The EMBO Journal vol.10 no.13 pp.4049-4060, 1991

A genetic and structural analysis of the yeast Vps15 protein kinase: evidence for a direct role of Vps15p in vacuolar protein delivery

Paul K.Herman[1], Jeffrey H.Stack[2] and Scott D.Emr[2]

California Institute of Technology, Division of Biology, Pasadena, CA 91125, USA

[1]Present address: Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720, USA
[2]Present address: Division of Cellular and Molecular Medicine, Howard Hughes Medical Institute, University of California, San Diego, School of Medicine, La Jolla, CA 92093-0668, USA

Communicated by D.Meyer

The yeast *VPS15* gene encodes a novel protein kinase homolog that is required for the sorting of soluble hydrolases to the yeast vacuole. In this study, we extend our previous mutational analysis of the *VPS15* gene and show that alterations of specific Vps15p residues, that are highly conserved among all protein kinase molecules, result in the biological inactivation of Vps15p. Furthermore, we demonstrate here that short C-terminal deletions of Vps15p result in a temperature-conditional defect in vacuolar protein sorting. Immediately following the temperature shift, soluble vacuolar hydrolases, such as carboxypeptidase Y and proteinase A, accumulate as Golgi-modified precursors within a saturable intracellular compartment distinct from the vacuole. This vacuolar protein sorting block is efficiently reversed when mutant cells are shifted back to the permissive temperature; the accumulated precursors are rapidly processed to their mature forms indicating that they have been delivered to the vacuole. This rapid and efficient reversal suggests that the accumulated vacuolar protein precursors were present within a normal transport intermediate in the vacuolar protein sorting pathway. In addition, this protein delivery block shows specificity for soluble vacuolar enzymes as the membrane protein, alkaline phosphatase, is efficiently delivered to the vacuole at the non-permissive temperature. Interestingly, the C-terminal Vps15p truncations are not phosphorylated *in vivo* suggesting that the phosphorylation of Vps15p may be critical for its biological activity at elevated temperatures. The rapid onset and high degree of specificity of the vacuolar protein delivery block in these mutants suggests that the primary role of Vps15p is to regulate the sorting of soluble hydrolases to the yeast vacuolar compartment.

Key words: lysosome/protein kinase/protein sorting/vacuole

Introduction

The cytoplasmic environment of eukaryotic cells is subdivided into a number of functionally distinct membrane-enclosed organelles. In order to maintain the functional integrity of these organelles, specific mechanisms must exist to allow the cell efficiently to sort and deliver proteins from their common site of synthesis in the cytoplasm to their appropriate final destinations. The secretory pathway of eukaryotic cells is responsible for the proper modification and delivery of proteins to the cell surface and to a variety of intracellular compartments (Pfeffer and Rothman, 1987). The delivery of proteins to the lysosomal, or vacuolar, compartment of eukaryotic cells is mediated by the secretory pathway and is one of the best characterized examples of an intracellular protein sorting process (Kornfeld and Mellman, 1989; Klionsky *et al.*, 1990). Following translocation across the membrane of the endoplasmic reticulum, lysosomal proteins transit to the Golgi complex together with proteins destined for secretion from the cell. Within a late Golgi compartment, lysosomal proteins are sorted away from the secretory protein traffic and are targeted to the lysosome. This routing of proteins from the default secretion path is an active process requiring specific sorting information present within lysosomal proteins.

In mammalian cells, the best characterized lysosomal sorting system involves the specific modification of soluble lysosomal enzymes with a mannose-6-phosphate moiety (Kaplan *et al.*, 1977; reviewed in Kornfeld and Mellman, 1989). This carbohydrate modification is recognized in the Golgi apparatus by specific membrane receptors that mediate delivery of the modified proteins to the lysosome. In contrast, the *cis*-acting sorting information present in several yeast vacuolar proteins is not associated with any specific carbohydrate modification and instead appears to reside within the polypeptide backbone of these proteins (Johnson *et al.*, 1987; Valls *et al.*, 1987; Klionsky *et al.*, 1988; Klionsky and Emr, 1990). Despite the gains made in our understanding of the initial recognition of lysosomal and vacuolar proteins, very little is presently known about the cellular components that function subsequently to bring about the proper packaging and delivery of these proteins to the lysosomal and vacuolar compartments.

In the yeast, *Saccharomyces cerevisiae*, an extensive genetic analysis of vacuolar protein localization has been undertaken in an attempt to develop a better understanding of the *trans*-acting machinery responsible for mediating protein delivery to the vacuole (reviewed in Klionsky *et al.*, 1990). Two independent genetic selections have identified a large number of yeast mutants defective in the localization of multiple soluble vacuolar hydrolases, including carboxypetptidase Y (CPY), proteinase A (PrA) and proteinase B (PrB) (Bankaitis *et al.*, 1986; Rothman and Stevens, 1986; Robinson *et al.*, 1988; Rothman *et al.*, 1989). Rather than delivering these proteins to the vacuole, *vps* mutants (for *v*acuolar *p*rotein *s*orting defective) missort these enzymes to the cell surface as Golgi-modified precursors. In addition to these localization defects, many of the *vps* mutants exhibit

additional phenotypes including defects in vacuole biogenesis and segregation, severe temperature-sensitive (*ts*) growth defects and defects in the sorting of vacuolar membrane proteins (Banta *et al.*, 1988; Robinson *et al.*, 1988; Herman and Emr, 1990; Raymond *et al.*, 1990). Complementation analyses between the *vps* mutants and other related sets of mutants, have demonstrated that there are at least 47 complementation groups required for the efficient targeting of vacuolar proteins in yeast (Klionsky *et al.*, 1990). This high level of genetic complexity indicates that vacuolar protein delivery is a complex process requiring the direct, or indirect, participation of a relatively large number of gene functions. In order to gain an understanding of the basic cellular processes underlying vacuolar and lysosomal protein delivery, we have initiated efforts to identify specific *VPS* gene products and to characterize, genetically and biochemically, the step(s) of the sorting pathway at which these products function (Banta *et al.*, 1990; Herman and Emr, 1990; Herman *et al.*, 1991).

Several experimental observations indicate that the yeast *VPS15* gene product may play a central role in regulating the sorting of soluble hydrolases to the vacuole (Herman *et al.*, 1991). First, mutations in the *VPS15* gene result in severe defects in the localization of several vacuolar hydrolases, including CPY, PrA and PrB (Robinson *et al.*, 1988; Herman *et al.*, 1991). These defects appear to be relatively specific for soluble constituents of the yeast vacuole as the localization of two vacuolar membrane proteins, alkaline phosphatase (ALP) and α-mannosidase, appears to be less defective in *vps15* mutants (Robinson *et al.*, 1988; Klionsky and Emr, 1989; Herman *et al.*, 1991). Second, the cloning and sequencing of the *VPS15* gene has indicated that the predicted Vps15 protein (Vps15p) sequence exhibits significant similarity to the catalytic domains of the serine/threonine family of protein kinases. Mutations altering highly conserved residues within the Vps15 protein kinase domain result in the biological inactivation of this protein and eliminate *in vivo* phosphorylation of Vps15p (Herman *et al.*, 1991). Finally, subcellular fractionation studies indicated that Vps15p may be associated with the cytoplasmic face of a late Golgi or vesicle compartment (Herman *et al.*, 1991). Since vacuolar protein sorting appears to occur within a late Golgi compartment (Graham and Emr, 1991; see Klionsky *et al.*, 1990), this intracellular location is consistent with Vps15p having a direct influence upon vacuolar protein sorting. On the basis of these observations, we proposed that Vps15p-mediated phosphorylation events may play a key role in regulating protein delivery to the yeast vacuole and that protein phosphorylation reactions, in general, might act as a molecular 'switch' within the eukaryotic secretory pathway to divert proteins from a default transit pathway (e.g. secretion) to an alternative pathway (e.g. to the vacuole).

In this study, we extend our mutational analysis of the Vps15p kinase domain and also examine the phenotypic consequences of alterations within the myristic acid attachment site and the large carboxy-terminal (C-terminal) domain of Vps15p. We find that relatively short C-terminal deletions of Vps15p result in a rapid, severe and highly specific, temperature-conditional defect in vacuolar protein delivery. Biochemical analysis of the *ts* vacuolar protein sorting defects, and their efficient reversal, indicates that Vps15p is directly involved in the delivery of soluble hydrolases to the yeast vacuolar compartment.

Results

Mutational analysis of the Vps15p kinase domain

Our initial analysis of the predicted Vps15p amino acid sequence suggested that this protein could be divided into three separate sequence domains (Herman *et al.*, 1991; Figure 1). The immediate N-terminal sequence of Vps15p contains a potential attachment site for myristic acid, a rare 14-carbon fatty acid (see below). The next ~300 amino acids of Vps15p exhibit significant sequence similarity to the catalytic domains of protein kinases. We assessed the functional significance of the Vps15p kinase domain sequences by altering two of the most conserved kinase motifs of Vps15p and analyzing the phenotypic consequences of these changes. Both of the alterations, D165R (i.e. D165 → R) and E200R, resulted in the biological inactivation of Vps15p (Herman *et al.*, 1991; see Figure 1). This mutational analysis suggested that Vps15p functions as a protein kinase *in vivo* and that specific protein phosphorylation reactions are required for the efficient delivery of proteins to the yeast vacuole. The third domain consists of the remaining C-terminal sequences of Vps15p (>1100 amino acids). This region of Vps15p exhibited no significant similarities to any other known protein sequence. The tripartite nature of the 1455 amino acid Vps15p is shown schematically in Figure 1.

The first kinase motif examined includes the Vps15p sequence DIKTEN and corresponds to the kinase domain region that exhibits the highest degree of sequence conservation between different protein kinases (Hanks *et al.*, 1988). In addition, the sequence conservation observed in this region of protein kinases also serves as an indicator of kinase substrate specificity (Hanks, 1987). The lysine residue within this sequence motif (K149 in the Vps15p sequence) is conserved in all known serine/threonine protein kinase sequences. In tyrosine-specific protein kinases, this lysine residue is replaced with either an alanine or an arginine residue (however, see Ben-David *et al.*, 1991; Featherstone and Russell, 1991). Using oligonucleotide-directed mutagenesis, we constructed a *VPS15* allele that encoded a protein with aspartic acid replacing this lysine at position 149 (K149D). This alteration resulted in the biological inactivation of Vps15p in each of three different assays; the K149D mutant exhibited a severe *ts* growth defect, mislocalized >95% of the newly synthesized CPY to the cell surface in a Golgi-modified precursor form (p2 CPY) and was defective for Vps15p phosphorylation *in vivo* (Figures 1 and 2).

Two additional alterations, D147R and E151R, were constructed within this kinase domain motif. The aspartic acid at position 147 of Vps15p is very highly conserved and is found in a corresponding position in all known protein kinases, both serine/threonine and tyrosine-specific (Hanks *et al.*, 1988). In addition, the aspartic acids D147 and D165 correspond to residues that appear to be conserved in a variety of phosphotransferase systems, including protein kinases and aminoglycoside phosphotransferases (Brenner, 1987). It was suggested that this common pair of aspartic acids could be involved in the binding of the ATP phosphate groups. The substitution of an arginine residue for the aspartic acid at position 147 resulted in a severe reduction in Vps15p activities. The D147R mutant exhibits a severe *ts* growth defect and mislocalizes ~90% of the newly synthesized CPY to the cell surface as p2 CPY (Figures 1 and 2). In addition, the D147R protein is very poorly

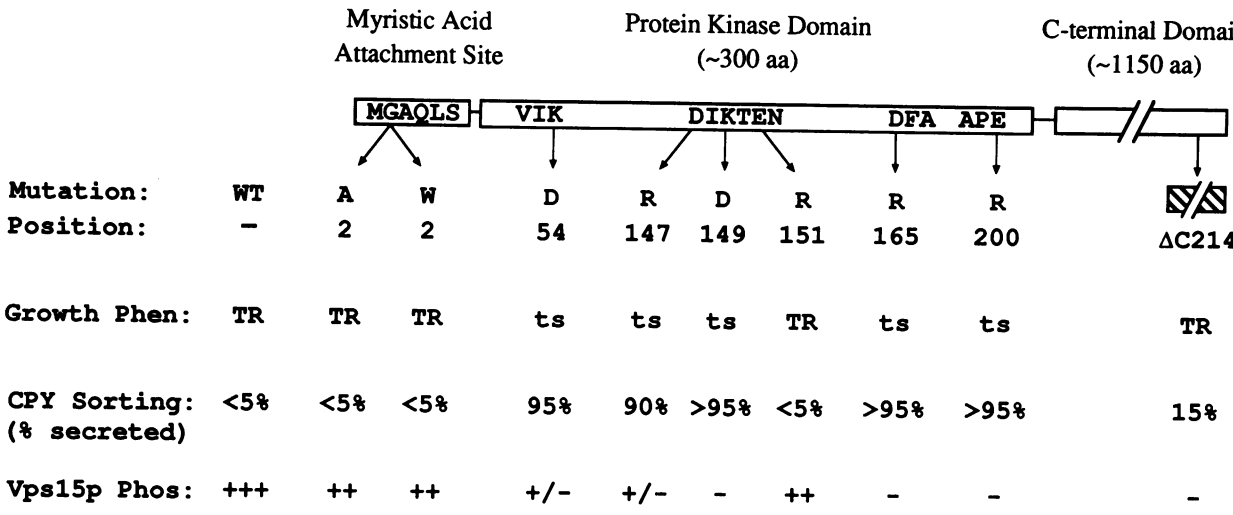

Fig. 1. Summary of the mutational analyses of *VPS15*. A schematic representation of the tripartite nature of the Vps15p sequence is shown. The alterations made at each residue are indicated below the bar diagram of Vps15p along with the amino acid position of this residue in the Vps15p sequence. The growth phenotype (GrowthPhen) indicates the ability of the different *vps15* mutants to grow at 38°C on YPD plates. The next row indicates the extent of the CPY sorting defect in the different mutants. To analyze CPY localization, the engineered *vps15* alleles were introduced into a Δ*vps15* yeast strain (PHY112) on a centromere-containing plasmid. Yeast spheroplasts were labeled with Tran^{35}S-label, fractionated into supernatant (extracellular) and pellet (intracellular) fractions and then immunoprecipitated with antisera specific for the vacuolar hydrolase CPY. The relative levels of the different forms of CPY in each fraction were determined and the values in the table indicate the percentage of the total CPY that is found as p2 CPY in the extracellular fraction. The final row (Vps15p Phos) is a relative measure of the extent of ^{32}P incorporation into Vps15p in the different mutants. The *vps15* alleles were introduced into the strain PHY112 on a 2μ multicopy plasmid. These different yeast strains were then labeled with [^{32}P]orthophosphate and immunoprecipitations were performed from yeast cell extracts with antisera specific for Vps15p. '++' indicates a signal ~30–50% of wild-type; '+', ~5–10% of wild-type; and '–' indicates that no detectable phosphorylation was observed. The relative synthesis rates and stability of each of the mutant Vps15 proteins were assessed in a series of pulse–chase immunoprecipitation experiments. Δ*vps15* yeast strains, expressing the appropriate Vps15p mutant protein, were pulse labeled with Tran^{35}S-label for 5–10 min and then chased for 0, 45 or 90 min following the addition of unlabeled methionine and cysteine. The relative levels of Vps15p present at each time point were determined by quantitative autoradiography following immunoprecipitation with antisera specific for Vps15p and separation on SDS–polyacrylamide gels. In addition, the steady-state levels of most of the altered Vps15p proteins were analyzed by immunoblotting experiments with antisera specific for Vps15p. Together, these analyses indicated that the steady-state level of each of the mutant proteins was very similar to that of wild-type Vps15p (data not shown; see Herman *et al.*, 1991).

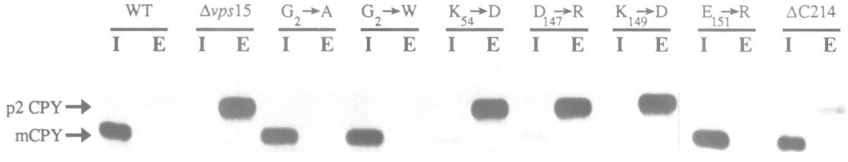

Fig. 2. Intracellular sorting of CPY in yeast *vps15* mutants. Yeast spheroplasts were labeled with Tran^{35}S-label for 20 min at 26°C and then chased for 30 min following the addition of methionine and cysteine to 25 mM. The labeled cultures were centrifuged for 2 min at 13 000 *g* and separated into pellet (I, intracellular) and supernatant (E, extracellular) fractions. The level of CPY in each fraction was assessed by quantitative immunoprecipitation with antisera to CPY. In each sample, the strain examined was PHY112 (Δ*vps15::HIS3*) carrying a particular *vps15* allele on a centromere-containing plasmid. WT refers to the wild-type allele (pPHY150) and Δ*vps15* to PHY112 harboring the vector plasmid (pPHYC18). For all other samples, the particular *vps15* allele present would encode a Vps15p protein with the indicated alteration (see Figure 1). The positions of mature (61 kDa) and p2 CPY (69 kDa) are indicated.

phosphorylated *in vivo*. The D147R protein incorporates only ~5–10% of the $^{32}PO_4$ incorporated by the wild-type Vps15p in a similar labeling experiment.

The final alteration of the DIKTEN sequence motif, E151R, was constructed on the basis of observations made with several serine/threonine-specific kinases that suggested that this residue was critical for kinase interaction with substrate (Taylor *et al.*, 1990). In general, protein kinases having a preference for basic amino acids preceding the substrate phosphorylation site have an acidic residue conserved at this position. Conversely, a basic amino acid tends to be present in kinases with a preference for acidic groups near the target phosphorylation site. We therefore assessed the phenotypic effects of substituting a basic residue, arginine, for the glutamic acid (E151) normally present at this position in Vps15p. As seen in Figure 1, this alteration had only a modest effect on Vps15p activity as judged by our three assays. While 38°C growth and CPY sorting appeared normal with the E151R mutant (Figures 1 and 2), the level of $^{32}PO_4$ incorporation into this altered Vps15p was only ~35–50% of that seen with the wild-type Vps15p *in vivo* (Figure 1).

Finally, we altered Vps15p at lysine residue K54 in the kinase domain. This residue corresponds to an invariant lysine observed in both serine/threonine and tyrosine protein kinases (Hanks *et al.*, 1988; Taylor *et al.*, 1990). This lysine

residue has been suggested to be within, or near, the ATP binding domain of protein kinases as it can be specifically labeled with ATP analogs such as *p*-fluorosulfonyl 5′-benzoyl adenosine (Zoller *et al.*, 1981; Kamps and Sefton, 1984). We constructed a mutant *VPS15* allele that encodes a protein with an aspartic acid replacing this lysine, K54D, and have found that this alteration dramatically reduces Vps15p activity *in vivo*. The K54D mutant exhibits a severe *ts* growth defect and mislocalizes ~95% of the newly synthesized CPY to the cell surface (Figures 1 and 2). In addition, this mutant is also very defective for the *in vivo* phosphorylation of Vps15p (Figure 1).

Vps15p is modified with myristic acid at its N terminus

The 14-carbon fatty acid, myristate, is added co-translationally to the N-terminal glycine residue of many cellular proteins following the removal of the initiating methionine (reviewed in Schultz *et al.*, 1988; Towler *et al.*, 1988b). Our previous studies indicated that the N-terminal sequence of Vps15p, (Met1)-Gly2-Ala3-Gln4-Leu5-Ser6-Leu7, was in good agreement with a proposed consensus sequence for myristic acid addition and therefore suggested that Vps15p could be myristoylated at its N terminus (Herman *et al.*, 1991). To analyze directly whether Vps15p was myristoylated *in vivo*, we radiolabeled yeast cells with tritiated myristic acid for 60 min and then performed immunoprecipitations with antisera specific to Vps15p. Vps15p was observed to be specifically labeled in these experiments suggesting that it is modified by the addition of myristic acid *in vivo* (Figure 3). Further support for this assertion was obtained from our analysis of two different *vps15* mutants that possessed alterations in the myristic acid attachment site. In these mutants, the critical glycine residue at position 2 was changed to either an alanine, G2A, or a tryptophan, G2W (Figure 1). Consistent with results from previous studies on the substrate specificity of the yeast myristoyl CoA:protein N-terminal myristoyltransferase (Towler *et al.*, 1987, 1988a), neither of the mutant Vps15p proteins were labeled by the [^{3}H]myristic acid in these experiments (Figure 3).

Although these two alterations abolished the myristoylation of Vps15p, the non-myristoylated Vps15 proteins appeared to possess near wild-type levels of biological activity *in vivo*. The G2A and G2W mutants both exhibited near wild-type growth rates at 38°C and neither mutant was defective for CPY delivery to the vacuole at 26°C (Figures 1 and 2). Previous cell-fractionation studies had indicated that Vps15p was associated with a membrane fraction of yeast cell extracts (Herman *et al.*, 1991). Since the N-terminal myristoylation of several eukaryotic proteins results in their association with a specific intracellular membrane, we examined whether the non-myristoylated Vps15p proteins remained associated with a membrane fraction. Differential centrifugation experiments demonstrated that, like wild-type Vps15p, the G2A mutant protein was associated with a 100 000 *g* pellet fraction and could be extracted from this fraction with 1% Triton X-100 (data not shown). Therefore, the membrane association of Vps15p does not appear to be mediated solely by the N-terminal myristic acid moiety. As with the kinase domain mutant, E151R (see above), the non-myristoylated Vps15 proteins were observed to be partially defective in the *in vivo* Vps15p phosphorylation reaction, as the phosphorylation signal was only ~35–50% of that observed with the wild-type protein (Figure 1).

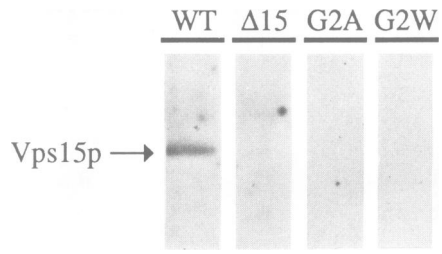

Fig. 3. *In vivo* myristoylation of the Vps15p protein. Yeast cells were labeled with [^{3}H]myristic acid for 60 min at 30°C and immunoprecipitated with antisera specific for Vps15p as described in Materials and methods. The strains examined were PHY112 (*Δvps15*) harboring the multicopy plasmids with the indicated *VPS15* alleles. WT refers to the wild-type *VPS15* allele (pPHY15E) and Δ15 to PHY112 harboring the vector alone (pPHYC18). The position of Vps15p (~170 kDa) is shown.

C-terminal Vps15p truncations exacerbate the effects of alterations in the Vps15p myristoylation site and kinase domain

In order to investigate the role of the large C-terminal domain in Vps15p function, we assessed the biological consequences of removing specific C-terminal sequences from Vps15p. The largest truncation mutant, removing the C-terminal 214 amino acids of Vps15p (ΔC214), exhibited a near wild-type growth rate at 38°C but was slightly defective for CPY sorting at 26°C (Figures 1 and 2). Interestingly, the ΔC214 protein was not phosphorylated *in vivo* suggesting that the C-terminal 214 amino acids of Vps15p either contain the site of phosphorylation or are specifically required for phosphorylation elsewhere within Vps15p (Figure 1; see below).

We subsequently introduced the G2A and E151R alterations into the ΔC214 truncated version of Vps15p (Figure 4A). Both double mutants, G2A/ΔC214 and E151R/ΔC214, exhibited a severe *ts* growth defect and missorted >95% of their CPY to the cell surface (Figure 4). Therefore, the ΔC214 truncation greatly exaggerates the effects of both the G2A and E151R alterations. The relative importance of the ΔC214 truncation in these double mutant interactions is illustrated by an analysis of a G2A/E151R double mutant. This double mutant exhibits a temperature-resistant growth phenotype and >95% of its CPY is properly sorted to the vacuole. Each of the single and double mutant Vps15p proteins described above were demonstrated to be associated with the P100 membrane fraction of yeast cell extracts indicating that their phenotypic effects were not due to the disruption of the Vps15p membrane association (data not shown).

We constructed a series of deletions at the 3′ end of the *VPS15* gene in order to map more precisely the C-terminal domain responsible for the observed synergy (see Materials and methods). These *vps15* deletion alleles encode truncated Vps15 proteins lacking either 167, 128 or 30 C-terminal amino acids. The deletions were constructed both in a wild-type *VPS15* background and in the G2A mutant. Interestingly, each of the double mutants, including G2A/ΔC30, exhibited an extreme *ts* growth defect (data not shown). As expected, each of the single ΔC deletion alleles was able to complement fully the *Δvps15 ts* growth phenotype. These results suggest that a C-terminal deletion of as little as 30 amino acids is sufficient for the synergistic interaction observed with the G2A alteration. In addition, these double

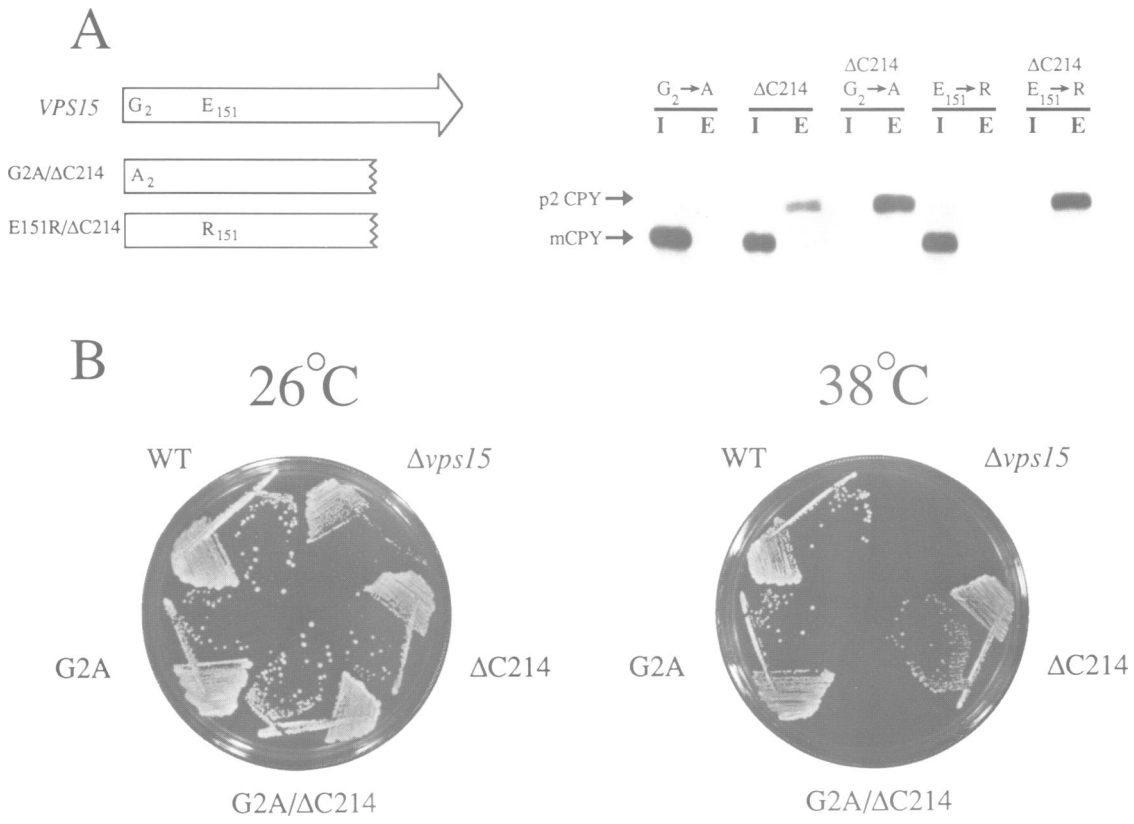

Fig. 4. Analysis of *vps15* double mutants. (**A**) Intracellular sorting of CPY in *vps15* double mutants. A schematic drawing illustrating the two *vps15* double mutants analyzed. A CPY sorting analysis is shown on the right-hand side. Yeast spheroplasts were labeled and processed as described in the legend to Figure 2. The strains were PHY112 harboring different *vps15* alleles on a low copy plasmid (pPHYC18). The mutants encoded Vps15p proteins with the single or double alterations as indicated. (**B**) Analysis of the *ts* growth defect of *vps15* double mutants. PHY112 cells harboring the indicated *vps15* alleles on a low copy yeast plasmid were streaked onto YPD plates and incubated at either 26 or 38°C. WT refers to the wild-type allele (pPHY15C) and Δ*vps15* refers to the vector control (pPHYC18).

mutant effects suggest a role for the Vps15p myristic acid modification in vacuolar protein sorting.

C-terminal deletion mutants of VPS15 C-terminal deletion mutants exhibit a rapid and specific temperature-conditional block in vacuolar protein sorting

The extreme defects observed with the G2A/ΔC214 and E151R/ΔC214 double mutants suggested that the C-terminal domain of Vps15p plays a significant role in Vps15p function. Although the C-terminal deletion mutants exhibited only a minor CPY sorting defect at 26°C, we found that this vacuolar protein sorting defect was greatly exaggerated at elevated growth temperatures. CPY sorting and/or processing in these mutants was analyzed in whole yeast cells at either 26 or 38°C as described in Materials and methods. In wild-type cells, all of the CPY radiolabeled at either 26 or 38°C was present at the 61 kDa mature species, indicative of efficient vacuolar delivery (Figure 5). In contrast, in Δ*vps15* cells all of the CPY was present as the 69 kDa Golgi-modified p2 precursor. In the case of the C-terminal deletion mutants, only a slight CPY processing defect was observed at 26°C, varying from ~5% p2 CPY with the ΔC30 mutant to ~15% with ΔC214 (Figure 5). However, at 38°C, all of the C-terminal deletion mutants were extremely defective for CPY maturation, accumulating >95% of the newly synthesized CPY in a p2 precursor form (Figure 5). These results suggested that each of these mutants exhibited an extreme *ts* defect in vacuolar protein delivery (see below). We have classified this type of allele as *tsf*,

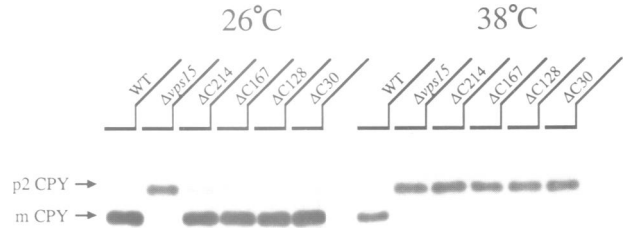

Fig. 5. Temperature-sensitive CPY processing in *vps15* C-terminal deletion mutants. Yeast cells were pre-incubated at either 26 or 38°C for 5 min prior to label addition. The cells were labeled with Tran^{35}S-label for 5 min, methionine and cysteine were added to a final concentration of 25 mM and the cells were chased for an additional 30 min. The labeling and chase were performed at the same temperature as the pre-incubation. The cells were then processed for immunoprecipitation with antisera specific for CPY as described in Materials and methods. All strains analyzed were PHY112 containing a low copy, centromere-containing yeast plasmid with the specific *vps15* allele indicated. WT refers to the wild-type allele (pPHY150) and Δ*vps15* to the vector control (pPHYC18). The positions of mature and p2 CPY are indicated.

or temperature-sensitive for function, to distinguish them from other *vps15* alleles (including null alleles) that result in a *ts* growth defect and a severe CPY missorting phenotype at both the permissive and non-permissive growth temperatures.

We analyzed the fate of the p2 CPY in these 38°C-blocked

cells in a series of pulse–chase temperature-shift experiments. In these experiments, yeast spheroplasts were briefly labeled with Tran^{35}S-label at 26°C and a chase was then initiated by the addition of unlabeled methionine and cysteine. One aliquot of the labeled culture was rapidly shifted to 38°C and chased for 30 min at that temperature. The remainder of the culture was kept at 26°C and also chased for 30 min. The cultures were then fractionated into an intracellular and an extracellular fraction and immunoprecipitations were performed with antisera specific for CPY. Following the short labeling period, the majority of the CPY was present as the ER-modified p1 form in both the wild-type and ΔC30 mutant (Figure 6). In wild-type cells, following the 30 min chase at either 26 or 38°C, all of the radiolabeled CPY was present in the intracellular fraction as a 61 kDa mature form, indicative of correct delivery to the vacuolar compartment. In the ΔC30 mutant, after 30 min of chase at 26°C, ~95% of the CPY was processed to its mature form and was found in the intracellular fraction (Figure 6). However, after 30 min of chase at 38°C, >95% of the radiolabeled CPY was present as the 69 kDa p2 precursor. Surprisingly, >90% of this p2 CPY was not secreted from the cell but was instead found associated with the spheroplast pellet (Figure 6). The observed block in p2 CPY secretion at 38°C was not specific to spheroplasts as we have performed similar temperature-shift experiments with whole yeast cells and have obtained identical results (see below).

In all previously examined *vps15* mutants (as well as all other *vps* mutants examined to date), p2 CPY was not retained within the cell pellet, but was instead efficiently secreted from the cells (Figure 2; Herman *et al.*, 1991). One possible explanation for the observed lack of p2 CPY secretion is that the ΔC30 mutant may exhibit a general block in protein secretion at 38°C. However, the observation that the ΔC30 mutant is able to grow at 38°C with a near wild-type growth rate is not consistent with this explanation. Nevertheless, we directly tested this possibility by examining the secretion of a 33 kDa cell wall protein, CWP33, in ΔC30 and wild-type yeast cells at 38°C. The CWP33 protein is a major constituent of the yeast cell wall and is released from the wall upon treatment with zymolase (Sanz *et al.*, 1987). The passage of this protein through the secretory pathway has been extensively studied (Sanz *et al.*, 1987; Toyn *et al.*, 1988) and it serves as a good marker of secretory protein flow to the cell surface. Using antibody directed specifically against this cell wall protein, we were able to show that ΔC30 mutant spheroplasts secrete the CWP33 protein into the media as efficiently as wild-type yeast at both 26 and 38°C (Figure 6). Therefore, the intracellular retention of p2 CPY in ΔC30 cells at 38°C is not due to a general block in protein secretion in these mutants.

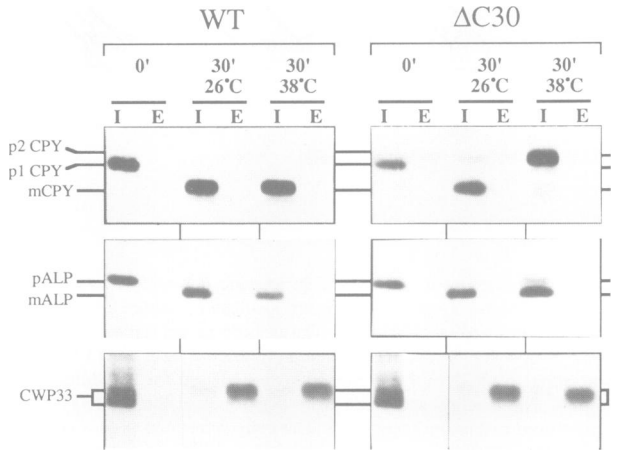

Fig. 6. Temperature-shift analysis of the *ts* defects associated with the ΔC30 mutant. Yeast spheroplasts were labeled with Tran^{35}S-label for 3 min at 26°C and methionine and cysteine were then added to 25 mM to initiate the chase period. One aliquot was chased at 26°C for 30 min and the other at 38°C for 30 min. The labeled cultures were then centrifuged for 2 min at 13 000 *g* and separated into a pellet (I, intracellular) and a supernatant (E, extracellular) fraction. The level of either CPY, ALP or CWP33 in each fraction was assessed by quantitative immunoprecipitation with antisera specific for the appropriate protein. WT refers to PHY112 cells harboring the pPHY150 plasmid (wild-type) and ΔC30 to PHY112 cells with a low copy plasmid carrying the indicated C-terminal deletion allele.

Altogether, our results suggest that the 38°C-induced block in the *vps15* ΔC30 mutant is specific for vacuolar protein traffic as protein secretion appears to continue unabated in this mutant at the elevated temperature. In order to examine the specificity of the vacuolar delivery block more carefully, we analyzed the fate of two additional vacuolar proteins, PrA and ALP, in ΔC30 mutant spheroplasts at 38°C. In identical temperature-shift experiments, we observed that the soluble hydrolase PrA behaved much like CPY; the majority of the radiolabeled PrA was associated with the cell pellet in a Golgi-modified precursor form (data not shown). In contrast, precursor ALP was processed to its mature form with near wild-type kinetics in the ΔC30 mutant blocked at 38°C (Figure 6). Therefore, the temperature-conditional block in vacuolar protein processing appears to be specific for soluble vacuolar hydrolases, as at least one vacuolar membrane protein is processed normally at 38°C. The efficient processing of ALP in ΔC30 cells at 38°C suggests that ALP is delivered to the vacuolar compartment since this proteolytic processing event is mediated by the vacuolar enzyme, PrA (Klionsky *et al.*, 1990). This assertion is further supported by indirect immunofluorescence experiments and biochemical analyses of ALP processing that indicate that ALP is efficiently localized to the vacuolar compartment in *vps15* mutants that display severe CPY localization defects (Klionsky and Emr, 1989; C.Raymond and T.Stevens, personal communication). In addition, as both CPY and ALP are processed in the vacuole in a PrA-dependent manner, the above results indicate that the proCPY accumulated at 38°C is not present in the same intracellular compartment as ALP and is therefore not likely to have reached the vacuolar compartment in temperature-shifted ΔC30 cells.

The onset of p2 CPY secretion is delayed in vps15 ΔC30 cells at 38°C

In a Δ*vps15* mutant essentially all of the CPY is present as the p2 precursor form and the great majority of this p2 CPY was secreted from the cell at both 26 and 30°C (Figure 2 and Herman *et al.*, 1991). We therefore decided to analyze p2 CPY secretion in a *vps15* null mutant at 38°C to test whether *vps15* mutants, in general, were competent for the secretion of vacuolar protein precursors at this elevated temperature. PHY112 (Δ*vps15*::*HIS3*) and wild-type yeast spheroplasts were labeled briefly at 26°C and then were chased at 38°C for 30 min as described above. Under these

conditions, all of the CPY in wild-type cells was processed to mature CPY and was associated with the spheroplast pellet (Figure 7). In the *Δvps15* yeast strain, >95% of the radiolabeled CPY was in its p2 precursor form and most of this was secreted from the cells into the media (Figure 7). Therefore, *vps15* mutants appear to be competent for p2 CPY secretion at 38°C.

In contrast to the above results with a *Δvps15* yeast strain, in the *vps15* ΔC30 mutant, >90% of the radiolabeled CPY was retained within the spheroplast pellet as p2 CPY after a 30 min chase at 38°C (see Figure 6). The fundamental difference between the *Δvps15* and ΔC30 mutants in these temperature-shift experiments is that prior to the imposition of the 38°C block *Δvps15* mutants are completely defective for vacuolar protein sorting while ΔC30 cells exhibit only a very slight CPY sorting defect (Figures 5 and 7). We reasoned that if ΔC30 cells were blocked at 38°C for an extended period of time it might be possible to saturate the intracellular compartment accumulating p2 CPY. At this point, ΔC30 cells would therefore begin to mimic *Δvps15* cells and missort newly synthesized CPY to the cell surface. Since the ΔC30 mutant is able to grow at the elevated temperature with a near wild-type growth rate, the 38°C block could be imposed for any desired length of time. To analyze the effects of pre-incubation at 38°C on p2 CPY secretion, we labeled ΔC30 and wild-type whole cells for 5 min at 38°C after 0, 30 or 60 min of pre-incubation at this temperature. The cultures were then chased for 30 min at 38°C, converted to spheroplasts and separated into media and spheroplast fractions. Immunoprecipitations were then performed with antisera specific to CPY.

In wild-type cells, essentially all of the radiolabeled CPY was present in the spheroplast fraction as mCPY for all three times of pre-incubation (data not shown). With the ΔC30 mutant, we observed that varying the length of the 38°C pre-incubation had a significant effect upon the fate of CPY trafficking through the secretory pathway. When no pre-incubation was performed, >95% of the radiolabeled CPY accumulated in an intracellular fraction as p2 CPY (Figure 7). However, when ΔC30 cells were pre-incubated for either 30 or 60 min prior to labeling, we observed that the majority of the newly synthesized CPY was secreted from the cells as the Golgi-modified p2 precursor (Figure 7). After 60 min of pre-incubation at 38°C, essentially all of the p2 CPY was detected in the extracellular fraction. Therefore, the ΔC30 mutant accumulates p2 CPY within a saturable compartment following a temperature shift to 38°C.

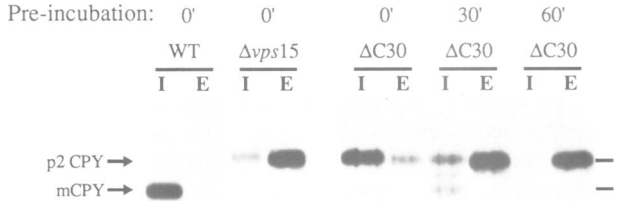

Fig. 7. p2 CPY secretion in the ΔC30 mutant. For the first two pairs of lanes, PHY112 spheroplasts harboring either pPHY150 (WT, wild-type allele) or pPHYC18 (*Δvps15*, vector) were labeled with Tran^{35}S-label for 3 min at 26°C. The cultures were then shifted to 38°C and chased for 30 min at this temperature following the addition of methionine and cysteine to 25 mM. The spheroplasts were processed as described below. For the final three pairs of lanes, PHY112 cells harboring a single copy yeast plasmid with the ΔC30 allele were pre-incubated for 0, 30 or 60 min at 38°C. The cells were then labeled with Tran^{35}S-label for 5 min at 38°C and chased for an additional 30 min at 38°C. The chase was initiated by the addition of methionine and cysteine to a final concentration of 25 mM. The whole cells were converted to spheroplasts, separated into a pellet (I, intracellular) and a supernatant (E, extracellular) fraction by centrifugation at 13 000 *g* for 2 min and immunoprecipitated with antisera specific to CPY. The positions of mature and p2 CPY are indicated.

The vps15 ΔC30 temperature-dependent block in CPY sorting is reversible

When the ΔC30 mutant is rapidly shifted to 38°C, p2 CPY and other soluble vacuolar precursors accumulate within an intracellular compartment. We examined the reversibility of this 38°C block in order to gain some insight into the nature of this compartment. As above, we briefly labeled ΔC30 spheroplasts at 26°C and then chased with unlabeled methionine and cysteine for 30 min at 38°C. After this chase period, ~95% of the radiolabeled CPY is cell-associated and in its p2 precursor form (Figure 8). This culture was subsequently split in half; one aliquot remained at 38°C for an additional 20 min and the other was placed at 26°C for 20 min. The additional 20 min chase at 38°C did not significantly alter the CPY distribution in the ΔC30 cells (Figure 8). The p2 CPY that was blocked after 30 min at 38°C remained blocked after an additional 20 min at 38°C. In contrast, >90% of the p2 CPY that accumulated at 38°C was processed to its mature form upon shifting the ΔC30

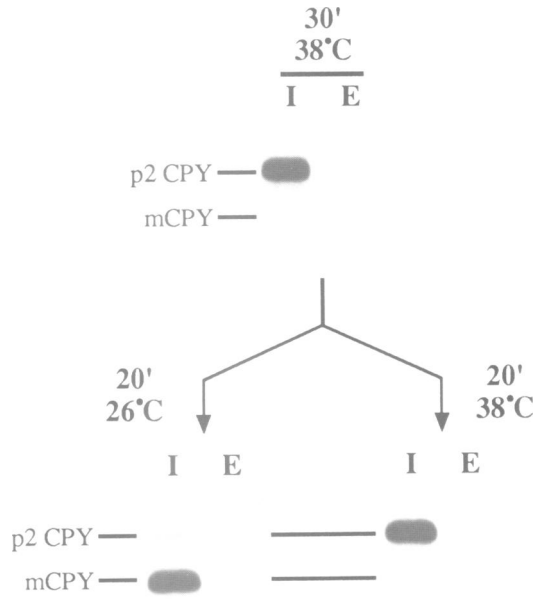

Fig. 8. Reversibility of *ts* vacuolar protein delivery defect of ΔC30 mutants. PHY112 spheroplasts harboring a single copy yeast plasmid with the ΔC30 allele were labeled for 3 min at 26°C and chased for 30 min at 38°C following the addition of methionine and cysteine to 25 mM. The labeled culture was then split into three and processed as follows. One aliquot was centrifuged for 2 min at 13 000 *g* and separated into a pellet (I, intracellular) and a supernatant (E, extracellular) fraction. The level of CPY in each fraction was assessed by quantitative immunoprecipitation with antisera to CPY. The second aliquot was chased for an additional 20 min at 38°C and the third for 20 min at 26°C. These latter two aliquots were then processed as the first. The positions of mature and p2 CPY are indicated.

cells back to 26°C suggesting that this CPY has been efficiently delivered to the yeast vacuolar compartment (Figure 8). Our results therefore indicate that the great majority of the p2 CPY that accumulates within 38°C-blocked ΔC30 cells remains competent for subsequent vacuolar delivery during the time that the block is imposed. Moreover, the efficient reversal of this temperature block suggests that the compartment housing the p2 CPY may in fact represent a normal functional intermediate in the vacuolar protein delivery pathway.

A short C-terminal domain of Vps15p is required for its phosphorylation in vivo

Vps15p has been demonstrated to be a phosphoprotein *in vivo* and mutational analyses of the Vps15p kinase domain have suggested that this phosphate incorporation may be due to a specific autophosphorylation reaction (Figure 1; Herman *et al.*, 1991). The ΔC214 truncated Vps15p protein was very defective for this *in vivo* phosphorylation reaction (Figure 1). Since the ΔC214 mutant also exhibits a *ts* CPY sorting defect, it is possible that the phosphorylation of Vps15p is important for it to achieve, or maintain, an active conformation at elevated growth temperatures. We therefore analyzed the *in vivo* phosphorylation of each of the C-terminal Vps15p truncation proteins. All of the C-terminal deletion mutants including ΔC30, were very defective for the *in vivo* phosphorylation of Vps15p (Figure 9). In order to determine if the observed lack of Vps15p phosphorylation was specifically due to the deletion of sequences within the C-terminal 30 amino acids of Vps15p (total of 1455 amino acids), we constructed an internal deletion of amino acids 1412–1427, ΔI16, and analyzed the phosphorylation of this Vps15p mutant protein. The ΔI16 protein was observed to be phosphorylated to a wild-type level (Figure 9). This result indicates that the lack of Vps15p phosphorylation in the C-terminal deletion mutants is not due to a general misfolding of Vps15p proteins that possess deletions in their C termini. Rather, our results suggest that a short C-terminal domain of Vps15p is specifically required for its phosphorylation *in vivo*. Furthermore, since deletions of this domain result in a *ts* vacuolar protein sorting defect, the phosphorylation of Vps15p may be essential for its biological activity at elevated temperatures.

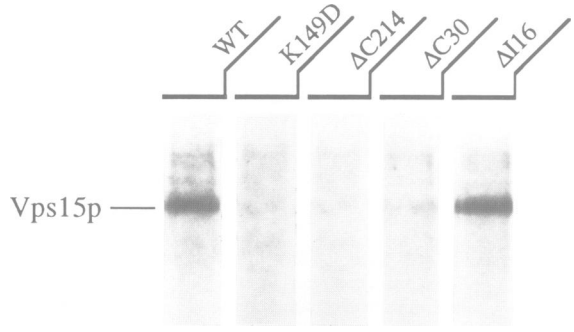

Fig. 9. *In vivo* phosphorylation of the *VPS15* C-terminal deletion mutants. Yeast cells were labeled with $^{32}PO_4$ for 30 min at 30°C and immunoprecipitated with antisera specific for Vps15p as described in Materials and methods. PHY112 cells harboring the indicated *vps15* alleles on multicopy plasmids were analyzed. WT refers to plasmid pPHY15E (wild-type allele). The position of Vps15p (~170 kDa) is shown.

Discussion

In a previous study, we identified a membrane-associated protein kinase, encoded by the yeast *VPS15* gene, that is essential for the efficient vacuolar delivery of multiple soluble hydrolases. The severe phenotypic consequences of mutations within the *VPS15* protein kinase domain, together with the specificity of *vps15* defects for soluble constituents of the vacuole, suggested that Vps15p might regulate specific protein phosphorylation reactions required for the delivery of soluble proteins to the yeast vacuole. In addition to the N-terminal 300 amino acid protein kinase domain, Vps15p also possesses a myristic acid addition site at its N terminus and a large C-terminal domain of >1100 amino acids that exhibited no significant similarity to any other known protein sequence. In this study, we have extended our previous mutational analysis of Vps15p and have now examined multiple alterations in each of these three Vps15p domains.

Vps15p kinase domain mutants

The Vps15p sequence exhibits a significant degree of similarity to the catalytic domains of the serine/threonine family of protein kinases. With this present study, we have now constructed mutations in four distinct Vps15p kinase subdomains and have altered specific amino acid residues that are highly conserved among all protein kinase molecules. This high level of conservation, together with observations from chemical modification experiments, suggests that these amino acids are directly involved in catalytic function (see Hanks *et al.*, 1988; Taylor *et al.*, 1990). Alterations within each of the four Vps15p kinase motifs result in severe defects in both the delivery of soluble hydrolases to the vacuole and the *in vivo* phosphorylation of Vps15p (Figure 1). Therefore these mutational studies suggest that the Vps15p kinase regulates Vps15p phosphorylation and that it may do so directly, by catalyzing a specific autophosphorylation reaction. We are currently attempting to demonstrate Vps15p kinase activity *in vitro* in order to determine if the *in vivo* phosphorylation of Vps15p does indeed correspond to an autophosphorylation reaction. Since the phenotype of *VPS15* kinase domain mutations (e.g. D165R in Figure 1) are generally more extreme than those associated with a loss of Vps15p phosphorylation (e.g. ΔC30 mutant in Figure 1), the Vps15p kinase domain likely regulates additional protein phosphorylation reactions *in vivo*. The severe vacuolar protein sorting defects associated with Vps15p kinase domain alterations would therefore be due to defects in multiple Vps15p-mediated protein phosphorylation reactions involving as yet unknown cellular substrates of the Vps15p kinase. Furthermore, the relatively efficient sorting of CPY to the vacuole in *vps15* mutants that are defective for the *in vivo* phosphorylation of Vps15p suggests that the phosphorylation of Vps15p is not essential for the delivery of soluble hydrolases to the yeast vacuole.

Vps15p is modified at its N terminus by the addition of myristic acid

Biochemical labeling experiments with [^{3}H]myristic acid indicated that Vps15p is modified *in vivo* by the addition of the 14-carbon fatty acid, myristate, at its N terminus. However, this lipophilic moiety does not appear to mediate the observed membrane association of Vps15p as non-myristoylated forms of this protein remain associated with a similar membrane fraction of yeast cell extracts. Although

the majority of myristoylated proteins are associated with specific intracellular membranes, there are several examples of proteins that are myristoylated but soluble, including the catalytic domain of the cAMP-dependent protein kinase (Towler *et al.*, 1988b). The biological significance of the myristic acid in these soluble proteins is generally not known. In the case of the cAMP-dependent protein kinase, the myristate does not appear to be important for catalytic activity as the myristoylated and non-myristoylated forms of the catalytic subunit have identical kinase activities *in vitro* (Slice and Taylor, 1989). We have found that the non-myristoylated form of Vps15p is phosphorylated to a lesser extent than the wild-type protein *in vivo*. If the observed *in vivo* phosphorylation of Vps15p is due to a specific autophosphorylation reaction, it will be interesting to determine if the myristate moiety added to the N terminus of Vps15p directly influences the catalytic activity of this enzyme.

C-terminal truncations of Vps15p result in a temperature-conditional defect in vacuolar protein sorting

The identification of conditional alleles of a given genetic locus generally allows for the development of a more complete understanding of the precise role and stage at which the gene product acts in the biochemical or developmental pathway under study. For example, in the analysis of the yeast *sec* mutants, the accumulation of specific intermediates at the non-permissive temperature allowed Schekman and his co-workers to assign particular gene functions to different positions throughout the secretory pathway (Schekman and Novick, 1982). Our initial genetic selection uncovered multiple *vps15* alleles that were *ts* for growth; however, all of these alleles resulted in an equally severe CPY sorting defect at both the permissive and non-permissive growth temperatures (Robinson *et al.*, 1988). During our present analysis of a series of C-terminal Vps15p deletions, we found that relatively short C-terminal truncations of Vps15p, removing as little as 30 amino acids, resulted in a severe *ts* defect in the delivery of CPY to the vacuole. This *ts* defect is especially dramatic in the ΔC30 mutant where CPY delivery is essentially wild-type at the permissive temperature but almost completely blocked when cells are shifted to the restrictive temperature of 38°C. Upon imposition of the temperature block, ΔC30 cells accumulate ~95% of the newly synthesized CPY within an intracellular compartment as a p2 precursor molecule. This intracellular retention of p2 CPY was a somewhat unexpected result as all of the previously characterized *vps15* mutants efficiently secrete p2 CPY. Furthermore, the *ts* vacuolar protein delivery defects associated with the ΔC30 mutant appear to be specific for soluble vacuolar hydrolases. ALP, a vacuolar membrane protein, is efficiently processed to its mature form at the non-permissive temperature in ΔC30 mutants. Since ALP and CPY are both processed in the vacuole in a PrA-dependent manner (reviewed in Klionsky *et al.*, 1990), the efficient maturation of ALP suggests that ΔC30 cells are processing-proficient and that CPY is apparently sequestered within an intracellular compartment distinct from the vacuole. Therefore the *ts* defects observed in the C-terminal deletion mutants are apparently due to a failure to deliver CPY to the vacuole properly.

The *ts* vacuolar protein delivery block in the Δ30 mutant exhibits an extremely rapid rate of onset as an essentially complete block in CPY processing can be established in < 1 min of incubation at the non-permissive temperature. The rapid onset of the mutant phenotype suggests that the *VPS15* gene product is quickly inactivated at 38°C and that this inactivation almost immediately manifests itself as a defect in CPY delivery to the vacuole. This, in turn, provides strong genetic evidence that Vps15p is directly involved in the sorting and/or delivery of proteins to the vacuole. Therefore the observed vacuolar protein sorting defects in *vps15* mutants are not likely to result as a secondary consequence of the loss of Vps15p function. Although the exact manner in which the C-terminal domain influences Vps15p activity is unclear, the observation that all of the C-terminal truncated forms of Vps15p, including ΔC30, are not phosphorylated *in vivo* suggests that the phosphorylation of Vps15p is important for its biological activity at elevated temperatures. The C-terminal 30 amino acids of Vps15p may either include the site of Vps15p phosphorylation or else constitute a specific domain required for phosphorylation elsewhere within this protein. We are presently attempting to map the specific site(s) of protein phosphorylation in Vps15p in order to determine how this phosphorylation, and the C-terminal domain in general, contribute to Vps15p function.

The immediate phenotypic consequence of a loss of Vps15p activity therefore appears to be the intracellular accumulation of Golgi-modified precursor forms of soluble vacuolar hydrolases, such as CPY and PrA. These precursors appear to be present within a specific intracellular compartment that is distinct from the vacuole. This compartment does not appear to represent an aberrant, dead-end structure since the resident p2 CPY can be efficiently processed to its mature form following a shift back to the permissive temperature. Moreover, the rapid and efficient reversal of the 38°C delivery block in ΔC30 cells suggests that the accumulated p2 CPY was present within a normal intermediate of the vacuolar protein transport pathway. Since very little is presently known about the transport intermediates functioning between the Golgi complex and the vacuole, the identification and characterization of this p2 CPY compartment could provide some fundamental insights into the vacuolar protein transport process. It is interesting to note that an electron microscopic analysis of *vps15* mutants indicated that 80 nm vesicles and abnormal membranous structures that resembled Golgi-derived Berkeley bodies accumulated within the cytoplasm of these cells (Banta *et al.*, 1988).

Models for Vps15p function in vacuolar protein sorting

CPY synthesized in ΔC30 cells immediately following a shift to 38°C accumulates within a saturable intracellular compartment as its p2 precursor form. This p2 CPY remains cell-associated during at least 50 min of subsequent incubation at 38°C. These data suggest that this p2 CPY is sequestered within a compartment that is unable to communicate with the cell surface. One possibility is that this p2 CPY has been sorted away from secretory protein traffic and packaged into a specific transport intermediate, possibly a membrane vesicle, committed for delivery to the vacuole. If Vps15p activity is required for either the delivery or fusion of these vesicles to the vacuole then the thermal

inactivation of Vps15p could result in the accumulation of p2 CPY within this intracellular compartment. In addition, this block in vacuolar protein delivery would also prevent the recycling of transport factors back to the Golgi where they could catalyze additional rounds of transport. In this model, p2 CPY would be packaged into vesicles until a cellular component essential for either this packaging, or the formation of the transport intermediate, became limiting. Once the system was saturated, p2 CPY would enter the default secretion pathway and be delivered to the cell surface. In an alternative model, Vps15p activity could be specifically required for the packaging of certain receptor–ligand (e.g. receptor-p2 CPY) complexes into transport vesicles destined for the vacuole. A loss of Vps15p activity would lead to an accumulation of p2 CPY, bound by its specific transmembrane receptor, in a late Golgi compartment. In this second model, the accumulated p2 CPY in the *vps15* ΔC30 mutant at 38°C would therefore be present within a late Golgi compartment. Secretion would then result from the saturation of these p2 CPY-specific receptors.

Although *vps15* mutants exhibit severe defects in the localization of several soluble vacuolar hydrolases, vacuolar membrane proteins, such as ALP, appear to be delivered to the vacuole in *vps15* cells. This observation has several interesting implications for Vps15p function within the vacuolar protein delivery pathway (Figure 10). ALP and CPY could transit together from a late Golgi compartment to the vacuole within the same vesicular transport intermediate. Since ALP is efficiently delivered to the vacuole in the *vps15* ΔC30 mutant, the cellular machinery required for the formation and delivery of these transport vesicles must be present and functional. Therefore, Vps15p would presumably function prior to these transport events and could be required for the packaging of p2 CPY into the vacuole-bound transport intermediates (Figure 10). On the other hand, CPY and ALP might be delivered to the vacuole by two independent pathways involving separate and distinct carriers. In this model, Vps15p could act at any step along the CPY delivery pathway but would not be required for transit along the ALP route (Figure 10). The intracellular compartment that accumulates p2 CPY in the ΔC30 *vps15* mutant is therefore a candidate for the transport intermediate trafficking between the Golgi and vacuolar compartments in the CPY-specific delivery pathway. Purification and compositional studies of this compartment could provide important insights into the precise mechanisms regulating vacuolar/lysosomal protein sorting in yeast and other eukaryotes.

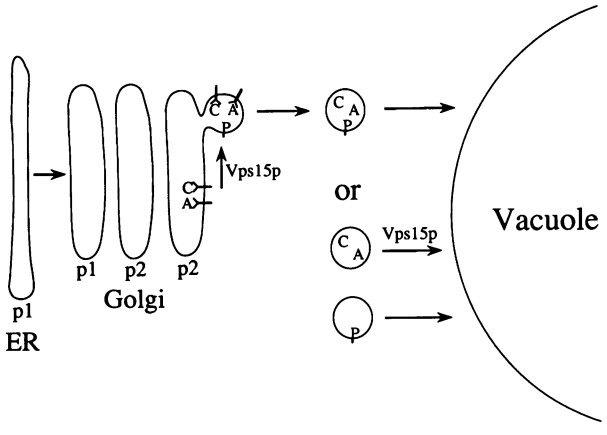

Fig. 10. Possible roles for Vps15p in vacuolar protein sorting. Two possible roles for Vps15p in the vacuolar protein delivery pathway are shown. In the top model, soluble vacuolar hydrolases, such as CPY and PrA, are shown trafficking to the vacuole within the same transport vesicle as vacuolar membrane proteins such as ALP. Vps15p is shown as functioning in the specific packaging of soluble vacuolar proteins into transport carriers. In the bottom model, soluble and membrane proteins are shown to be transiting to the vacuole via distinct transport intermediates (e.g. vesicles). In this model, Vps15p is specifically required for the recognition or fusion of the soluble hydrolase-containing vesicles with the vacuolar compartment. The different vacuolar proteins are indicated as follows: C, CPY; A, PrA; and P, ALP. The p1 and p2 designations refer to the form of CPY or PrA present in the indicated secretory pathway compartment.

Materials and methods

Strains and media

Escherichia coli strains BW313 (*F' lysA/dut ung thi-1 relA spoT1*; Kunkel, 1985) and CJ236 (*dut1 ung1 thi-1 relA1*/pCJ105 [cam^{r}F1]; Kunkel *et al.*, 1987) were used for the oligonucleotide-directed mutagenesis experiments and JM101 (*F' traD36 lacI^{q}ZΔM15 proAB/supE thiDlac Δlac-pro*; Miller, 1972) was used for all other purposes. The *S.cerevisiae* strains PHY112 (*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9 Δvps15::HIS3*; Herman *et al.*, 1991) and SEY6210 (*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801 suc2-Δ9*; Robinson *et al.*, 1988) were used for all experiments described in this study. Standard yeast and *E.coli* media were used and supplemented as needed (Miller, 1972; Sherman *et al.*, 1979).

Yeast methods

Standard yeast genetic methods were used throughout this study (Sherman *et al.*, 1979). Yeast cells were transformed by the method of alkali cation treatment (Ito *et al.*, 1983) and transformants were selected on the appropriate SD media.

Plasmid construction and oligonucleotide-directed mutagenesis

Recombinant DNA manipulations were performed as described previously (Ausubel *et al.*, 1987; Maniatis *et al.*, 1982). Two yeast shuttle vectors, pPHYC18 (*CEN, URA3*; Herman and Emr, 1990) and pJSY324 (2μ, *TRP1*) were used for most of the plasmid constructions described within this study. The yeast vector pJSY324 was constructed by subcloning the 1.6 kb *Hpa*I–*Hin*dIII fragment of YEp13, containing the yeast 2μ plasmid origin of replication, into the *Aat*II site of pRS304 (Sikorski and Hieter, 1989). The *Hin*dIII 3′ recessed ends were filled in with Klenow polymerase and the appropriate deoxynucleotides prior to ligation. The yeast *VPS15* plasmids pPHY15-1, pPHY15C and pPHY15E were described previously (Herman *et al.*, 1991).

Oligonucleotide-directed mutagenesis of the *VPS15* gene was performed using *dut$^-$ ung$^-$ E.coli* as described previously (Kunkel, 1985; Ausubel *et al.*, 1987). Two different mutagenesis procedures were used to generate the mutants described in Figure 1. The first scheme was used to produce the G2 → A and E151 → R alterations and was described previously (Herman *et al.*, 1991). For the second, the *VPS15* gene was subcloned as a 4.8 kb *Sca*I–*Sna*BI fragment from pPHY15-1 into the *Sma*I site of both pJSY324 and pBluescriptII KS$^+$ (Stratagene) to produce pJSY324.15 and pBP.15, respectively. These *VPS15*-containing plasmids were introduced into the *dut$^-$ ung$^-$ E.coli* strain CJ236 and single-stranded plasmid DNA was isolated and mutagenized as described in the Bio-Rad MutaGene kit manual. The mutagenized *VPS15* DNA was subsequently subcloned as a *Bam*HI–*Xho*I fragment into pPHYC18 and pJSY324 as required. The wild-type *Sca*I–*Sna*BI fragment was also cloned into the *Sma*I site of pPHYC18 to generate pPHY150.

The *VPS15* C-terminal deletion plasmids were constructed by subcloning the appropriate *VPS15* restriction fragments from pPHY15-1 into the *Sma*I site of pPHYC18. The following restriction fragments were gel-isolated and, if required, were treated with either T4 DNA polymerase to remove 3′ overhangs, or Klenow polymerase to fill in 3′ recessed ends: *Sca*I–*Bsp*HI (ΔC30), *Sca*I–*Hgi* AI (ΔC128) and *Sca*I–*Xmn*I (ΔC167). For the double mutants combining either the G2 → A or E151 → R alteration with a specific C-terminal deletion, the above fragments were excised from either pPHYC15-G2A (G2 → A) or pPHYC15-E151R (E151 → R), respectively.

The largest C-terminal deletion, removing 214 amino acids of the Vps15p, was constructed as follows. First, the 3.6 kb *ClaI* fragment of pPHY15-1 was subcloned into the *ClaI* site of pPHYC18 to produce the plasmid pPHY127. The 1.8 kb *XbaI* fragment from M13V15 RF DNA (see Herman *et al.*, 1991), wild-type or mutagenized, was gel-isolated and used to replace the *XbaI* fragment of pPHY127, thereby reconstructing a *VPS15* gene truncated at the 3′ internal *ClaI* site.

Cell labeling and immunoprecipitation

Immunoprecipitations from whole yeast cells labeled with Tran^{35}S-label (ICN Radiochemicals) were performed as described previously (Herman and Emr, 1990; Herman *et al.*, 1991). For the analysis of the temperature-conditional CPY processing defect in the *VPS15* C-terminal deletion mutants, yeast cells were pre-incubated at either 26 or 38°C for 5 min prior to labeling. The cells were then labeled with Tran^{35}S-label for 5 min and chased for 30 min at the same temperature. The chase was initiated by the addition of unlabeled methionine and cysteine to 25 mM. CPY fractionation immunoprecipitations from radiolabeled yeast spheroplasts were performed as described previously (Robinson *et al.*, 1988).

For the *ts* shift experiments, typically 20 OD_{600} cell equivalents of yeast spheroplasts were labeled for 3 or 4 min at 26°C with Tran^{35}S-label in 2.5 ml of spheroplast labeling media (Wickerham's minimal proline media supplemented with 1.3 M sorbitol; Wickerham, 1946). The culture was then divided into two aliquots of 1.5 and 1.0 ml. To the first aliquot an equal volume of 47–48°C chase solution (spheroplast labeling media plus 50 mM methionine, 50 mM cysteine and 0.4% yeast extract) was added to rapidly bring up the culture temperature. The chase was then continued for 30 min at 38°C. To the second aliquot, an equal volume of 26°C chase solution was added and 1.0 ml was removed and fractionated for subsequent immunoprecipitation as follows. The spheroplast cultures were centrifuged at 13 000 *g* for 10 s and the supernatant was carefully removed and TCA was added to a final concentration of 5%. The spheroplast pellet was resuspended in 1.0 ml of 5% TCA. The TCA precipitations were held on ice for at least 20 min. The remaining half of the second aliquot was chased for 30 min at 26°C. After 30 min of chase, 1.0 ml of both the 38 and 26°C cultures were fractionated as described above. One-half, or 1.0 ml, of the 38°C culture was shifted to 26°C and the other half was kept at 38°C for an additional 20 min. After this additional chase, the samples were processed as described above. For the whole cell temperature-shift experiments, a similar experimental protocol was followed except that the labeled aliquots removed for analysis were added to an equal volume of ice-cold stop solution (50 mM Tris–HCl, pH 7.5, 2 M sorbitol, 40 mM NaN_3, 40 mM NaF, 20 mM dithiothreitol) and held on ice for 5 min. Zymolyase-100T (Seikagako Kogyo Co.) was added to 20 $\mu g/OD_{600}$ cell equivalents and spheroplasting was carried out for 30 min at 30°C. The cultures were then fractionated as described above for the spheroplast labelings. In some experiments, the chase solution was supplemented by the addition of 0.04 vol of 1 M Tris–HCl, pH 7.0, and/or α_2-macroglobulin to a final concentration of 1 mg/ml. The antiserum specific for CWP33 was a generous gift from P.Sanz.

The *in vivo* phosphate labeling of yeast cells and the subsequent immunoprecipitation of Vps15p were performed as described previously (Herman *et al.*, 1991) except that the protein A–Sepharose beads were washed as follows: once with Tween-20 IP buffer (0.5% Tween-20, 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1 mM EDTA) and once with IP buffer 2 (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1 mM EDTA).

For the analysis of Vps15p myristoylation, yeast cells were grown to mid-logarithmic phase in Wickerham's minimal media supplemented with 0.2% yeast extract (Wickerham, 1947; Robinson *et al.*, 1988). Five OD_{600} units of cells were collected by centrifugation and resuspended in 1 ml of the same media. Cerulenin (Sigma) was added to a final concentration of 20 μg/ml and the cells were incubated for 20 min at 30°C. Following this pre-incubation, 1.0 mCi of [9,10(*n*)-^{3}H]myristic acid (New England Nuclear) was added and the cells were labeled for 60 min at 30°C. The relatively short labeling period was chosen to minimize the conversion of the labeled myristic acid to other fatty acids, such as palmitate. The labeling was terminated by the addition of TCA to a final concentration of 5%. The protein A–Sepharose beads were washed as described above for the *in vivo* phosphorylations.

Functional analysis of vps15 mutants

For this study, we assayed the biological activities of the engineered *vps15* alleles by introducing these alleles into a Δ*vps15* yeast strain (PHY112) on either a low copy centromere-containing plasmid (pPHYC18), or multicopy 2μ circle derivative (pJSY324). The low copy plasmids were tested for their ability to complement the severe *ts* growth defect (at 38°C) and the CPY localization defects associated with Δ*vps15* yeast strains. The *in vivo* phosphorylation of the mutant Vps15p proteins was assayed by labeling Δ*vps15* yeast strains, carrying the appropriate *vps15* alleles on a 2μ multicopy plasmid, with [^{32}P]orthophosphate and then immunoprecipitating with antisera specific for Vps15p.

Acknowledgements

We thank members of the Emr laboratory for critically reading the manuscript and for many helpful discussions during the course of this work. This work was supported by the Howard Hughes Medical Institute and a grant from the National Science Foundation (to S.D.E.). This work is dedicated to the memory of E.W.Herman.

References

Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. (1987) *Current Protocols in Molecular Biology.* John Wiley and Sons Inc., New York.

Bankaitis,V.A., Johnson,L.M. and Emr,S.D. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 9075–9079.

Banta,L.M., Robinson,J.S., Klionsky,D.J. and Emr,S.D. (1988) *J. Cell Biol.*, **107**, 1369–1383.

Banta,L.M., Vida,T.A., Herman,P.K. and Emr,S.D. (1990) *Mol. Cell. Biol.*, **10**, 4638–4649.

Ben-David,Y., Letwin,K., Tannock,L., Bernstein,A. and Pawson,T. (1991) *EMBO J.*, **10**, 317–325.

Brenner,S. (1987) *Nature*, **329**, 21.

Featherstone,C. and Russell,P. (1991) *Nature*, **349**, 808–811.

Graham,T.R. and Emr,S.D. (1991) *J. Cell Biol.*, **114**, 207–218.

Hanks,S.K. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 388–392.

Hanks,S.K., Quinn,A.M. and Hunter,T. (1988) *Science*, **241**, 42–52.

Herman,P.K. and Emr,S.D. (1990) *Mol. Cell. Biol.*, **10**, 6742–6754.

Herman,P.K., Stack,J.H., DeModena,J.A. and Emr,S.D. (1991) *Cell*, **64**, 425–437.

Ito,H., Fukuda,Y., Murata,K. and Kimura,A. (1983) *J. Bacteriol.*, **153**, 163–168.

Johnson,L.M., Bankaitis,V.A. and Emr,S.D. (1987) *Cell*, **48**, 875–885.

Kamps,M.P. and Sefton,B.M. (1986) *Mol. Cell. Biol.*, **6**, 751–757.

Klionsky,D.J. and Emr,S.D. (1989) *EMBO J.*, **8**, 2241–2250.

Klionsky,D.J. and Emr,S.D. (1990) *J. Biol. Chem.*, **265**, 5349–5352.

Klionsky,D.J., Banta,L.M. and Emr,S.D. (1988) *Mol. Cell. Biol.*, **8**, 2105–2116.

Klionsky,D.J., Herman,P.K. and Emr,S.D. (1990) *Microbiol. Rev.*, **54**, 266–292.

Kornfeld,S. and Mellman,I. (1980) *Annu. Rev. Cell Biol.*, **5**, 483–525.

Kunkel,T.A. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 488–492.

Kunkel,T.A., Roberts,J.D. and Zakour,R.A. (1987) *Methods Enzymol.*, **154**, 367–382.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Miller,J. (1972) *Experiments in Molecular Genetics.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Pfeffer,S.R. and Rothman,J.E. (1987) *Annu. Rev. Biochem.*, **56**, 829–852.

Raymond,C.K., O'Hara,P.J., Eichinger,G., Rothman,J.H. and Stevens,T.H. (1990) *J. Cell Biol.*, **111**, 877–892.

Robinson,J.S., Klionsky,D.J., Banta,L.M. and Emr,S.D. (1988) *Mol. Cell. Biol.*, **8**, 4936–4948.

Rothman,J.H. and Stevens,T.H. (1986) *Cell*, **47**, 1041–1051.

Rothman,J.H., Howald,I. and Stevens,T.H. (1989) *EMBO J.*, **8**, 2057–2065.

Sanz,P., Herrero,E. and Sentandreu,R. (1987) *Biochim. Biophys. Acta*, **924**, 193–203.

Schekman,R. and Novick,P. (1982) In Strathern,J.N., Jones,E.W. and Broach,J.R. (eds), *The Molecular Biology of the Yeast Saccharomyces.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 361–393.

Schultz,A.M., Henderson,L.E. and Oroszlan,S. (1988) *Annu. Rev. Cell Biol.*, **4**, 611–647.

Sherman,F., Fink,G.R. and Lawrence,L.W. (1979) *Methods in Yeast Genetics: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sikorski,R.S. and Hieter,P. (1989) *Genetics*, **122**, 19–27.

Slice,L.W. and Taylor,S.S. (1989) *J. Biol. Chem.*, **264**, 20940–20946.

Taylor,S.S., Buechler,J.A. and Yonemoto,W. (1990) *Annu. Rev. Biochem.*, **59**, 971–1005.

Towler,D.A., Adams,S.P., Eubanks,S.R., Towery,D.S., Jackson-

Machelski,E., Glaser,L. and Gordon,J.I. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 2708–2712.
Towler,D.A., Adams,S.P., Eubanks,S.R., Towery,D.S., Jackson-Machelski,E., Glaser,L. and Gordon,J.I. (1988a) *J. Biol. Chem.*, **263**, 1784–1790.
Towler,D.A., Gordon,J.I., Adams,S.P. and Glaser,L. (1988b) *Annu. Rev. Biochem.*, **57**, 69–99.
Toyn,J., Hibbs,A.R., Sanz,P., Crowe,J. and Meyer,D.I. (1988) *EMBO J.*, **7**, 4347–4353.
Valls,L.A., Hunter,C.P., Rothman,J.H. and Stevens ,T.H. (1987) *Cell*, **48**, 887–897.
Wickerham,L.J. (1946) *J. Bacteriol.*, **52**, 293–301.
Zoller,M.J., Nelson,N.C. and Taylor,S.S. (1981) *J. Biol. Chem.*, **256**, 10837–10842.

Received on July 26, 1991; revised on September 9, 1991