Protein Transport to the Vacuole and Receptor-mediated Endocytosis by Clathrin Heavy Chain-deficient Yeast

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Abstract. Clathrin heavy chain-deficient mutants (chcl) of Saccharomyces cerevisiae are viable but exhibit compromised growth rates. To investigate the role of clathrin in intercompartmental protein transport, two pathways have been monitored in chcl cells: transport of newly synthesized vacuolar proteins to the vacuole and receptor-mediated uptake of mating pheromone. Newly synthesized precursors of the vacuolar protease carboxypeptidase Y (CPY) were converted to mature CPY with similar kinetics in mutant and wild-type cells, chcl cells did not aberrantly secrete CPY and immunolocalization techniques revealed most of the CPY in chcl cells within morphologically identifiable vacuolar structures. Receptor-mediated internalization of the mating pheromone α-factor occurred in chcl cells at 36-50% wild-type levels. The mutant cells were fully competent to respond to pheromone-induced cell-cycle arrest. These results argue that in yeast, clathrin may not play an essential role either in vacuolar protein sorting and delivery or in receptor-mediated endocytosis of α-factor. Alternative mechanisms ordinarily may execute these pathways, or be activated in clathrin-deficient cells.

AT least two avenues of intracellular protein traffic service lysosomes in mammalian cells and lysosome-like vacuoles in yeast. Newly synthesized lysosomal and vacuolar enzymes follow the secretory pathway from the endoplasmic reticulum to the Golgi body (II, 39). After traversing Golgi body cisternae, the enzymes are diverted from the secretory pathway at an undefined location and delivered to their final residence. One branch of the endocytic pathway also leads to lysosomes (or vacuoles). In mammalian cells, both fluid-phase and receptor-mediated endocytosis involve transfer of extracellular molecules to an endosomal compartment (19). Endosomal sorting reactions direct traffic between several pathways, one of which leads to lysosomes. Although not as extensively characterized in yeast, both fluid-phase and receptor-mediated internalization have been described (7, 22, 36).

Transfer of molecules between compartments along both lysosome-oriented routes is thought to be mediated by transport vesicles (II, 19). Clathrin-coated membranes and vesicles have been implicated as key intermediates in the formation of these transport vesicles (15, 35). The most compelling example of an association between clathrin and transport vesicle generation has been provided by studies of receptor-mediated endocytosis in mammalian cells (15, 16). Initially, receptor-bound ligands are concentrated along indented domains (pits) of the plasma membrane that are decorated on the cytoplasmic surface with a polygonal protein lattice. The predominant component of the lattice is clathrin. Clathrin-coated pits invaginate and bud, forming clathrin-coated vesicles laden with receptors and associated ligands. Vesicles quickly shed their coat before fusion with the subsequent endocytic compartment. Less extensive biochemical and morphological evidence has been garnered in support of a similar role for clathrin during vesicle generation in other intercompartmental transport pathways, including the route followed by newly synthesized lysosomal enzymes (3, 4, 12, 34).

In vitro, clathrin exhibits properties that are uniquely suited for participation in membrane vesiculation. The polyhedral lattice on purified clathrin-coated vesicles can be depolymerized to yield “triskelions” composed of three clathrin heavy chains (each 180 kD) and three clathrin light chains (usually two species varying in size from 30-40 kD) (26, 47). Triskelions can assemble to form empty lattice cages (26) or rebind to vesicles previously stripped of their clathrin coats (46).

From these studies, a model emerged which suggested that polymerization of clathrin triskelions into polyhedral cages drives formation of coated vesicles from coated membrane regions (17, 35). In addition, specific interactions between the clathrin coats, or an associated protein, and membrane proteins and receptors carrying ligands could insure collection of cargo proteins at membrane sites engaged in vesicle formation (16, 31, 35). In this view, clathrin plays a pivotal role in the biogenesis and selectivity of transport vesicles.
In earlier reports we have described the generation of clathrin heavy chain-deficient mutants of *Saccharomyces cerevisiae* (32, 33). Yeast strains harboring null alleles of the single clathrin heavy chain gene (CHC1) are viable but exhibit reduced growth rates. Viable cells devoid of clathrin-coated vesicles provide a means to determine whether clathrin is required for various intracellular transport reactions. Such a direct test of biological function has not been available previously. Here we investigate the integrity of two transport pathways in clathrin-deficient cells: transfer of newly synthesized vacuolar proteins to the vacuole and receptor-mediated internalization of peptide mating pheromone.

**Materials and Methods**

**Strains, Media, and Genetic Methods**

Yeast strains used in this study are listed in Table I. YPD is a medium (1% Bacto-Yeast extract, 2% Bactopeptone; Difco Laboratories, Inc., Detroit, MI) with 2% glucose. Solid YPD medium contained 2% agar. For 35S-labeled α-factor, labeling, cells were grown in Wickerham's minimal medium (51) except sulfate salts were replaced by chloride salts and ammonium sulfate. Labeling was initiated by adding 200 μCi of [35S]SO42-- (1,200 Ci/mmol) and 0.2 mM PMSF (inhibitor medium). 35S-labeled α-factor was added to 10^{-6} M and the cultures incubated with shaking at 24°C. At time intervals, 100 μl samples were removed and diluted into 2 ml of ice-cold 50 mM sodium citrate, pH 2. After 20 min on ice, cells were collected onto glass filters (GFC Whatman, Inc., Clifton, NJ) and washed with 20 ml of the citrate buffer. Filters were dried and counted in a liquid scintillation counter for 10 min with Beta-Max scintillant (West Chem, San Diego, CA). 200 μOD600/ml of strain GPY74-29B internalized 63% of 35S-labeled α-factor in 75 min at 24°C. This value was taken as the percentage of 35S-labeled peptide that was biologically active. In several experiments, replacement assays were conducted on 20-min time point samples to determine the amount of biologically active α-factor remaining in the medium. Cells were removed by centrifugation and GPY74-29B cells added to 200 OD600/ml and incubated at 24°C for 75 min. In each case (CHC1 *MATa* and *chcl* *MATa* strains), the sum of the counts internalized by the cells in the first 20-min incubation and GPY74-29B cells in the replacement incubation corresponded to the amount of biologically active α-factor initially present in the samples. Halo assays were conducted on 20-min time point samples to determine whether clathrin is required for the internalization of biologically active α-factor.

**Uptake and Binding Assays**

α-Factor uptake and binding were measured by applying a modification of the assay used by Chvatchko et al. (7). Cells were grown overnight in YPD to an OD600 of 0.8%, then washed, and resuspended at 30 OD600/ml in pH 5.5 50 mM sodium phosphate, pH 7.2, 135 mM sodium chloride, 1% Triton X-100, and 0.4% SDS. Immunoprecipitation techniques and SDS-PAGE analysis are described in Stevens et al. (45). Densitometry has been previously reported (33). Each form of CPY in Fig. 2 is expressed as the percentage of the total density value of all three forms at the time point. The total values varied up to 20% between time points.

**Radiolabeling, Preparation of Lysates, and Immunoprecipitations**

Cells were grown overnight into midlogarithmic phase at 30°C in minimal medium plus 2% glucose and 200 μM ammonium sulfate. For labeling, cells were harvested by centrifugation, washed in minimal medium, and resuspended at 2 OD600 per ml in minimal medium plus 2% glucose. Labeling was initiated by adding 200 μCi of [35S]SO42-- (1,200 Ci/mmol; ICN Biochemicals, Inc., Irvine, CA) per OD600. After 30 min, 30% ammonium sulfate (3 mM), and methionine (0.01%). Samples were collected at designated time intervals, placed on ice, and sodium azide added to 10 mM. Upon completion of the time course, cells were lysed by agitation with glass beads and SDS as described (33). To measure both internal and secreted CPY, after labeling, cells were mixed with an equal volume of minimal medium with 2% glucose, 500 μg/ml ovalbumin or BSA and ammonium sulfate, cysteine, and methionine at concentrations cited above. Ovalbumin and BSA stabilize secreted CPY (45). At each time point, cells were collected by centrifugation, the medium removed, and 10 mM sodium azide added to the cell pellet, and both fractions placed on ice. Cell lysates were prepared as described above. Medium fractions received SDS to a final concentration of 0.8%. All samples were heated at 100°C for 3 min, then adjusted to final concentrations of 20 mM sodium phosphate pH 7.2, 35 mM sodium chloride, 1% Triton X-100, and 0.4% SDS. Immunoprecipitation techniques and SDS-PAGE analysis are described in Stevens et al. (45). Densitometry has been previously reported (33). Each form of CPY in Fig. 2 is expressed as the percentage of the total density value of all three forms at the time point. The total values varied up to 20% between time points.

**Table I. Yeast Strains Used in this Study**

| Yeast strain | Genotype | Reference |
|--------------|----------|-----------|
| GPY1100      | MATa leu2-3,-112 ura3-52 his4-519 trp1 can1 | Strain TD4 from G. R. Fink* |
| GPY1101      | chcl-Δ8::LEU2 transformant of GPY1100 | 32 |
| GPY1103      | chcl-Δ8::LEU2 transformant of GPY1100 | 32 |
| GPY74-29B    | MATa leu2-3,-112 ura3-52 pep4::URA3 his4-519 trp1 prbl slt | This study |
| GPY79.1      | chcl-Δ10::LEU2 transformant of GPY74-29B | This study |
| GPY79.2      | chcl-Δ10::LEU2 transformant of GPY74-29B | This study |
| GPY68        | MATa chcl-Δ10::LEU2 leu2-3,-112 ura3-52 his4-519 trp1 prbl | This study |
| GPY56-26C    | MATa leu-2-3,112, ade2 his6, trpl, met1, ssl prbl | This study |
| GPY70.3      | chcl-Δ10::LEU2 transformant of GPY56-26C | This study |

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Electron Microscopy

The procedures for fixation, embedding in Lowicryl resins, and immunolabeling are detailed in van Tuinen and Riezman (49).

Results

A general strategy has been adopted to assess the role of clathrin in various intercompartmental traffic routes. Sets of congenic clathrin-deficient (chcl) and wild-type (CHC1) strains were generated by using single-step gene transplacement to disrupt CHC1 in haploid cells (33). chcl Transformants obtained by this technique were isogenic with the parental CHC1 haploid, except at the CHC1 locus. Mutant strains grew two to three times more slowly than their wild-type counterparts. Pairs of congenic strains were then subjected to phenotypic analyses.

ProCPY Is Matured at Wild-type Rates in chcl Cells

The vacuolar protease CPY provides a diagnostic tool to characterize transit of newly synthesized proteins to the vacuole. CPY is translated as an inactivezymogen that acquires four core oligosaccharides upon translocation into the endoplasmic reticulum (18, 44). By SDS-PAGE, this form (pl) has a mobility corresponding to 67 kD. During transit through the Golgi apparatus, a discrete addition of mannose residues produces the 69-kD p2 species (44). Proteolytic maturation of p2 CPY to enzymatically active CPY (m form, 61 kD) most likely takes place in the vacuole (9, 44, 53). Conversion of pl to p2 to mCPY can easily be monitored by SDS-PAGE.

The time course of proCPY modification was examined in congenic sets of CHC1 and chcl strains using a pulse-chase regimen. Cells were labeled for 5 min with [35S]SO42- and then allowed to continue growth in the presence of excess unlabeled SO42-, cysteine, and methionine. Total cell lysates were prepared from cells collected at intervals after implementation of the chase. CPY was immunoprecipitated from each lysate and resolved on SDS-polyacrylamide gels. As shown in Fig. 1, top, proCPY synthesized in wild-type cells undergoes the anticipated, ordered series of mobility shifts. In mutant cells (Fig. 1, bottom), mCPY appears at a wild-type rate, although the conversion of pl to p2 CPY is somewhat abnormal. Densitometric analyses of the autoradiograms in Fig. 1 substantiate these conclusions (Fig. 2). In both mutant and wild-type cells, 50% of the proCPY has been matured at ~9 min of chase (Fig. 2 A). Compared with CHC1 cells, a slight delay in the processing of pl CPY is evident in mutant cells: t, is 3.2 min in CHC1 cells and 5.8 min in chcl cells (Fig. 2 B). Also, the p2 form in mutant cells accumulates to only 50% of the wild-type level at the 5-min chase points. The same results were obtained with two other sets of congenic strains. These experiments argue that, as assayed by proCPY maturation, a lack of clathrin heavy chain does not alter the rate of CPY delivery to the vacuole. There appears to be, however, a slight but reproducible anomaly in the ability of mutant cells to convert pl to p2 CPY.

ProCPY Is Not Secreted by chcl Strains

Mislocalization of proCPY has been observed in three instances: overexpression of CPY from a multicopy plasmid carrying the CPY structural gene PRC1 (45); mutation of the vacuolar address determinant on CPY (24, 48); mutation of other genes involved in directing traffic to the vacuole (2, 37). In each experiment, at least 40% of the proCPY escaped sorting, was packaged into secretory vesicles, and secreted as p2 CPY into the medium. By comparison, wild-type strains secrete 1–5% of newly made proCPY.

To examine the possibility that chcl cells fail to segregate proCPY from the secretory pathway, CPY secreted into the medium of mutant and wild-type strains was quantified. After a 15-min labeling period, congenic strains were incubated for 45 min in chase medium. Samples were harvested at 0 and 45 min of chase and CPY was immunoprecipitated from cell lysates (Fig. 3 C) and medium fractions (Fig. 3 M). SDS-PAGE analysis of precipitated CPY, shown in Fig. 3, revealed that wild-type and mutant strains export similarly low levels of CPY. When corrected for the amount loaded on the gel (see legend to Fig. 3), <5% of the total CPY was detected in each strain. Analyses conducted with three other pairs of congenic partners gave similar results. Also, chcl cells did not secrete another vacuolar protein, proteinase A (not shown). In each experiment, parallel cultures of wild-type strains bearing PRC1 on a multicopy plasmid exported ~50% of the total CPY as the p2 form. Thus, clathrin-deficient cells retain the ability to sort vacuolar proteases from the secretory pathway with high fidelity.
**CPY in chc1 Cells Is Present in Vacuoles**

Immunolocalization of CPY in thin sections from mutant cells was undertaken to identify the subcellular residence of CPY. Fig. 4, A-C show electron micrographs of thin sections of a chc1 strain stained with affinity-purified anti-CPY antibodies and protein A-gold complexes. For comparison, Fig. 4 D depicts a similarly processed congenic CHC1 strain. Vacuoles were located by their dense, granular contents enclosed within a membrane bilayer. This distinctive characteristic, revealed by uranylacetate and lead acetate (or lead citrate) staining, is a commonly used criterion for vacuole identification (37, 40, 49). Clathrin-deficient mutant cells routinely exhibit accumulated membrane organelles and vacuolar structures (arrowheads) that appear more fragmented and are more frequently multivesicular in cross section than vacuoles in wild-type cells (33). In spite of the different internal morphologies of mutant and wild-type cells, most of the CPY in both cell types resides within vacuolar structures. In both mutant and wild-type cells, a low degree of labeling was present over organelles (arrows) smaller than the major vacuolar compartments. At present, the precise identification of these smaller structures is uncertain. They could represent smaller autonomous vacuoles, convolutions of the vacuole that appear as distinct structures in cross section, or another distinct membrane compartment. Small numbers of protein A-gold complexes in the cytoplasm represent background also observed in cells in which PRC1 has been deleted (not shown).

It is clear, in summary, that clathrin-deficient cells can sort newly synthesized CPY from the secretory pathway and deliver it to vacuoles.

**Endocytosis by chc1 Cells**

As described in the Introduction, studies of receptor-mediated endocytosis have provided the single demonstration of clathrin's association with membrane vesiculation. Recent reports have presented evidence for internalization of the peptide pheromone α-factor by MATa yeast. α-Factor uptake bears the hallmarks of receptor-mediated endocytosis: uptake depends on time, temperature, energy, and specific cell–surface receptors (7, 22). Furthermore, peptide uptake was accompanied by a concomitant loss of cell–surface receptor activity, presumably due to receptor–ligand internalization (22).

A modified version of the assay described by Chvatchko et al. (7) was used to measure uptake of radiolabeled α-factor...
Figure 4. Immunolocalization of CPY. Strains GPYII03 (A–C) and GPYII00 (D) were processed for CPY immunolocalization (see Materials and Methods). Thin sections of cells were incubated with affinity-purified anti-CPY antibodies. Bound antibodies were decorated with protein A-gold complexes. Large arrowheads point to vacuolar structures labeled with protein A-gold. Note the multiple vacuolar structures in A–C that often appear multivesicular. In contrast, the CHCI strain in D exhibited a single large vacuole. Small arrows indicate undefined membrane organelles containing label. n, the nucleus. Bars, 0.5 μm.

by wild-type and clathrin-deficient cells. Purified 35S-labeled α-factor was added to chcl sstl MATa or CHCI sstl MATa cells (10⁻⁸ M α-factor, 3 × 10⁸ cells/ml). The sstl mutation eliminates a secreted protease which degrades α-factor (6, 8). After incubation at 24°C, cells were collected and washed with pH2 buffer. pH2 treatment strips α-factor bound to receptors at the cell surface leaving only internalized peptide associated with cells (7; Baker, D., unpublished results). In our first experiments, despite the sstl mutation, exogenous α-factor was rapidly inactivated by chcl, but not CHCI, cells. Viable staining with methylene blue indicated that ~5% of the mutant cells were inviable whereas <0.1% of the wild-type cells were dead. It seemed possible that α-factor inactivation resulted from degradation by proteases released by inviable chcl cells. To circumvent this problem, genetic and biochemical strategies were applied. First, prbl (52) and pep4 (20) mutant alleles were introduced into chcl and CHCI strains to lower the level of endogenous vacuolar pro-
Figure 5. Uptake and binding of α-factor by MATa chcl and MATa CHC1 strains. (A) chcl cells internalize α-factor. MATa CHC1 strain GPY74–29B (solid squares) and MATa chcl strain GPY79.1 (open squares) were suspended at 3 × 10⁶ cells/ml in YP plus 5% glucose, 5 mM TAME, and 0.2 mM PMSF. α-Factor was added to 10⁻⁸ M and cells incubated at 24°C. At the designated times, cells were harvested and assayed for internalized α-factor by washing with pH2 buffer as described in Materials and Methods. (B) α-Factor uptake is energy and receptor dependent. α-Factor was measured after 20 min at 24°C as described in A. Samples without energy contained 10 mM sodium azide and 10 mM sodium fluoride in place of glucose. The bars indicate average values obtained from multiple experiments using MATa CHC1 strain GPY74–29B, chclΔ10 derivatives 79.1 and 79.2, and chclΔ10 MATa strain GPY68. In each experiment the amount of α-factor internalized by the MATa CHC1 strain was taken as 100%. (C) α-Factor binding. GPY74–29B (CHC1) and GPY79.1 (chclΔ10) were incubated with 10⁻⁸ M α-factor in YP medium plus 10 mM potassium fluoride, 10 mM sodium azide, 5 mM TAME, and 0.2 mM PMSF for 25 min at 24°C. Bound α-factor was measured by washing with the incubation medium as described in Materials and Methods.

Our results argue that receptor-mediated endocytosis can occur in the absence of clathrin heavy chain, albeit at reduced rates. chcl null alleles do not alter the cellular responses of MATa cells to α-factor and, accordingly, MATa chcl cells mate at near wild-type efficiencies (not shown).
Discussion

Aspects of two intracellular transport pathways have been examined in clathrin heavy chain-deficient yeast. Mutant cells properly localize newly synthesized vacuolar proteases and carry out receptor-mediated uptake of α-factor mating pheromone. Clathrin is therefore, unexpectedly, not required for either pathway, or can be replaced by a functional homologue in clathrin-deficient cells.

Transport of Newly Synthesized Proteins to the Vacuole

Comparison of CPY transport in chcl and CHCI strains has shown that mCPY appears at identical rates. Precursor CPY molecules are efficiently diverted from the secretory pathway and immunolocalization studies located most CPY molecules in vacuolar structures. These findings argue that clathrin does not provide a necessary function during vacuolar protein sorting and delivery. However, a slight defect was apparent in the conversion of pl to p2 CPY in mutant cells. The reduced rate of glycosyl maturation of pl CPY in chcl cells could represent an effect of clathrin deficiency on mannosyltransferase activity or organization, but if pl CPY were transported from the Golgi apparatus to the vacuole, lower molecular mass forms of mCPY would be expected. mCPY from CHCI and chcl migrate identically on SDS-PAGE (Payne, G. S., unpublished data). While the anomalous delay in glycosyl maturation of CPY in chcl strains remains unexplained, there appears to be no compromise in the rate of formation of the proteolytically mature enzyme.

These results contrast sharply with the characteristics of yeast mutant strains selected for mislocalization of vacuolar proteins (vpl mutants [37]; vpt mutants [2]). Mutations in as many as 50 genes have been obtained by selecting for efficient secretion of carboxypeptidase Y in a precursor or hybrid protein form. Many of these mutant strains secrete a large fraction of CPY in a proenzyme form and the kinetics of transport are comparable to that observed for a typical secreted protein. In spite of a dramatic reduction in the intracellular levels of soluble vacuolar proteins, many of these mislocalization mutants exhibit normal rates of growth. Clearly, the clathrin heavy chain gene is not a member of the vacuole protein sorting gene family.

Protease A is required for maturation of proCPY (1), yet when it is mislocalized along with proCPY in vpl or vpt mutants, both molecules persist in a precursor form (2, 37). Once again, in contrast to this phenotype, chcl mutant cells produce and retain mature protease A (unpublished data).

It is unlikely that mature CPY and protease A are formed before the vacuolar protein sorting event in chcl mutant cells. The essential sorting determinant has been identified in the propeptide segment of both proteases (24, 27, 48). Deletion of this signal(s) results in secretion of the mutant protease. Hence, premature processing of proCPY would remove the localization signal and result in mature, secreted CPY.

Although our results imply that clathrin is dispensable in transport of vacuolar proteins, they do not exclude the possibility that newly made proteins pass through a clathrin-coated compartment in route to the vacuole. This seems likely in the lysosome-oriented pathway in mammalian cells. Lysosomal hydrolases and the cation-independent, 212-kD mannose-6-phosphate receptor (involved in selective targeting of lysosomal protein to lysosomes) have been identified in purified clathrin-coated vesicles (4, 5, 41). In addition, immunocytochemical studies have revealed lysosomal proteins and mannose-6-phosphate receptors in coated regions of the Golgi apparatus and in coated vesicles (3, 13, 14). Similar studies of yeast vacuolar proteins await more purified preparations of clathrin-coated vesicles and elucidation of the intracellular distribution of clathrin-coated membranes.

Endocytosis

We have measured internalization of α-factor to assess receptor-mediated endocytosis by chcl cells. Jenness and Spatrick (22) and Chvatchko et al. (7) have recently provided evidence for uptake of α-factor via an endocytic pathway. Internalization of α-factor is temperature, energy, and receptor dependent and is accompanied by a loss of surface binding sites. Mutant yeast strains defective in fluid-phase accumulation of Lucifer Yellow CH are also unable to take up α-factor. Uptake of α-factor into the vacuole has been confirmed by direct visualization of 3H-labeled pheromone as autoradiographic grain tracks on thin sections (Chvatchko, Y. E. van Tuinen, and H. Riezman, unpublished results). Furthermore, internalized α-factor is not degraded in strains lacking active vacuolar proteases (pep4). Proof of an endocytic mechanism awaits the detection of a vesicular intermediate in the uptake of these molecules.

α-Factor is internalized by yeast cells in which clathrin has been specifically and completely eliminated. Although this result was unexpected, ports have been reported in studies of mammalian cells. Huet et al. (21) stimulated endocytosis of human fibroblast histocompatibility antigens with an anti-
body directed against β2-microglobulin. Immunoelectron microscopy has shown that uptake occurs through surface invaginations and endocytic vesicles that do not display clathrin coats. Another group (29) reported that inhibition of clathrin-coated pit formation in human Hep2 cells by intracellular K+ depletion completely blocks transferrin internalization but reduces endocytosis of ricin toxin by only 50%. Both of these studies suggest the existence of a clathrin-independent endocytic pathway. In a more direct approach, two groups have microinjected anticalasin antibodies into living cells and measured the endocytosis of molecules known to enter cells through clathrin-coated intermediates. In one case, uptake of α2-microglobulin, no effect was observed (50). In the second study, a 50% diminution of Semliki forest virus endocytosis was obtained (10).

Based on the studies using mammalian cells, and the data presented here, it appears that clathrin is not essential for receptor-mediated endocytosis. Thus, clathrin cannot be the sole mediator of plasma membrane vesiculation during endocytosis. Our studies do not distinguish between three possible interpretations of this conclusion: (a) clathrin does not directly provide the force necessary to generate a vesicle from the plasma membrane but, instead, expedites endocytosis through other capacities such as clustering certain surface receptors; (b) clathrin normally is the factor responsible for vesicle formation during receptor-mediated endocytosis but another protein can provide complementing function; (c) both clathrin-dependent and -independent pathways exist and α-factor is normally internalized by a clathrin-independent route.

The reduced uptake of α-factor by chcl cells is difficult to interpret and could reflect any of the scenarios just listed. If clathrin serves an auxiliary role in all endocytosis or if a heavy chain functional homologue exists, then elimination of CHC1 could expose less efficient endocytic pathways. On the other hand, α-factor may normally enter cells through a clathrin-independent route and the reduced internalization in chcl cells may be an indirect consequence of defects in other transport pathways. Isolation of conditionally defective CHC1 alleles and attempts to identify clathrin heavy chain homologues should aid in distinguishing these possibilities.

Recently, the sequence of the putative structural gene for the α-factor receptor, STE2, has been determined (30). The deduced amino acid sequence reveals a protein with seven potential membrane-spanning domains. This structural motif is shared by mammalian cell β-adrenergic receptors (see reference 43 for review). In contrast, other growth factor and nutrient receptor polypeptides, such as epidermal growth factor and low density lipoprotein receptors, are predicted to span the membrane only once. With this distinction in mind, it may be relevant that, unlike epidermal growth factor and low density lipoprotein receptors, β-adrenergic receptors are internalized (termed sequestration) by an undefined mechanism (43). In particular, it is unclear whether internalization occurs through clathrin-coated intermediates.

Lemmon and Jones (28) have identified a unique yeast strain that is inviable when CHC1 is disrupted. Inviability is due to a single genetic locus, named SCD1, which is unlinked to CHC1. In contrast, we have surveyed >10 strains from four separate laboratories without observing a locus that leads to inviability when combined with chcl (33). It thus seems likely that the strain described by Lemmon and Jones harbors an infrequent, possibly altered allele of SCD1. This allele could mark a gene whose product can normally substitute for clathrin directly, or indirectly as a component of a parallel pathway. However, other less relevant explanations could apply and further information is needed to establish the nature of SCD1 (see reference 33 for a detailed discussion). The existence of SCD1 does not affect our conclusions that transport to the vacuole and endocytosis can be sustained in the absence of clathrin.

Finally, we caution against concluding from our studies that clathrin does not play an important role in intracellular protein transport. Yeast cells devoid of clathrin heavy chain display compromised growth rates, accumulation of membrane organelles, a Golgi body or post-Golgi body delay in export of a secreted protein, and a reduced ability to internalize α-factor (32, 33). Preliminary results also indicate that another late Golgi or nascent secretory vesicle event, proteolytic maturation of the α-factor precursor, is disturbed in MATα chcl cells. Hence, although clathrin is not required for the essential functions originally postulated, it may facilitate a subset of intracellular transport processes. Furthermore, alternative mechanisms that back up clathrin function in normal cells may be pressed into service or even enhanced in clathrin-deficient cells.

Immunoelectron microscopy was carried out in the laboratory of Howard Riezman, supported by a grant from the Swiss National Science Foundation. We thank Rachel Sterne, Jeremy Thorner, Joel Rothman, and members of Jasper Rine's and our laboratories for helpful discussion and advice. Stephen P. Bell was responsible for the initial stages of the α-factor binding and endocytosis experiments. Joel Rothman and Tom Stevens generously provided antibody to proteinase A. We acknowledge Raymond Deshaies and Michael Basson for comments on the manuscript and Peggy McCutchen Smith for help in preparing the manuscript.

G. Payne was a senior fellow of the American Cancer Society, California Division. This work was supported by grants from the National Institute of General Medical Sciences of the National Institutes of Health and the National Science Foundation.

Received for publication 25 November 1987, and in revised form 11 December 1987.

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