cytosol. Cytosol or HCS were separated in an SDS–10% polyacrylamide gel at the same relative concentrations (70.0 μg versus 24.0 μg per lane) as used in the fusion reactions presented in (A), transferred to a PVDF membrane and immunodecorated with affinity-purified Sec18p antibodies. This 10% polyacrylamide gel resolved the two forms of Sec18p ($M_r$ 82 000 and 84 000) which have been previously described (Eakle *et al.*, 1988).

molecular mass cut-off (Figure 7A). Preliminary data show that the factor is trypsin-sensitive and therefore a protein (not shown). The fusion stimulation by this factor represents the authentic fusion reaction, as known inhibitors of the cytosol- or Sec18p-supported reaction, such as Gdi1p, anti-Ypt7p and anti-Sec18p, inhibited the fusion

stimulation by heated cytosol supernatant (Figure 7A). Again, it could be excluded that Sec18p itself is reactivating by competing for bound antibodies, as it is quantitatively removed by the heat treatment and subsequent centrifugation (Figure 7B). The fusion stimulation by 1.8 μg of HCS is about the same as fusion stimulation by 9.0 μg cytosol per reaction, representing an ~5-fold increase in specific activity.

## Discussion

It has been assumed for several years that Sec18p (NSF) and Sec17p (α-SNAP) may be employed in all intracellular fusion events. However, these proteins are not required for yeast homotypic ER-membrane fusion *in vitro* (Latterich and Schekman, 1994), whereas a Sec18p homolog, Cdc48p, is required (Latterich *et al.*, 1995). Fusion of mammalian mitotic Golgi fragments does depend on NSF/SNAP, but also on p97, a Cdc48p homolog (Acharya *et al.*, 1995; Rabouille *et al.*, 1995). Finally, basolateral transport in MDCK cells is dependent upon NSF, SNAP and Rab proteins whereas apical transport is not (Ikonen *et al.*, 1995). These exciting findings suggested that (most) heterotypic fusion events may require NSF/Sec18p, whereas homotypic fusion may require the p97/Cdc48p system or related components (Mellman, 1995). Also, NSF(Sec18p)-mediated fusion may be proposed to depend on GTPases of the Rab/Ypt protein families whereas Cdc48p/p97-mediated membrane fusion may not. In contrast to this hypothesis, homotypic fusion of early endosomes *in vitro* can be inhibited by the addition of a monoclonal NSF antibody which inhibits both intra-Golgi transport (Diaz *et al.*, 1989) and ER-to-Golgi fusion (Beckers *et al.*, 1989) in a mammalian system. The homotypic nature of endosome fusion, however, is still being debated; it may actually consist of homotypic and heterotypic components (Mellman, 1995).

### *Sec17p/Sec18p*

We find that homotypic vacuole-to-vacuole fusion depends on functional Sec17p and Sec18p. (i) Affinity-purified antibodies to Sec17p and Sec18p strongly inhibit vacuole fusion, and this inhibition can be prevented by preincubation of the antibodies with the respective antigen. (ii) Purified recombinant Sec18p drives the fusion reactions at least as efficiently as cytosol and is inhibited by NEM. (iii) Fusion stimulation by purified Sec18p was inhibited by the same reagents as the cytosol-driven reaction (including antibodies to Sec17p and Ypt7p), suggesting that the same fusion pathways are being used with either reagent. Together, these data clearly demonstrate that the yeast homologs of α-SNAP and NSF are critical components of a homotypic intracellular fusion event. Our data also support the hypothesis that a requirement for Ypt/Rab-GTPases coincides with involvement of SNAP and NSF in the same fusion reaction.

It is noteworthy that freshly harvested vacuoles can fuse efficiently in the absence of cytosol, obviously supported by Sec17p and Sec18p already present on the vacuolar membrane. Other fusion reactions such as endosome–endosome fusion and intra-Golgi transport are also efficiently supported by organelle-bound NSF (Glick and Rothman, 1987; Rodriguez *et al.*, 1994).

### *Acyl-CoA*

Coenzyme A and acyl-CoAs stimulate homotypic vacuole fusion *in vitro*, providing a second example (Glick and Rothman; 1987; Pfanner *et al.*, 1989, 1990) of CoA stimulation of fusion. It has been suggested that acyl-CoA stimulates Golgi trafficking via protein palmitoylation. Interestingly, SNAP-25, a protein involved in synaptic vesicle fusion, is palmitoylated *in vivo* (Hess *et al.*, 1992). Also, fusion-relevant yeast synaptobrevin homologs (Snc1p, Snc2p) are palmitoylated *in vivo* (Couve *et al.*, 1995), and a 62 kDa protein is hyperacylated in mitotic mammalian cells where vesicular transport is inhibited (Mundy, 1995). Our data indicate that the transfer of acyl groups is critical for the stimulation of vacuole fusion. Enhancement of fusion by palmitoyl-CoA was still strictly dependent on the presence of active Sec18p, just as functional NSF was required for fusion stimulation during *in vitro* Golgi transport (Pfanner *et al.*, 1989).

(Palmitoyl-) CoA may stimulate vacuole fusion *in vitro* by providing acylation of a vacuole membrane target that is already partially acylated. As palmitoyl-CoA may partition into vacuole membranes and reach relatively high local concentrations, further studies will be needed to address the physiological relevance of the stimulation seen in this and similar systems (Quesnel and Silvius, 1994). Considering that palmitoylation of viral surface proteins, e.g. influenza virus hemagglutinin, is crucial for the fusion of these viruses with target cells (Lambrecht and Schmidt, 1986; Naeve and Williams, 1990), investigation of the role of acyl-CoAs in membrane fusion may provide important insights into how acylation regulates membrane functions.

### *Sec18p-activating factor*

When vacuoles have been preincubated with anti-Sec18p, we find that ATP and recombinant Sec18p can support the release of Sec17p from vacuole membranes, whereas ATP alone cannot (unpublished). These data suggest that, on vacuoles pretreated with anti-Sec18p, most of the vacuolar Sec17p is not complexed with vacuolar Sec18p. Rather, it will interact with externally added Sec18p and can be released by this Sec18p plus ATP. As release of Sec17p seems to be a prerequisite for vacuole fusion (Mayer *et al.*, 1996), we speculate that the cytosolic 'Sec18p-activating factor' supports the release of Sec17p by converting the vacuole-bound 'dormant' Sec18p to an active form. This active form would thereby become sensitive to inactivation by anti-Sec18p, whereas the dormant form would remain resistant. Further studies are needed to test this hypothesis. Remarkably, a similar 'activation' phenomenon has been described previously. Homotypic endosome fusion is inhibited when endosomes and cytosol are incubated together with NEM. A cytosol preparation can almost completely restore fusion activity to pretreated vacuoles, even when the NSF in the cytosol had been inactivated (Rodriguez *et al.*, 1994). Yeast cytosol, whether it was isolated from a *SEC18* or a *sec18-1* strain, could functionally substitute for mammalian cytosol in this reaction. Furthermore, both cytosol and endosomes were needed for efficient fusion inactivation at low NEM concentrations (Rodriguez *et al.*, 1994). Similarly, immunodepletion of cytosolic NSF did not affect transport from endosomes to the *trans*-Golgi network in a cell-free

mammalian system (Goda and Pfeffer, 1991). Using NEM pretreated reactions, these authors showed that a glycerol gradient fraction of cytosol, corresponding to 50–100 kDa, could re-activate the NEM-inhibited system. The involvement of NSF in either the fusion reaction or the re-activation step was unclear, as attempts to inhibit this transport step by NSF antibodies yielded inconclusive results (Goda and Pfeffer, 1991). In line with these observations, Wattenberg *et al.* (1992) reports that the NSF on functional intra-Golgi transport vesicles is no longer sensitive to NEM. We suggest that NSF/Sec18p may be present on the membranes of endosomes and vacuoles at all times with an activation step supported by cytosol being needed for sensitivity to either low NEM concentrations or Sec18p antibodies. Whether restoration of endosome fusion activity (or endosome-to-Golgi transport activity) was in fact due to the activation of a 'dormant' NEM-insensitive membrane-bound form of NSF or, as Rodriguez *et al.* (1994) and Goda and Pfeffer (1991) suggest, largely due to the presence of a second NEM-sensitive factor, remains to be shown (for example, by using more specific reagents than NEM, such as NSF antibodies).

## Materials and methods

### Yeast strains

*S.cerevisiae* strains used in this study were BJ3505 (*Mat***a** *pep4::HIS3 prb1-Δ1.6R HIS3 lys2-208 trp1-Δ101 ura3-52 gal2 can*; Moehle *et al.*, 1986) and DKY6281 (*Mat***a** *leu2-3 leu2-112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 pho8::TRP1*; from Dr D.Klionsky, University of California, Davis) (Haas *et al.*, 1995). K91-1A (*Matα ura3 pho8::pAL134 pho13::pH13 lys1*) was from Dr Y.Kaneko, Institute of Fermentation, Osaka, Japan. The SEC18 strain used was RSY249 (*Mat***a**, *his4-619, ura3-52*), its sec18 derivative was RSY272 (*Mat***a**, *his4-619, ura3-52 sec18-1*), both from Dr R.Schekman, University of California, Berkeley.

### Vacuole isolation, cytosol isolation, and in vitro fusion reactions

These were as described in Haas (1996) with vacuole concentrations of 0.17 or 0.20 mg protein/ml reaction (in 30 μl standard reactions). All fusion experiments were carried out in siliconized tubes. The vacuole isolation procedure is based on a discontinuous ficoll density gradient centrifugation (Bankaitis *et al.*, 1986), leading to a 45- to 50-fold enrichment of vacuolar protein with respect to the total cell protein applied to the gradient. Only trace amounts of cytosolic and ER markers are found in these vacuole preparations (Bankaitis *et al.*, 1986). Cytosol preparations (Haas, 1996) from *SEC18* and the isogenic *sec18-1* strains were prepared from cells grown at the permissive temperature (23°C) with the lysis buffer containing DTT (2 mM) and ATP (1 mM). The ultracentrifugation was modified to a 45 min step at 245 000 *g* (max). In fusion reactions, fusion reaction buffer [100 mM K(OAc), 50 mM KCl, 20 mM PIPES–KOH pH 6.8, 250 mM sorbitol, 5 mM $MgCl_2$] was supplemented with 100 μM DTT and 0.04× PIC [protease inhibitor cocktail; 1× PIC is 2 μM Pefabloc SC (Boehringer Mannheim Inc.), 10 ng/ml pepstatin A (Sigma Chemical Corp.), 2 ng/ml leupeptin (Sigma) and 10 μM *o*-phenanthroline (Sigma)]. After the fusion reactions, the alkaline phosphatase-containing cytosol was removed by two large-volume washes of the vacuoles as described previously (Haas, 1996) and enzymatic activity was determined in the pellet fractions. 'Background' fusion activities were defined as the sum of the separate alkaline phosphatase activities of the reagents used in the experiment.

### Protein purification

Amino-terminal $His_6$-tagged Sec17 and Sec18 proteins were purified from *Escherichia coli* XL1-blue harboring the vector pQE9 (Qiagen) with the complete *SEC17* or *SEC18* coding sequences respectively; the constructs were kindly provided by Drs T.Söllner and J.Rothman (Sloan-Kettering Institute, New York). The recombinant proteins were purified from *E.coli* lysates using an $Ni^{2+}$-nitriloacetic acid (NTA) agarose-affinity column (Qiagen) as described in Whiteheart *et al.* (1994). The resulting proteins were desalted using a G100 column (Pharmacia) equilibrated with fusion reaction buffer containing 2 mM ATP, 1 mM DTT and 10% (v/v) glycerol. Dilutions of these protein stocks for fusion experiments were done immediately before the fusion experiments in the same buffer lacking glycerol. Fusion proteins containing protein A plus a portion of Sec17p or containing a fusion between *E.coli* β-galactosidase and a portion of Sec17p were described previously (Griff *et al.*, 1992) and the respective plasmids were kindly provided by Dr C.Kaiser (Massachusetts Institute of Technology). The fusion proteins were purified as described (Griff *et al.*, 1992) by either affinity purification on an IgG column (Pharmacia Biotechnology; for protein A hybrid) or by purifying inclusion bodies and separating their proteins in an SDS–polyacrylamide gel (β-galactosidase hybrid).

### Antibody production and affinity-purification

Antibodies against the protein A Sec17p fusion protein were raised in white New Zealand rabbits by repeated injection of 50 μg protein. Bleeds were taken 10 days after each boosting. Antibodies were affinity-purified by first separating inclusion body proteins, including the β-galactosidase Sec17p fusion protein, in a 15% SDS–polyacrylamide gel and transferring the proteins to a PVDF membrane (Bio-Rad). The membrane strip containing the fusion protein was incubated with anti-protein A Sec17p serum and the Sec17p-specific antibodies were eluted from the membrane as described in Griff *et al.* (1992), but using 0.12 vol. 1 M Tris–HCl pH 9.4 for the neutralization step. For our initial studies, an aliquot of an affinity-purified Sec17p antibody that had already been tested in other fusion systems (Griff *et al.*, 1992; Latterich *et al.*, 1994) was generously provided by Dr C.Kaiser. Antibodies against $His_6$-tagged Sec17p or $His_6$-tagged Sec18p were raised by injecting the complete, native recombinant proteins (40–100 μg per injection). These antibodies were purified on columns containing immobilized $His_6$-tagged Sec17p or $His_6$-tagged Sec18p. To prepare these columns, 4.5 (Sec17p) or 3.0 (Sec18p) mg protein was coupled to cross-linked agarose using the AminoLink Immobilization Kit (Pierce). To purify specific antibodies, 5 ml of the respective immune serum was centrifuged in an Eppendorf microcentrifuge (14 000 r.p.m., 4°C, 5 min), the supernatant adjusted to 20 mM potassium phosphate (pH 8.0; PPB) from a 1 M stock and applied to the affinity columns equilibrated with 20 mM PPB. The flow-through was reloaded five times. The columns were extensively washed with 20 mM PPB and the bound specific antibodies eluted with 10 ml glycine–HCl (200 mM, pH 2.5). Fractions (1 ml) were collected and immediately neutralized with 1 M Tris–HCl, pH 9.4. The protein-containing fractions were pooled and concentrated in Centricon C-30 (Amicon) spin concentrators. The buffer was subsequently exchanged to fusion reaction buffer by repeated addition of reaction buffer and concentration of the samples. Concentrated antibody samples were kept at –20°C. IgG was purified as described previously (Haas *et al.*, 1995). Fab fragments of anti-Sec18p IgG were produced using the Immuno-Pure Fab preparation kit (Pierce), followed by exchange of buffer as described above for affinity-purified antibodies. Immunoblot analysis was performed as in Conradt *et al.* (1992) with modifications as published (Haas *et al.*, 1995).

### Immunodepletion of yeast cytosol

The cytosol used in these immunodepletion experiments was isolated using a method based on the protocol for the isolation of active recombinant $His_6$-Sec18p (Whiteheart *et al.*, 1994). K91-1A cells were grown and treated as in the standard vacuole isolation protocol (Conradt *et al.*, 1992), including exposure to alkaline DTT solution and spheroplast formation using oxalyticase (Enzogenetics, Corvallis, OR). Spheroplasts were resuspended in 1.0 ml lysis buffer [100 mM K(OAc), 250 mM KCl, 20 mM PIPES–KOH pH 6.8, 5 mM $MgCl_2$, 250 mM sorbitol, 1× PIC, 1 mM PMSF, 2 mM DTT, 1 mM ATP] per 1000 $OD_{600}$ units of starting cell material and lysed by two passages through a French press at 8000 p.s.i. and 4°C. The lysate was centrifuged (14 000 r.p.m., 4°C, 5 min, microfuge) and the supernatants were centrifuged in a Beckman Optima TLX ultracentrifuge (TLA-100.2 rotor, 150 000 *g*, 1 h, 4°C). Supernatants were pooled, frozen in liquid nitrogen and stored at –80°C. For each immunodepletion sample, 5.0 ml of either Sec18p preimmune serum, Sec18p immune serum or Sec17p immune serum were centrifuged in a microfuge at 14 000 r.p.m. and 4°C for 5 min. The supernatants for each serum were combined and adjusted to 20 mM PPB using a 1 M stock solution. For each sample, 1.5 ml of fresh protein A–Sepharose CL4B beads (Pharmacia Inc., Piscataway, NJ) were washed repeatedly with 20 mM PPB (pH 8.0), placed in a 12 ml polycarbonate tube, mixed with serum and gently shaken at 4°C for 120 min. The Sepharose resin was separated from the serum by a brief (2 min) centrifugation step in

a clinical centrifuge at 1000 *g*, followed by several washes with lysis buffer and reisolation of the resin. 1.0 ml of cytosol (prepared as described above) was centrifuged in a microfuge (5 min, 14 000 r.p.m., 4°C) and the supernatant was diluted with 1.5 ml of fusion reaction buffer containing 0.5 mM DTT, 0.25 mM ATP and 0.5× PIC. This mixture was added to 1.5 ml of Sepharose resin (prepared as above) and gently shaken in 12 ml polycarbonate tubes at 4°C for 120 min. Resins were reisolated by a centrifugation step (as above) and supernatant recovered. Each pretreated cytosol solution was then incubated briefly (15 min, 4°C) with 0.2 ml of fresh protein A–Sepharose resin in the same buffer to remove any remaining free antibodies. After centrifugation in a clinical centrifuge (as above), pretreated cytosols were recovered, frozen in liquid nitrogen, and stored at –80°C. IgG and their bound antigens were recovered by repeated large-volume washes (PPB) of the resins, followed by elution of bound material with 1.0 ml and then 0.2 ml glycine–HCl (200 mM, pH 2.5). The supernatants were pooled and protein precipitated by trichloroacetic acid, followed by acetone washes and resuspension of each precipitate in 1.0 ml 2× sample buffer containing protease inhibitors and heating for 5 min at 95°C (Haas *et al.*, 1995).

### *Treatment of cytosol with NEM*

Cytosol (Haas, 1996) in 1× fusion reaction buffer/2 mM DTT/4 mM ATP was centrifuged in a microfuge for 5 min at 14 000 r.p.m. (4°C). Spin columns were prepared by placing 1 ml of G25 bed volume (equilibrated with the same buffer) into 1 ml syringes. Cytosol (125 µl per column) was desalted on these columns and an aliquot was set aside. The remaining material was divided into two equal aliquots. Aliquot #1 received 10 mM freshly prepared NEM, while aliquot #2 received a mixture of 10 mM NEM and 10 mM DTT (in both cases, in addition to the 2 mM DTT already present). The samples were kept on ice for 30 min. Aliquot #1 received DTT to a final concentration of 10 mM and both samples were immediately desalted on G25 columns as above. Protein concentrations were determined for all desalted cytosols (Bio-Rad Assay Reagent Kit), and standard fusion reactions were performed with the indicated amounts of cytosol.

### *Pretreatment of vacuoles with antibodies*

Vacuoles (0.25 or 0.30 mg protein/ml) isolated from BJ3505 and DKY 6281 were mixed in a 1:1 ratio in fusion reaction buffer and diluted in fusion reaction buffer to a final concentration of 0.17 or 0.20 mg protein/ml. Anti-Sec18p IgG or preimmune IgG (60 µg/ml) were added to 1.5 ml reaction tubes containing between 800 and 1400 µl of the above vacuole mixture. The mixtures were incubated at 30°C for 10 min, chilled on ice for 3 min and the vacuoles reisolated by centrifugation (microcentrifuge, 10 000 r.p.m., 4°C, 45 s). The vacuoles were resuspended in fresh reaction buffer to a final concentration of 0.17 or 0.20 mg protein/ml and used directly in the fusion reactions. The same procedure was used in the absence of antibodies to increase the dependence of the fusion reaction on added cytosol (Haas, 1996).

### *Other materials*

Polyclonal rabbit antiserum against Cdc48p, as described in Fröhlich *et al.* (1991) and Latterich *et al.* (1995), was generously provided by Dr K.-U.Fröhlich (Universität Tübingen, Germany). Affinity-purified anti-Ypt7p was a gift from Drs D.Scheglmann and D.Gallwitz (Göttingen, Germany; as described in Haas *et al.*, 1995). A complex form of thioredoxin (Xu and Wickner, 1996) was kindly provided by Dr Z.Xu (this laboratory). All other reagents were from the commercial sources or prepared as decribed in Haas *et al.* (1994, 1995) and Haas (1996). Centricon and microcon separators were from Amicon.

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