A role for clathrin in the sorting of vacuolar proteins in the Golgi complex of yeast

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We have investigated the role of clathrin in vacuolar protein sorting using yeast strains harboring a temperature-sensitive allele of clathrin heavy chain (*chc1-ts*). After a 5 min incubation at the non-permissive temperature (37°C), the *chc1-ts* strains displayed a severe defect in the sorting of luminal vacuolar proteins. Sorting of a vacuolar membrane protein, alkaline phosphatase, and transport to the surface of a cell wall protein, was not affected at 37°C. In *chc1-ts* cells incubated at 37°C, secretion of the missorted luminal vacuolar protein carboxypeptidase Y (CPY) was blocked by the *sec1* mutation which prevents fusion of secretory vesicles to the plasma membrane. Unexpectedly, *chc1-ts* cells incubated for extended periods at 37°C regained the ability to sort CPY. Cells carrying deletions of the *CHC1* gene (*chc1Δ*) also sorted CPY to the vacuole even when subjected to temperature shifts. Vacuolar delivery of CPY in *chc1Δ* cells was not blocked by *sec1* suggesting that transport does not occur by secretion and endocytosis. These results provide *in vivo* evidence that clathrin plays a role in the Golgi complex in sorting of vacuolar proteins from the secretory pathway. With time, however, yeast cells lacking functional clathrin heavy chains are able to adapt in a way that allows restoration of vacuolar protein sorting in the Golgi complex. These conclusions clarify previous studies of *chc1Δ* cells which raised the possibility that clathrin is not involved in vacuolar protein sorting. **Key words:** clathrin/Golgi complex/sorting/vacuole/yeast

Introduction

Eukaryotic cells are organized into structurally and functionally diverse membrane-delineated compartments, each containing a distinct set of proteins. This elaborate organization relies on the accurate distribution of newly synthesized proteins to their resident compartments (Pfeffer and Rothman, 1987). In the case of the major hydrolytic compartments, lysosomes in mammalian cells and vacuoles in yeast, the resident luminal proteins are synthesized as precursors which follow the secretory pathway into the Golgi complex (Kornfeld and Mellman, 1989; Rothman *et al.*, 1989; Klionsky *et al.*, 1990). In a late Golgi compartment, the precursors are sorted from proteins destined for the cell surface and directed into a pathway leading to the hydrolytic organelles. If the sorting process is disrupted, the precursors continue along the secretory pathway and are released into the extracellular environment. Thus, the identification of components responsible for segregating lysosomal/vacuolar

precursors from the secretory pathway is central to understanding the sorting process.

Studies of mammalian cells have implicated clathrin-coated membranes in the sorting of lysosomal precursors (Kornfeld and Mellman, 1989). The well-characterized role of clathrin in receptor-mediated endocytosis serves as a paradigm for its involvement in lysosomal sorting (Pearse and Robinson, 1990). During endocytosis, transmembrane receptors for extracellular ligands cluster in clathrin-coated pits along the plasma membrane. The coats consist of clathrin heavy and light chain subunits and complexes of associated proteins. Clathrin-coated pits invaginate and pinch off, carrying the receptors and their ligands into the resulting clathrin-coated vesicles. The clathrin coat is then rapidly disassembled to allow the vesicle to fuse with the next endocytic compartment. The selectivity inherent in endocytosis is mediated in part by the cytoplasmic domains of the receptors; mutations in the tails of receptors eliminate clustering in coated pits and reduce receptor endocytosis (Pearse and Robinson, 1990). Evidence for the functional importance of clathrin in endocytosis has been provided by the finding that receptor-mediated endocytosis is inhibited by introduction into living cells of antibodies directed against clathrin coat components (Doxsey *et al.*, 1987; Chin *et al.*, 1989).

Clathrin coats along Golgi complex membranes could play an analogous role in lysosomal precursor sorting. Lysosomal precursors are recognized by transmembrane receptors (mannose-6-phosphate receptors, M6PR) by virtue of mannose-6-phosphate moieties on the oligo-saccharide side chains of the precursors (Kornfeld and Mellman, 1989). Morphological and biochemical studies suggest at least one form of M6PR (cation-independent) associates with clathrin coats (Dahms *et al.*, 1989). These observations have led to the proposal that clustering of M6PR and bound lysosomal precursors in clathrin-coated domains of the Golgi complex leads to selective incorporation of the precursors into clathrin-coated vesicles targeted for the lysosomal pathway. Consistent with this idea is the finding that certain mutations in the cytoplasmic domain of M6PR reduce lysosomal precursor sorting (Lobel *et al.*, 1989). However, in contrast to endocytosis, there is no direct functional evidence for clathrin's role in lysosomal precursor sorting.

Characterization of *Saccharomyces cerevisiae* mutants harboring clathrin heavy chain gene deletions (*chc1Δ*) has provided a means to assess the functional involvement of clathrin coats in various intracellular transport processes (Payne, 1990). When vacuolar protein sorting was monitored in *chc1Δ* yeast strains, no defect was observed (Payne *et al.*, 1988). This presented a paradox because of the similarity of the pathways followed by vacuolar precursors in yeast and lysosomal precursors in mammalian cells. Here we show that sorting of vacuolar precursor proteins is rapidly and severely inhibited when strains carrying a temperature-sensitive allele of *CHC1* (*chc1-ts*) are shifted to the

non-permissive temperature. However, over time, at the non-permissive temperature, cells regain the ability to sort vacuolar precursors. These findings resolve the apparent discrepancy by providing direct *in vivo* evidence that clathrin plays a role in vacuolar precursor sorting in yeast.

Results

Missorting of carboxypeptidase Y in *chc1-ts* cells

In order to determine the immediate consequences of impairing clathrin function we examined the ability of *chc1-ts* cells to sort the precursor form of the soluble vacuolar protease, carboxypeptidase Y (CPY). As CPY is transported to the vacuole of wild-type (WT) cells, it undergoes a series of characteristic modifications that can be used to monitor its progress (Stevens *et al.*, 1982). Translocation into the endoplasmic reticulum, signal cleavage and addition of core oligosaccharides yields the p1 form of CPY (67 kDa). Limited extension of the core oligosaccharides in the Golgi complex causes a 2 kDa increase in apparent mol. wt (MW) to produce the p2 form. p2 CPY is sorted from the secretory pathway and delivered to the vacuole where proteolytic maturation converts p2 CPY to the enzymatically active form (mCPY, 61 kDa). Under normal circumstances, at least 95% of p2 CPY is properly sorted from the secretory pathway. The remaining 5% travels to the cell surface and is secreted as the p2 form. Sorting can be perturbed by abnormally high levels of CPY expression (Stevens *et al.*, 1986) or mutation of the CPY vacuolar targeting signal (Valls *et al.*, 1987). In addition, mutations in >50 complementation groups (*vps*, vacuolar protein sorting) also affect CPY sorting (Rothman *et al.*, 1989; Klionsky *et al.*, 1990). In all cases increased levels of p2 CPY are secreted.

The fidelity of CPY transport to the vacuole was assayed in a congeneric set of *CHC1* and *chc1-ts* strains using a pulse-chase regimen. Cells incubated at the permissive temperature (24°C), or shifted to the non-permissive temperature (37°C) for 5 min or 3 h (Figure 1), were metabolically labeled for 10 min with [³⁵S]methionine and [³⁵S]cysteine. At this point, an aliquot of cells was harvested (pulse). Labeling of the remaining cells was quenched by addition of excess non-radioactive amino acids and the incubation continued for an additional 20 min (chase). Each sample was separated into intracellular (Figure 1, I) and extracellular fractions (Figure 1, E). Since the cell wall acts to retain many secreted proteins in the periplasmic

compartment, the cell wall was removed by treatment with β 1-3 glucanase. The resulting spheroplasts were sedimented and the supernatant containing the released periplasmic proteins was combined with the culture medium to generate a complete extracellular fraction. Lysis of the spheroplasts yielded the intracellular fraction. CPY was then immunoprecipitated from each fraction. In both WT and *chc1-ts* cells labeled at 24°C, both p1 and p2 CPY were present after the pulse (Figure 1, lanes 1 and 5). Following the 20 min chase incubation, all of the CPY was proteolytically matured indicating delivery to the vacuole (Figure 1, lanes 3 and 7). The absence of detectable CPY in the extracellular fractions indicates that efficient sorting occurred in both strains at 24°C (Figure 1, lanes 2, 4, 6 and 8). In WT cells shifted to 37°C for 5 min, CPY was also efficiently sorted. At 37°C CPY was already distributed between p1, p2 and mCPY forms after the labeling period (Figure 1, lane 9), due to an increased transport rate at the higher temperature. In the following 20 min, complete sorting and maturation occurred (Figure 1, lanes 11 and 12). In contrast, the *chc1-ts* cells exhibited an obvious sorting defect after 5 min at 37°C. Whereas the relative proportions of p1 and p2 in the mutant cells were similar to those observed in WT cells after the 10 min labeling (Figure 1, lanes 9 and 13), the amount of mature CPY in the mutant cells was barely detectable and there was a corresponding appearance of p2 CPY in the extracellular fraction (Figure 1, lanes 13 and 14). The sorting defect was more apparent after the 20 min chase period (Figure 1, lanes 15 and 16); 50% of the CPY was secreted and the majority of the cell-associated CPY was still present as the p2 form. A longer chase period resulted in secretion of this intracellular pool of p2 CPY (see Figure 2A), indicating that it was in transit to the cell surface. The same sorting defect has been observed in other *chc1-ts* strains (M. Seeger, unpublished data). The rapid and severe effect of *chc1-ts* on CPY sorting argues that clathrin plays a direct role in the sorting process.

Surprisingly, the ability of *chc1-ts* cells to sort CPY improved after longer incubations at the non-permissive temperature. An example of cells incubated at 37°C for 3 h before labeling is shown in Figure 1, lanes 21–24. Following the 10 min labeling, mature CPY was present in the mutant cells and no extracellular CPY was detected. In the 20 min chase samples, the majority of the CPY (65%) was matured. This experiment shows that *chc1-ts* cells incubated for extended times at the non-permissive

Fig. 1. The precursor form of CPY is secreted immediately upon shifting *chc1-ts* cells to the non-permissive temperature. *CHC1* (GPY382) and *chc1-ts* (GPY383) cells were grown in SD CAA-ura at 24°C. Cells were labeled for 10 min with [³⁵S]methionine and cysteine directly at 24°C (lanes 1–8) or were incubated for 5 min (lanes 9–16) or 3 h (lanes 17–24) at 37°C prior to 10 min labeling at 37°C. After the 10 min labeling period, excess unlabeled amino acids were added and an aliquot was harvested from each sample (0' chase). The remaining cells were incubated for an additional 20 min at the labeling temperature (20' chase). Intracellular (I) and extracellular (E) fractions were prepared as described in Materials and methods and CPY was immunoprecipitated from each fraction. Immunoprecipitates were subjected to SDS-PAGE and autoradiography. The ER and early Golgi form of CPY is labeled as p1, the late Golgi form is labeled as p2 and the mature form is labeled as mCPY (see text for details).

temperature resemble both *chc1* Δ and WT cells in their ability to sort CPY.

The *chc1-ts* mutation specifically affects sorting of soluble vacuolar proteins

We examined the extent and specificity of the sorting defect in *chc1-ts* cells by monitoring the sorting of another soluble vacuolar protease, proteinase A (PrA), and a vacuolar membrane protein, alkaline phosphatase (ALP). A pulse-chase protocol similar to that used in Figure 1 was employed after shifting WT and *chc1-ts* cells to 37°C for 5 min. The defect in CPY sorting in *chc1-ts* cells was reproduced in this experiment (Figure 2A, lanes 5–8); after the 40 min chase period 71% of the CPY was secreted in the p2 form, 19%

was present intracellularly as the mature form and 10% remained intracellular as the p2 form.

PrA has a biosynthetic itinerary similar to CPY, including corresponding p1, p2 and mature forms (Klionsky *et al.*, 1990). Sorting and vacuolar maturation of PrA was also affected by *chc1-ts*, although to a lesser extent than CPY (Figure 2B). At the 40 min time point, only 35% of PrA was matured in *chc1-ts* cells, compared with 93% in WT cells. The remainder of the PrA in *chc1-ts* cells (65%) was present as the p2 form, 57% intracellular and 8% extracellular. By comparison, only 10% of the p2 CPY was intracellular after the 40 min chase period (Figure 2A, lane 7). Although the basis of this difference is not known, it is not a novel phenotype; many of the *vps* mutants display a similar difference (Robinson *et al.*, 1989). Nevertheless, the results indicate that *chc1-ts* cells are defective in the sorting and delivery of both proteases to the vacuole.

In the same experiment we assessed transport to the vacuole of the vacuolar membrane protein, ALP (encoded by *PHO8*) (Kaneko *et al.*, 1987). Like the soluble proteases, ALP is sorted from the secretory pathway in the Golgi complex and undergoes proteolytic maturation in the vacuole (Klionsky and Emr, 1989). As shown in Figure 2C, ALP was proteolytically matured at the same rate in WT and *chc1-ts* cells, indicating that ALP transport was not affected by *chc1-ts*.

In *chc1-ts* cells, the conversion of the p1 forms of CPY and PrA to the p2 forms and the secretion of p2 CPY and PrA argues that the secretory pathway through the Golgi complex is not perturbed by the mutation. As an additional means to evaluate the secretory pathway we examined the secretion of a 33 kDa cell wall protein (CWP) (Sanz *et al.*, 1987). Although there are no discernible stage-specific modifications that can be used to follow the progress of CWP through the secretory pathway, enzymatic removal of the cell wall with β -1,3-glucanase releases cell surface CWP into the extracellular fraction. Accordingly, CWP was immunoprecipitated from the same intracellular and extracellular fractions used to evaluate the vacuolar proteins (Figure 2D). At the 40 min chase point, the same amount of CWP was detected in the extracellular fractions of WT and *chc1-ts* cells. Another secreted protein, α -factor, was also exported at wild-type rates from *chc1-ts* cells (M. Seeger and G.S. Payne, *J. Cell. Biol.*, in press). These results support the conclusion that *chc1-ts* does not alter the rate of protein

Fig. 2. The *chc1-ts* mutation affects the sorting of soluble but not membrane-bound vacuolar proteins. *CHC1* (GPY382) and *chc1-ts* (GPY383) cells were grown at 24°C, shifted to 37°C for 5 min and subjected to a pulse-chase regimen as described in the legend to Figure 1, except the chase period was 40 min. CPY (A), proteinase A (B), alkaline phosphatase (C) and a 33 kDa cell wall protein (CWP) (D) were precipitated with specific antisera and analyzed as in the legend to Figure 1. In (B) the protein which migrates slightly slower than PrA is not related to PrA but is precipitated by the antibody.

Fig. 3. The *sec1* mutation blocks secretion of p2 CPY in *chc1-ts sec1* cells. Pulse-chase analysis of CPY in *chc1-ts SEC1* (GPY441, lanes 1–4) and *chc1-ts sec1* (GPY440, lanes 5–8) cells was carried out as described in the legend to Figure 2 except that cells were shifted to 37°C for 30 min prior to labeling to ensure imposition of the *sec1* block.

movement through the Golgi complex. Together, the analyses of these four proteins indicate that *chc1-ts* has a specific effect on the sorting of soluble vacuolar precursors rather than generally perturbing the functional integrity of the Golgi complex.

Missorted CPY travels to the cell surface of *chc1-ts* cells by the secretory pathway

Secretion of p2 CPY and PrA by *chc1-ts* cells suggests that, in the absence of sorting, the vacuolar proteins accompany secreted protein into secretory vesicles budding from the Golgi complex. Fusion of the secretory vesicles with the plasma membrane would then expel the missorted vacuolar proteins from the cell. To test this model, we made use of *sec1*, a temperature-sensitive mutation which blocks fusion of secretory vesicles to the plasma membrane at 37°C (Novick and Schekman, 1979). The *sec1* mutation only affects secretory vesicles, it has no effect on the transport pathway to the vacuole (Stevens *et al.*, 1982). If the missorted vacuolar proteins are traveling to the cell surface in secretory vesicles, then a strain with both *chc1-ts* and *sec1* would be expected to accumulate the p2 precursor forms within the cells at 37°C.

We obtained a *chc1-ts sec1* double mutant strain from the meiotic progeny of a diploid strain generated by crossing a *sec1* strain with a *chc1-ts* strain. For comparative purposes we also isolated a *chc1-ts* strain from the same set of progeny. CPY sorting was analyzed in these two strains using a variation of the pulse-chase, fractionation and immunoprecipitation regimen described above. In this experiment, the cells were shifted to 37°C for 30 min before labeling to ensure that the *sec1* block was in effect. Like the *chc1-ts* cells used in the experiments shown in Figures 1 and 2, the new *chc1-ts* strain exhibited a dramatic sorting defect (Figure 3, lanes 1–4). On the other hand, the *chc1-ts sec1* cells did not secrete p2 CPY (Figure 3, lanes 5–8). Instead, p2 CPY accumulated within the cells (Figure 3, lane 7). After the 40 min chase period a small amount of mCPY was present in the *chc1-ts sec1* cells. This could be due to limited maturation of CPY which is concentrated with other missorted vacuolar proteases in the secretory vesicles. Alternatively, a minor fraction of CPY could reach the vacuole when accumulated within *chc1-ts sec1* cells. Nevertheless, the substantial accumulation of p2 CPY within the double mutant cells argues that the missorted vacuolar proteins reach the cell surface in secretory vesicles.

The *chc1-ts* sorting defect is reversible

As a further characterization of the sorting defect in *chc1-ts* cells, we determined whether the temperature-induced sorting block could be reversed by returning cells to the permissive temperature. WT and *chc1-ts* cells were shifted to 37°C for 5 min and labeled for 10 min. Labeling was quenched with excess unlabeled amino acids and a portion of each sample was harvested at this point to evaluate CPY transport and sorting. Of the residual cells, half were returned to 24°C and half were left at 37°C for an additional 40 min. In WT cells, the CPY synthesized in the 10 min labeling period (Figure 4, lanes 1 and 2) was efficiently sorted to the vacuole at both 24°C and 37°C (Figure 4, lanes 3–6). The severe sorting defect in the *chc1-ts* cells resulted in secretion of 80% of the newly synthesized CPY when

the chase was continued at 37°C (Figure 4, lanes 9 and 10). Only 8% of the CPY was present as the mature form. However, when the mutant cells were transferred to 24°C after the 10 min labeling period, 30% of the CPY was correctly sorted and matured (Figure 4, lanes 11 and 12). Correspondingly, the amount secreted was reduced to 54%.

In considering the significance of the increased sorting after the shift to 24°C, the distribution of CPY in the secretory pathway at the time of shift must be taken into account. That is, when the cells are returned to 24°C, even if the sorting capacity is completely re-established, only CPY which has not passed the Golgi complex sorting compartment is available for delivery to the vacuole. By this logic, all of the p1 CPY could still be sorted. The p2 CPY, however, is distributed in unknown amounts between Golgi compartments and secretory vesicles. Thus, only a portion of the p2 CPY is available for sorting. After the 10 min labeling, 19% of the CPY was present as the p1 form and 61% was present as the p2 form, giving an upper limit of 80% available for sorting. Making the reasonable assumption that restoration of sorting does not occur immediately at 24°C, the 22% increase in sorting which occurred when cells were returned to 24°C (30% mature at 24°C after 40 min chase period compared to 8% at 37°C) suggests that the missorting defect is substantially reversible. In fact, if only the p1 CPY is considered, the defect was completely reversible.

Sorting of CPY in *chc1Δ* cells

Previous experiments using *chc1Δ* cells did not reveal a CPY sorting defect (Payne *et al.*, 1988). The sorting defect exhibited by *chc1-ts* cells immediately after the temperature shift prompted us to re-examine CPY transport in *chc1Δ* cells. WT and *chc1Δ* cells were shifted to either 30°C or 37°C for 5 min and subjected to the same pulse-chase analysis applied to *chc1-ts* cells (Figure 5). The temperature shifts had no effect on sorting of CPY in WT cells (Figure 5, *CHC1* panel). There was a slight effect of temperature shifts on sorting of CPY in the *chc1Δ* cells. Sixteen per cent of the CPY was secreted at 24°C (Figure 5, *chc1Δ*, lanes 3 and 4), 35% was secreted at 30°C (Figure 5, *chc1Δ*, lanes 7 and 8) and 25% was secreted at 37°C (Figure 5, *chc1Δ*, lanes 11 and 12), after correcting for cell lysis (5–10%) based on the presence of cytoplasmic phosphoglycerate kinase (PGK) in the extracellular fraction (see Materials and methods). The intracellular CPY in each case was predominantly mature. This contrasts with *chc1-ts* cells (Figures 2 and 4) where 70–80% of the CPY was secreted and <15% was mature and intracellular. Also, whereas only p2 CPY was detected in the extracellular fraction of *chc1-ts* cells, another species of CPY, which migrated slightly more slowly than mature CPY (see the arrowhead in Figure 5, *chc1Δ* panel), was observed in the extracellular fractions of the *chc1Δ* cells (Figure 5, *chc1Δ* panel, lanes 4, 8 and 12). The novel extracellular CPY form (arrowhead) is due to the *prb1* mutation which eliminates proteinase B (Mohle *et al.*, 1987). In *chc1Δ PRB1* strains, the novel species was not present (see Figure 6). Missorting of CPY has been observed with other *chc1Δ* cells although the absolute amount of missorting at different temperatures, and the CPY species present extracellularly, vary from strain to strain (see Figure 6; M. Seeger and G.S. Payne, unpublished observations). The distribution of CPY forms in the *chc1Δ* cells shifted to 37°C was similar to that observed in *chc1-ts*

Fig. 4. The CPY sorting defect in *chc1-ts* cells is reversible. *CHC1* (GPY382) and *chc1-ts* (GPY383) cells were subjected to a modified pulse–chase protocol. After shift to 37°C, labeling for 10 min, and initiation of the chase, a sample was immediately harvested (0' chase). The remaining cells were divided into two aliquots. One part was incubated for an additional 40 min at 37°C while the second part was incubated for 40 min at 24°C. CPY was immunoprecipitated and analyzed as in the legend to Figure 1.

cells incubated at 37°C for 3 h prior to labeling (cf. Figure 5, *chc1*Δ, lanes 11 and 12 and Figure 1, lanes 23 and 24). This similarity further substantiates the proposal that an adaptation improves sorting of soluble vacuolar proteins in cells lacking clathrin heavy chain.

sec1 does not block delivery of CPY to the vacuole in *chc1*Δ cells

Although *chc1*Δ cells transport most of the CPY to the vacuole, it is possible that the newly synthesized CPY is, in fact, missorted, but the CPY that reaches the cell surface is retrieved by endocytosis and delivered to the vacuole. Since *sec1* would be expected to block any vacuolar pathway that required transport to the cell surface, the mutation allowed us to determine whether delivery of CPY to the vacuole in *chc1-ts* cells occurred by this type of 'secretion–recapture' process (Hickman and Neufeld, 1972). CPY transport was analyzed in a *chc1*Δ *sec1* double mutant strain and a *chc1*Δ *SEC1* strain by the same pulse–chase protocol described previously for analysis of the *chc1-ts* *sec1* strain. The *chc1*Δ *SEC1* strain used in this experiment exhibited sorting properties similar to the *chc1*Δ strain used in the experiment described above (Figure 6, lanes 1–4). At 24°C, 20% of the CPY was secreted, distributed between p2 and the mature forms (Figure 6, lanes 1 and 2). At 37°C, virtually all the secreted CPY (25% of the total) was present as the mature form (Figure 6, lanes 3 and 4). The extracellular CPY was not due to lysis as judged by immunoprecipitation of PGK. The *chc1*Δ *sec1* strain missorted 6% of CPY at 24°C (Figure 6, lanes 5 and 6). At 37°C there was no secretion of CPY (Figure 6, lane 8) and the bulk (78%) of the intracellular CPY was mature (Figure 6, lane 7). The amount of p2 CPY accumulated in the double mutant cells at 37°C (22%) corresponds well to the amounts of CPY secreted at 37°C by *chc1*Δ strains used in this experiment and the one described in the previous section. This correspondence argues that the p2 CPY in the double mutant strain at 37°C is missorted CPY which has been trapped by *sec1* in secretory vesicles *en route* to the cell surface. An effect of the temperature shift on sorting probably accounts for the observation that more p2 CPY was accumulated at 37°C than was secreted at 24°C in the *chc1*Δ *sec1* strain (Figure 6, lanes 5–8). Maturation of the majority of the newly synthesized CPY in the presence of the *sec1*

Fig. 5. Temperature shifts do not dramatically affect sorting of CPY in *chc1*Δ cells. *CHC1* (GPY55-15B, top panel) and *chc1*Δ (GPY68.1, bottom panel) cells were grown at 24°C in SDYE and then labeled directly for 10 min at 24°C or shifted to 30 or 37°C, for 5 min prior to labeling for 10 min at the respective temperatures. Samples were analyzed for CPY as in the legend to Figure 1. Arrowhead indicates the novel extracellular CPY form (see text).

block shows that CPY is not delivered to the vacuole by way of the cell surface.

Discussion

We have observed a rapid, reversible and selective inhibition of vacuolar protein sorting from the Golgi complex in strains expressing a temperature-sensitive mutant form of clathrin heavy chain. This inhibition is most likely a direct consequence of temperature-induced inactivation of the mutant heavy chain since shifting *chc1*Δ cells from 24°C

Fig. 6. The *sec1* mutation does not block maturation of CPY in *chcl1Δ* cells. *chcl1Δ SEC1* (GPY50-2A) and *chcl1Δ sec1* (GPY50-2D) cells were analyzed for CPY transport using a pulse-chase protocol as described in the legend to Figure 3 except that the intracellular fraction represents whole cells not spheroplasts (see Materials and methods).

to 37°C does not result in a similar sorting defect. The observation that targeting of a vacuolar membrane protein is not altered suggests that the effect of the *chcl1-ts* mutation is specific for the soluble vacuolar proteins. These findings provide evidence that clathrin plays a direct role in the sorting of newly synthesized soluble vacuolar proteins.

Previous studies demonstrated that the fidelity of vacuolar protein sorting was not perturbed by a deletion of *CHC1* (Payne *et al.*, 1988). Two interpretations of these results were put forward: either clathrin is not directly involved in vacuolar protein sorting, or clathrin-independent sorting mechanisms can substitute when clathrin function is eliminated in *chcl1Δ* cells. The results obtained with the *chcl1-ts* cells offer strong support for the latter interpretation. The rapid and severe sorting defect in *chcl1-ts* cells argues that clathrin plays a direct role in sorting, and the restoration of sorting in *chcl1-ts* cells incubated for extended times at 37°C indicates that clathrin-independent sorting does arise in the absence of functional clathrin heavy chain. Our discovery of the *in vivo* importance of clathrin in yeast vacuolar protein sorting, in combination with morphological and biochemical studies of lysosomal protein sorting in mammalian cells, (reviewed by Kornfeld and Mellman, 1989), demonstrates a conserved function of clathrin in directing proteins from the Golgi complex to the hydrolytic compartment in eukaryotic cells.

Several observations suggest that the effect of *chcl1-ts* on soluble vacuolar protein targeting is not due to a general perturbation of Golgi complex function. First, sorting of the vacuolar membrane ALP is not altered in the mutant cells at early times after shift to the non-permissive temperature. Second, the transport of a cell wall protein to the surface is not affected at the non-permissive temperature. Third, the Golgi-specific glycosylation of CPY and PrA responsible for conversion of the p1 to p2 forms is also unaffected. Fourth, the temperature-induced defect is rapidly reversible. Finally, it should also be noted that the sorting defect occurs several hours before the growth rate of the *chcl1-ts* cells is affected (M. Seeger and G.S. Payne, in preparation). Together, these results indicate that the immediate impairment of clathrin function causes a specific defect in the sorting of soluble vacuolar proteins, implicating clathrin as a component of the sorting apparatus. Additionally, the insensitivity of ALP sorting to *chcl1-ts* raises the possibility that a clathrin-independent mechanism is responsible for sorting of this vacuolar membrane protein from the Golgi complex.

Of the protein trafficking pathways examined, only vacuolar protein sorting is differentially affected in *chcl1-ts*

Fig. 7. Model for the role of clathrin in the sorting of soluble vacuolar proteins. (A) Sorting in WT cells. (B) Sorting defect in *chcl1-ts* cells after 5 min at 37°C. (C) Sorting in *chcl1-ts* cells incubated for extended periods at 37°C and in *chcl1Δ* cells. V, vacuole; G, Golgi complex; IC, putative intermediate compartment; (●) soluble vacuolar protein; (Y) putative sorting receptor; (TTT) clathrin. See text for details.

and *chcl1Δ* cells (M. Seeger and G.S. Payne, *J. Cell. Biol.*, in press; P.K. Tan *et al.*, in preparation). In both cell types the rate of protein secretion occurs at near WT rates. Endocytosis, which occurs at 35–50% of WT levels in *chcl1Δ* cells, is reduced to 45% of WT levels in *chcl1-ts* cells within 10 min at the non-permissive temperature. The defect in retention of the Golgi membrane proteins Kex2p and dipeptidyl aminopeptidase A which was observed in *chcl1Δ* cells also appears in *chcl1-ts* cells soon after imposing the non-permissive temperature. Finally, after 2 h at 37°C the growth rate of *chcl1-ts* cells slows to that characteristic of *chcl1Δ* strains. Thus, the recovery of the vacuolar sorting process in *chcl1-ts* cells is specific and does not reflect a global adaptation to the loss of clathrin function.

The finding that a subset of Golgi membrane proteins are rapidly mislocalized to the cell surface upon shift of *chcl1-ts* cells to the non-permissive temperature (M. Seeger and G.S.

Table I. Yeast strains used in this study

Strain	Genotype	Source
GPY50-2A	<i>MATα chcl-Δ8::LEU2 leu2-3,112 ura3-52 his4 trp1-289</i>	this study
GPY50-2B	<i>MATα, leu2 ura3-52 his4-519 trp1-289 sec1-1</i>	this study
GPY50-2D	<i>MATα chcl-Δ8::LEU2 sec1-1 leu2-3,112 ura3-52 his4 trp1-289</i>	this study
GPY55-15B	<i>MATα leu2-3,112 ura3-52 his4-519 trp1 prb1 gal2</i>	Payne and Schekman (1989)
GPY68.1	<i>MATα leu2-3,112 ura3-52 his4-519 trp1 prb1 gal2 chcl-Δ10::LEU2</i>	Payne and Schekman (1989)
GPY268	<i>MATα ura3-52 leu2-3,112 his3Δ200 dpp2Δ::HIS3 chcl-Δ10::LEU2 trp1 YCpchcl-521TRP</i>	this study
GPY382	pCHCc102 transformant of GPY268 cured of YCpchcl-521TRP	this study
GPY383	pBP6 transformant of GPY268 cured of YCpchcl-521TRP	this study
GPY440	<i>MATα trp1 ura3-52 pBP6 leu2-3,112 chcl-Δ10::LEU2 sec1-1</i>	this study
GPY441	<i>MATα trp1 ura3-52 pBP6 leu2-3,112 chcl-Δ10::LEU2</i>	this study

Payne, *J. Cell. Biol.*, in press) indicates that missorting of soluble vacuolar proteins is not the only Golgi complex defect in *chcl-ts* cells. Clathrin may therefore provide two separate functions at the Golgi complex. However, it is not clear that clathrin's role in Golgi membrane protein retention is exerted at the Golgi complex. Clathrin could instead act at a post-Golgi compartment to recycle the membrane proteins to the Golgi complex (Payne and Schekman, 1989).

A model for the function of clathrin in vacuolar protein sorting and the response of cells to a loss of clathrin function is presented in Figure 7. The view of vacuolar protein sorting in WT yeast cells (Figure 7A) is analogous to models of M6PR-mediated lysosomal protein sorting in mammalian cells (see Introduction). We posit transmembrane receptors for soluble vacuolar proteins and an intermediate compartment between the Golgi complex and the vacuole. Although no direct evidence for a vacuolar protein receptor analogous to M6PR has been obtained, the definition of a four amino acid sequence in the CPY precursor which is necessary for efficient sorting is consistent with a receptor-mediated sorting mechanism (Valls *et al.*, 1987). The existence of an intermediate compartment is also uncertain. A pre-vacuolar compartment has been identified in the endocytic pathway in yeast but its relationship to the exocytic pathway has yet to be determined (Singer and Riezman, 1990). Our model proposes that the putative vacuolar protein sorting receptors are clustered into clathrin-coated regions of the Golgi complex. Through this association with clathrin, the receptor-bound vacuolar proteins are packaged into clathrin-coated vesicles targeted (in an unknown fashion) to the intermediate compartment (IC in Figure 7A). In the IC, the vacuolar precursors dissociate from the receptors and are delivered to the vacuole while the receptors are recycled to the Golgi complex (dotted arrow in Figure 7A).

Upon a shift of *chcl-ts* cells to the non-permissive temperature, the formation of clathrin-coated vesicles would be blocked (Figure 7B). We present the possibility that the Golgi membrane clathrin coats cannot progress to coated vesicles. A defect at this stage could explain the recovery of sorting (see below) but an equally plausible scenario is the the Golgi membrane clathrin coats disassemble at the non-permissive temperature. In the model in Figure 7B, the sorting receptors accumulate in the Golgi complex where they quickly become saturated with newly synthesized vacuolar proteins. Excess vacuolar proteins would then be free to continue along the secretory pathway to the cell surface. The saturation of the receptors would have to occur quickly since most (70–80%) of the newly synthesized CPY is secreted by *chcl-ts* cells after 5 min at 37°C. A potentially

germane observation (Figure 1, lanes 9–16) indicates that delivery to the vacuole in WT cells is complete by the 20 min chase point, but at the same time point much of the p2 CPY has not been secreted by *chcl-ts* cells. Perhaps this slow export of p2 CPY from *chcl-ts* cells reflects slow dissociation of accumulated, receptor-bound CPY in the Golgi complex of the mutant cells. If the model that clathrin coats disassemble at the non-permissive temperature is considered, then the receptors and bound vacuolar proteins could accompany excess vacuolar proteins along the secretory pathway to the cell surface.

After extended times at 37°C, *chcl-ts* cells regain the ability to direct newly synthesized CPY to the vacuole. This unexpected finding suggests that transport of soluble vacuolar proteins to the vacuole can occur in the absence of functional clathrin heavy chain. In support of this hypothesis, previous experiments using cells devoid of clathrin heavy chain (due to a deletion of *CHC1*) did not reveal a defect in CPY sorting (Payne *et al.*, 1988). The recovery of vacuolar protein sorting in *chcl-ts* cells and the efficient sorting in *chclΔ* cells is diagrammed in Figure 7C. If the temperature-sensitive clathrin heavy chain is unable to form coated vesicles as shown in Figure 7B then it might occlude transport to the vacuole by blocking membrane vesiculation. If the non-functional clathrin coats disassemble or are destroyed over time at 37°C then the generation of transport vesicles could resume. This model presumes that clathrin heavy chain is not necessary for vesiculation or targeting of the vesicles to the vacuolar pathway but acts primarily as a molecular scaffold to collect receptors at sites undergoing vesiculation. Accordingly, in the absence of clathrin, the receptors would not be efficiently collected into the transport vesicles. The ensuing limited accumulation of receptors in the Golgi complex could result in a small amount of excess vacuolar proteins that escape sorting. On the other hand, it is also possible that, in the absence of clathrin, a completely different pathway takes over delivery of soluble vacuolar proteins to the vacuole. A candidate for this substituting pathway is the clathrin-independent route followed by the vacuolar membrane protein, ALP.

A large number of vacuolar protein sorting mutants (*vps*) have been isolated on the basis of soluble vacuolar protein secretion (Rothman *et al.*, 1989; Klionsky *et al.*, 1990). It is unlikely that *chcl* mutants are present in this collection since the *vps* mutants constitutively secrete vacuolar proteins at all temperatures tested. Given the model presented in Figure 7, we envision that a subset of the *VPS* gene products participate in vesiculation of the Golgi membrane while clathrin functions to collect receptors. Elimination of one

of the *VPS* genes would prevent vesiculation and lead to receptor saturation and secretion of soluble vacuolar proteins. The *vps* mutants would not adapt like the *chc1* strains either because there is no opportunity for vesiculation to resume or because the presence of clathrin coats restricts the receptors from moving to an alternative pathway. As probes become available for *VPS* gene products, the relationship between the *Vps* proteins and clathrin coats can be established and their roles in vacuolar protein sorting determined.

Materials and methods

Materials

Unless noted otherwise, all reagents were purchased from Sigma Chemical Co., St Louis, MO.

Strains, media and genetic methods

The genotypes of strains used in this work are shown in Table I. GPY440 and GPY441 are meiotic progeny from a cross of GPY50-2B and GPY383.

YPD medium is 1% Bacto-Yeast extract, 2% Bactopeptone (Difco Laboratories, Inc., Detroit, MI) without amino acids and 2% dextrose. SD is 0.67% yeast nitrogen base (Difco), 2% dextrose. Supplemented SD is SD with 20 μ g/ml histidine, uracil, tryptophan and 30 μ g/ml leucine, adenine and lysine. SD CAA medium is SD with 5 mg/ml vitamin assay casamino acid mix (Difco) with 15 μ g/ml adenine and 20 μ g/ml methionine, histidine, uracil and tryptophan. SD CAA-ura is SD CAA without uracil. SDYE is SD with 0.2% yeast extract. Cell densities in liquid culture were measured in a 1 cm plastic cuvette using a Beckman Instruments DU-62 spectrophotometer. One A_{500} unit is equivalent to 2.3×10^7 cells/ml.

Yeast mating, sporulation and tetrad analyses were conducted as described by Sherman *et al.* (1974). DNA transformations were performed by the lithium acetate procedure (Ito *et al.*, 1982) or by spheroplast transformation (Hinnen *et al.*, 1978).

YCpChc-521TRP is a derivative of YCpChcC102TRP (Munn *et al.*, 1991) which carries *chc1-521*, a temperature-sensitive allele of *CHC1* generated by hydroxylamine mutagenesis. Isolation and characterization of *chc1-521* will be described in detail elsewhere. The region of *chc1-521* which is responsible for the *ts* phenotype was introduced into *CHC1* carried on YCp50 (selectable marker *URA3*) to generate pBP6 (Munn *et al.*, 1991). pChcC102 is *CHC1* in YCp50 (Payne *et al.*, 1987).

Metabolic labeling and immunoprecipitation

For metabolic labeling, cells were grown to mid-logarithmic phase at 24°C in SD CAA-ura (strains with plasmids) or SDYE. Cultures were shifted to 30 or 37°C for various times. Cells (5×10^6) were harvested and washed twice in supplemented SD. Cells were resuspended in 250 μ l of supplemented SD plus 0.2 mg/ml ovalbumin. Labeling was initiated by addition of 50 μ Ci of Trans³⁵S-label (ICN, Irvine, CA) and incubated at the appropriate temperatures. Labeling was terminated by the addition of 0.3% cysteine and methionine in $10 \times$ CAA-ura to a final concentration of 0.03%. After chase periods of 20 or 40 min, Na₂S₂O₃ (10 mM) was added and samples were placed on ice. Labeled cells were sedimented and 200 μ l of media removed for immunoprecipitation. Five microliters of 2% SDS and 0.2 mg/ml ovalbumin were added and the sample heated to 100°C for 3 min. Samples were brought to 1 ml with phosphate-buffered saline (PBS) containing 1% Triton X-100 and 0.2 mg/ml ovalbumin (PT). Cells were washed in PBS, and resuspended in 100 μ l spheroplast buffer (1.4 M sorbitol, 50 mM Tris-HCl, pH 7.4, 10 mM Na₂S₂O₃, 40 mM β -mercaptoethanol). Oxalyticase (50 U/ A_{500} , Enzymogenetics, Corvallis, OR) was added and cells were incubated at 30°C for 30 min. Spheroplasts were sedimented and the supernatant removed. Five microliters of 2% SDS was added to the supernatant, the samples were heated at 100°C for 3 min and then added to the media samples to yield the extracellular (E) fraction. Spheroplasts were washed once in spheroplast buffer without β -mercaptoethanol then lysed by addition of 100 μ l of 2% SDS. Lysed cells were heated at 100°C for 3 min. Lysates were brought to 1 ml with PT.

In the experiment with *chc1Δ* and *chc1Δ sec1* cells shown in Figure 6, in order to avoid cell lysis during the spheroplast preparation, the cells were directly lysed with glass beads (Munn *et al.*, 1991). Thus, the intracellular fraction consists of both intracellular and periplasmic compartments.

All samples subjected to immunoprecipitation received 50 μ l of a 10% *Staphylococcus aureus* suspension (IgSorb; The Enzyme Center, Malden, WA) and were incubated on ice for 15 min. The IgSorb was sedimented by centrifugation at 16 000 g for 15 min. To the supernatant was added

antisera against CPY, PrA, ALP, CWP or PGK. Following an overnight incubation at 4°C, protein A-Sepharose (20% suspension; Pharmacia, Piscataway, NJ) was added to collect antibody. Beads were washed once with PBS, 1% Triton X-100, 0.1% SDS (PTS), once with 2 M urea, 2 M NaCl, 1% Triton X-100, 100 mM Tris-HCl, pH 7.4, again with PTS and finally twice with 10 mM NaCl, 10 mM Tris, pH 6.8. Beads were resuspended in 25 μ l Laemmli sample buffer (Laemmli, 1970) and heated at 100°C for 3 min. All samples were subjected to SDS-PAGE. Gels were incubated in 25% methanol, 10% acetic acid, then treated with Entensify (NEN/DuPont, Boston, MA). Gels were exposed to X-ray film (Kodak, X-OMAT AR) at -70°C.

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