The Light Chain Subunit Is Required for Clathrin Function in
Saccharomyces cerevisiae*

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Clathrin, a multimeric protein involved in intracellular protein trafficking, is composed of three heavy chains (Chc) and three light chains (Clc). Upon disruption (clc1Δ) of the single Clc-encoding gene (CLC1) in yeast, the steady state protein levels of Chc decreased 5–10-fold compared with wild type cells; consequently, phenotypes exhibited by clc1Δ cells may result indirectly from the loss of Chc as opposed to the absence of Clc. As an approach to directly examine Clc function, clc1Δ strains were generated that carry a multicopy plasmid containing the clathrin heavy chain gene (CHC1), resulting in levels of Chc 5–10-fold elevated over wild-type levels. As with deletion of CHC1, deletion of CLC1 results in defects in growth, receptor-mediated endocytosis, and maturation of the mating pheromone α-factor. However, elevated Chc expression in clc1Δ cells partially suppresses the growth and α-factor maturation defects displayed by clc1Δ cells alone. Biochemical analyses indicate that trimerization and assembly of Chc are perturbed in the absence of Clc, resulting in vesiculation defects. Our results demonstrate that the light chain subunit of clathrin is required for efficient Chc trimerization, proper formation of clathrin coats, and the generation of clathrin-coated vesicles.

Distinct compartments in eukaryotic cells are maintained through the selective transport of proteins carried out by small vesicular carriers. Generation of these vesicles involves assembly of proteinaceous coats on the cytoplasmic surface of donor compartment membranes, which leads to the budding of coated vesicles. Although a variety of proteins are transported through vesicular movement, a subset of specific trafficking events are mediated by clathrin-coated vesicles. These include the retention of resident Golgi membrane proteins, receptor-mediated endocytosis, and the sorting of lysosomal/vacuolar proteins from the secretory pathway to the lysosome/vacuole (1, 2).

The clathrin molecule, or triskelion, is a hexamer composed of three heavy chain subunits (Chc) and three light chain subunits (Clc) (1). In mammals, there are two forms of light chain, LCa and LCb, which share 60% amino acid identity and appear to be randomly distributed in clathrin trimers. In yeast, there is only one form of light chain encoded by the CLC1 gene (3). Formation of a clathrin-coated vesicle is initiated by binding of clathrin-associated protein complexes (APs)3 to the donor membrane. Triskelions associate with the APs and polymerize into polygonal lattice structures. Such clathrin-coated membrane segments, known as coated pits, are thought to collect specific cargo proteins through interactions between the cargo protein cytoplasmic domains and the AP complexes. The clathrin-coated pit then invaginates and pinches off to form a clathrin-coated vesicle carrying the selected cargo proteins. Formation of the vesicle may involve rearrangement of subunits assembled on the coated pit, or it may be driven by the polymerization of new clathrin subunits into a polyhedral cage. Once the vesicle has formed, the clathrin lattice is depolymerized into triskelions to allow fusion of the vesicle with the target organelle membrane. The resulting triskelions are then available for another round of vesicle formation.

Conformational differences in clathrin triskelions at each stage of the assembly and disassembly cycle can allow for specific homotypic and heterotypic associations that contribute to regulation of the cycle. This regulation is likely to occur at a number of levels to ensure the appropriate temporal and spatial sequence of molecular interactions necessary for selective protein transport by clathrin-coated vesicles. Clc exhibits several properties that suggest it may act as a regulatory subunit (4). In vitro, Clc can bind calmodulin, Hsc70, and calcium (5–7). LCa can be phosphorylated in vitro by casein kinase II and is phosphorylated at the same sites in vivo (8). Calcium binding and Hsc70 recognition by Clc have been proposed to play a role in depolymerization of clathrin coats (6), although this view has recently been challenged (9). The roles of calmodulin binding and phosphorylation have not been determined. Differences between LCa and LCb suggest that each may act in separate regulatory functions; i.e., LCb contains a phosphorylation site, while LCa does not. Furthermore, there are neuronal isoforms of Clc, generated through differential mRNA splicing, that are presumed to be involved in more specialized secretory and endocytic functions (10).

In order to evaluate the role of Clc in regulating clathrin function in vivo, we have initiated a genetic approach in the yeast Saccharomyces cerevisiae. Yeast Clc has only 18% amino acid identity with mammalian light chains, in contrast to the 50% sequence identity between clathrin heavy chains (3). Despite the sequence divergence, yeast Clc shares many biochemical properties with mammalian Clc including stoichiometric

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1 The abbreviations used are: AP, clathrin-associated protein; LSB, Laemmli sample buffer; CPY, carboxypeptidase Y; MES, 4-morpholineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; DSP, dithiothreitol/0.1M dithiothreitol; MSP, medium speed pellet; HSP, high speed pellet; TNG, trans-Golgi network; WT, wild type; ALP, alkaline phosphatase.
association with Chc in triskelions, size, overall acidic composition, heat and acid stability, and the ability to bind calmodulin and calcium (3, 4). The evolutionary conservation of these properties argues that Chc in yeast and mammals carries out similar functions. Deletion of CLC1 in yeast results in a slow growth phenotype similar to that observed in strains lacking the Chc gene (CHC1), suggesting that Chc is critical to clathrin function (3). Here we present phenotypic characterization of protein trafficking pathways as well as clathrin structure and distribution in cells carrying the CLC1 disruption (clc1Δ) and in ccl1Δ cells expressing elevated levels of Chc1p. Our results suggest that Chc plays a role in stabilizing clathrin trimers and in formation of clathrin coats and clathrin-coated vesicles.

**EXPERIMENTAL PROTOCOLS**

**Plasmids**—Plasmid constructions were carried out using standard molecular biology techniques (11). An AhsHI to BamHI fragment containing CLC1 was inserted into pBTM73 to generate pgalCLCURA3 (Gal-CLC1). pRS424/CLC1 was created by inserting a 1.5-kilobase pair BamHI fragment containing CLC1 into the BamHI site of pRS414. pRS424/CHC1/e and pRS425/CHC1/e were created by inserting a 6-kilobase pair BamHI fragment containing CHC1 into the BamHI site of pRS424 or pRS425.

**Yeast Strains and Media**—Yeast strains used in this study are listed. Yeast growth, mating, sporulation, and tetrad analyses were conducted as described by Sherman et al. (12). DNA transformations were performed by the lithium acetate procedure (13).

**Growth**—Yeast growth, mating, sporulation, and tetrad analyses were conducted as described by Sherman et al. (12). DNA transformations were performed by the lithium acetate procedure (13). GPY1118 MATα ura3–52 leu2–3,112 trp1 his4-519 can1 pep4::LEU2 YEpCHCCLURA3 was grown in liquid medium at 30°C with shaking (100 rpm) in a 5-ml sterile screw-cap tube. Cells were harvested by spinning at 10,000 g for 10 min and washed once in water before resuspension in Buffer A. Cells were resuspended in the appropriate medium and grown to the appropriate OD600. Cells were subjected to differential centrifugation analysis (14) or to metabolic labeling and immunoprecipitation analysis (15) as described by Sherman et al. (12). DNA transformations were performed by the lithium acetate procedure (13).

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Elevated expression of Chc1p in clc1Δ cells partially suppresses the growth defect. A, immunoblot of Chc1p protein levels. Protein extracts were prepared from congenic clc1Δ cells containing vector alone (Δ), single copy CLC1 (WT), or multicopy CHC1 (ΔMC CHC1). Extracts were subjected to SDS-PAGE (6% acrylamide), transferred to nitrocellulose, and probed with monoclonal antibody to Chc1p. Lanes 4 and 5 were loaded with one-tenth the amount of extract loaded in lanes 2 and 3, respectively. B, relevant genotypes of strains shown in C. C, growth of clc1Δ cells overexpressing CHC1. Congenic strains Δ, WT, and ΔMC CHC1 carrying GAL-CLC1 were grown on galactose or dextrose media at 30°C. Glucose represses expression of Clc1p from GAL-CLC1.

Resuspended in volumes equal to those of supernatants. Tris extraction of pellet fractions was done by resuspending the pellets after centrifugation in volumes of 0.8 M Tris-HCl, pH 7.5, equivalent to supernatants. Portions of each fraction were heated at 100°C in LSB and analyzed by SDS-PAGE and immunoblotting.

**Chemical Cross-linking—** Yeast clathrin was purified from GPY1118. Cells were grown 8 × 10⁶ cells/ml in YPD and subjected to galactose lysis in 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. The lysate was precipitated with 35% ammonium sulfate. The resulting pellet was resuspended in the same buffer and subjected to chromatography on a 2.5 × 100-cm Sepharose CL-4B column. Fractions (8 ml each) enriched for clathrin were resuspended in volumes equal to those of supernatants. Protein extracts were prepared from congenic clc1Δ cells containing vector alone (Δ), single copy CLC1 (WT), or multicopy CHC1 (ΔMC CHC1). Extracts were subjected to SDS-PAGE (6% acrylamide), transferred to nitrocellulose, and probed with monoclonal antibody to Chc1p. Lanes 4 and 5 were loaded with one-tenth the amount of extract loaded in lanes 2 and 3, respectively. B, relevant genotypes of strains shown in C. C, growth of clc1Δ cells overexpressing CHC1. Congenic strains Δ, WT, and ΔMC CHC1 carrying GAL-CLC1 were grown on galactose or dextrose media at 30°C. Glucose represses expression of Clc1p from GAL-CLC1.

Deletion of CLC1 causes a severe growth defect commensurate with that of clc1Δ cells (3). To determine whether elevation of Chc1p levels influences the growth of cells lacking Clc1p, we compared the growth rates of ΔMC CHC1 cells to wild-type and clc1Δ cells. In these experiments, strains were used that also harbored a plasmid carrying a copy of CLC1 under control of the GAL1 promoter (GAL-CLC1). When grown on galactose-containing medium to maintain expression of GAL-CLC1, all three strains exhibited equivalent growth (Fig. 1C, Galactose). This result indicates that the elevated expression of Chc1p in the ΔMC CHC1 strain does not affect cell growth if Clc1p is present. On dextrose-containing medium, where GAL-CLC1 expression is repressed, the poor growth of Δ cells became apparent (Fig. 1C, Dextrose). On this medium, ΔMC CHC1 cells also did not achieve wild-type growth but exhibited improved growth relative to the Δ cells. ΔMC CHC1 cells similarly exhibited intermediate growth rates when growth was monitored in liquid glucose-containing medium. The ΔMC CHC1 cells doubled in number in 2.8 h, while the WT cells doubled in 1.8 h and the Δ cells doubled in 4.5 h. These observations reveal that Clc1p is important for cellular growth. Furthermore, the partial restoration of growth by increased levels of Chc1p in ΔMC CHC1 cells suggests that Chc1p can act to some degree without Clc1p.

**RESULTS**

Increased Expression of CHC1 Partially Suppresses the Growth Defect Caused by clc1Δ—The half-life of Chc1p is reduced from greater than 2 h to approximately 1 h in clc1Δ strains (23). Chc1p levels were examined by immunoblotting to determine if this instability results in reduced steady state levels of protein. In clc1Δ cells, levels of Chc1p are approximately one-tenth the amount expressed by cells carrying a single copy of CLC1 (Fig. 1A, lane 1, compared with lanes 2 and 5). Consequently, defects in clc1Δ strains may result indirectly from the reduction of Chc1p as opposed to the absence of Clc1p. To circumvent the coincident loss of Chc1p, we constructed a set of isogenic clc1Δ strains that carry a plasmid vector with no insert (Δ), a single copy plasmid with CLC1 (WT), or a multicopy plasmid with CHC1 (ΔMC CHC1). The ΔMC CHC1 strain overexpresses Chc1p about 5-fold compared with the strain expressing single copy levels of Clc1p (Fig. 1A, lanes 2–4). The ΔMC CHC1 strain thus allowed us to assess the phenotypic consequences of Clc1p deficiency under conditions where Chc1p is not a limiting factor for clathrin function.

The absence of Chc1p results in an α-factor maturation defect. Cells were grown at 30°C and metabolically labeled with [35S]cysteine and [35S]methionine for 30 min. α-Factor was immunoprecipitated from the culture supernatant and subjected to SDS-PAGE (15% acrylamide). Cells were then harvested by centrifugation and washed in PBS. Extracts were prepared from congenic strains carrying vector alone (Δ), single copy CLC1 (WT), or multicopy CHC1 (ΔMC CHC1). Extracts were subjected to SDS-PAGE (6% acrylamide), transferred to nitrocellulose, and probed with monoclonal antibody to Chc1p. Lanes 4 and 5 were loaded with one-tenth the amount of extract loaded in lanes 2 and 3, respectively. B, relevant genotypes of strains shown in C. C, growth of clc1Δ cells overexpressing CHC1. Congenic strains Δ, WT, and ΔMC CHC1 carrying GAL-CLC1 were grown on galactose or dextrose media at 30°C. Glucose represses expression of Clc1p from GAL-CLC1.
glycosylated precursor (Fig. 2, lane 3). Elevated expression of Chc1p in ΔMC CHC1 cells resulted in a slight but reproducible suppression of the clc1Δ α-factor processing defect. This strain secreted 65% unprocessed α-factor (Fig. 2, lane 2). Overexpression of Chc1p in a wild-type (CHC1 CLC1) strain had no effect on α-factor processing (data not shown). The defective α-factor maturation in the ΔMC CHC1 strain indicates that Clc1p is required for the efficient processing of α-factor, most likely by functioning in the localization of Kex2p. However, the partial suppression of the α-factor maturation defect in the ΔMC CHC1 strain compared with the clc1Δ strain parallels the relative growth defects in the two strains and supports the conclusion that Chc1p retains some activity in the absence of Clc1p.

Vavcular Protein Sor[ing Is Efficient in clc1Δ Cells—To determine the effect of CLC1 deletion on another transport event that occurs at the Golgi complex, sorting and transport to the vacuole of the soluble vacuolar protease CPY was examined. CPY is normally synthesized in a precursor form that is translocated into the ER, where it is subject to signal sequence cleavage and core glycosylation. This produces a 67-kDa p1 form of the protein (25). This p1 form is then transported to the Golgi complex, where further glycosylation yields a 68-kDa p2 form (25). After sorting from the secretory pathway at the TGN and delivery to the vacuole, the p2 form is proteolytically processed to the 61-kDa mature protein (25). chc1-ts cells shifted to the nonpermissive temperature for short time periods fail to sort CPY at the TGN; thus, the p2 form of CPY is secreted from the cell (18). This observation suggests a role for Chc1p in vacuolar protein sorting. However, in chc1Δ cells or in chc1-ts cells shifted to the nonpermissive temperature for long time periods, sorting of CPY occurs normally (18, 26). It therefore appears that cells recover the ability to sort CPY following Chc inactivation, although the basis for this recuperation is not known.

Transport of CPY to the vacuole was monitored in the congenic clc1Δ strains using a pulse-chase regimen. Cells were labeled with [35S]cysteine and [35S]methionine for 15 min at 30 °C, and then excess unlabeled amino acids were added to quench labeling (pulse). Cells were then incubated for 0, 15, 30, or 45 min (chase), and CPY was immunoprecipitated from the extracellular (E) and intracellular (I) fractions at each time point (Fig. 3). The WT strain exhibits normal CPY sorting and transport with essentially complete maturation (86% mature, 4% p2 intracellular) within 15 min after commencing the chase (Fig. 3, WT). A small amount of mature CPY was also detected in the medium and most likely reflects a low level of cell lysis that occurs during preparation of extracellular fractions. For Δ cells, a subtle delay in processing of CPY was detected. p2 represented 18% of the total after the 15-min time point and 7% after the 45-min time point (Fig. 3, Δ). Sorting was relatively efficient; only about 6% of the CPY was secreted as the p2 form. An increasing percentage of mature CPY, from 2% at the 0 time point to 12% at 45 min, was also found in the extracellular fraction. This is consistent with our observations that chc1Δ and chc1-ts cells are more prone to lysis. For ΔMC CHC1 cells, there was little difference in the kinetics of processing or in the sorting of CPY in comparison with the Δ strain. Compared with the Δ cells, less mature CPY was found extracellularly in the ΔMC CHC1 cells, probably because the ΔMC CHC1 cells are less prone to lysis during preparation of the extracellular fractions. Based on these results, it is evident that CPY processing is only slightly perturbed in clc1Δ cells and that elevated expression of Chc1p has little or no effect on CPY transport.

Clc1p Is Required for Efficient Receptor-mediated Endocytosis—Loss of Chc function results in an immediate reduction in the rate of receptor-mediated internalization of the α-factor mating pheromone (20). In chc1Δ cells or in chc1-ts cells at the nonpermissive temperature, radiolabeled α-factor uptake decreases 2–4-fold (20). The role of Clc1p in endocytosis was examined by measuring α-factor internalization by Δ, ΔMC CHC1, and WT strains. In the Δ strain, levels of α-factor uptake were diminished to levels comparable with that previously found for chc1Δ cells, approximately 3–5-fold less than that of WT cells (Fig. 4 and data not shown). The ΔMC CHC1 strain exhibited an identical defect in α-factor uptake. It should be noted that when Chc1p was overexpressed in the WT strain, α-factor internalization proceeded as it does in the wild-type strain (data not shown). Hence, overexpression of Chc1p itself does not interfere with normal α-factor internalization. Therefore, Clc1p is required for the efficient uptake of α-factor, and elevated expression of Chc1p does not provide detectable function in the absence of Clc1p.

Chc1p Is Able to Form Trimers in the Absence of Clc1p—Analysis of clathrin-mediated transport pathways indicates that the absence of Clc1p causes defective clathrin function even in the presence of excess Chc1p. To examine the basis for these defects, we employed the ΔMC CHC1 strain to conduct biochemical studies of Chc1p, since the reduced levels of Chc1p in the Δ strain were difficult to detect. Trimerization of Chc1p in the absence of Clc1p was probed by chemical cross-linking of cytosol from the WT and ΔMC CHC1 strains using the reversible cross-linker DSP. Kirchhausen and Harrison (22) have demonstrated trimer organization through chemical cross-linking of purified bovine brain clathrin triskelions. When a similar cross-linking procedure was performed on purified yeast clathrin, the same pattern of cross-linked species was observed after immunoblotting with anti-Chc1p antibody (Fig. 5A, lanes 1, 2, and 8). Increasing the amount of DSP resulted in conversion of lower molecular weight species into the higher trimer form. These cross-linked species correspond to forms previously identified as Chc monomer (M0), Chc monomer plus one light chain (M1), Chc dimer forms with 0, 1, or 2 associated light chains (D0, D1, and D2), and a Chc trimer form (T). The pattern produced by immunoblotting with antibodies directed against yeast Clc1p was the same as that with anti-Chc1p antibodies.
except for the absence of M₀ and D₀ (Fig. 5A, lane 6). To examine the trimerization status of Chc1p in the WT and ΔMC CHC1 strains, cells were converted to spheroplasts and lysed by homogenization, and 100,000 × g supernatant fractions (cytosol) were prepared. Cross-linked WT cytosol, when probed for Clc1p (Fig. 5A, lane 7) or Chc1p (Fig. 5A, lanes 3–5), revealed a banding pattern similar to purified triskelions. The bands are slightly more diffuse, which may be due to interactions with other cytosolic proteins or to less efficient cross-linking caused by quenching of the DSP by other extract components. When ΔMC CHC1 cytosol was cross-linked, we observed a pattern consistent with trimer organization. The M₀ species cross-linked to two prominent higher molecular weight bands with increasing amounts of reagent (Fig. 5A, lanes 9–12). These forms correspond to the D₉ and T sizes. As expected for a strain lacking Clc1p, the light chain-containing forms M₁, D₁, and D₂ were not detected. To determine if the banding pattern resulted from specific interactions of Chc1p in triskelions, cross-linking was performed on extracts from clc1Δ strains that overexpress a truncated form of Chc1p lacking the trimerization domain ΔMC CHC1(Tri−). At DSP concentrations sufficient to convert most of the monomeric Chc1p in WT and ΔMC CHC1 extracts to dimeric and trimeric species (Fig. 5A, lanes 5 and 12), almost all of the truncated Chc1p remained
monomeric (Mₚ), and no trimeric form was observed (Fig. 5A, lane 14). A minor band (Fig. 5A, lane 14, asterisk) migrating more slowly than the monomer was detected, which could be due to the association of two Chc1p molecules through interactions along the arm domains similar to interactions that occur in assembled clathrin coats. The presence of a specific trimmer band in the ΔMC CHC1 strain provides strong evidence for the existence of Chc1p trimers in the absence of Clc1p.

Sepharose CL-4B size exclusion chromatography was also performed on extracts from the strains to evaluate the trimerization status of Chc1p. In cytosol prepared from WT cells, Chc1p peaked at fractions 12 and 14, where purified triskelions elute (Fig. 5B, WT, and data not shown). The Chc1p elution pattern from cytosol of ΔMC CHC1 was distinct, with Chc1p peaking in fractions 16 and 18 (Fig. 5B, ΔMC CHC1 cytosol). Fractions 16 and 18 correspond to the elution position of monomeric Chc1p, established by chromatography of the truncated form of Chc1p lacking the trimerization domain. However, the Chc1p from ΔMC CHC1 was also detected in earlier fractions (fractions 10–14), suggesting the presence of multimers. These findings, combined with the results from the cross-linking experiments, suggest that Chc1p can associate into trimers in the absence of Clc1p but that the association is inefficient or unstable when compared with Clc1p-containing trimers.

**Differential Fractionation of Chc1p Is Altered in the Absence of Clc1p**—We carried out differential centrifugation of WT and ΔMC CHC1 strains as an approach to determine if the trimers formed in ΔMC CHC1 cells are capable of assembly. Cells were converted to spheroplasts and lysed by homogenization. Extracts were then subjected to a low speed spin (1,500 × g for 5 min) to remove unbroken cells. Centrifugation of the low speed supernatant at 26,000 × g for 30 min yielded a medium speed pellet (MSP) and a medium speed supernatant. The medium speed supernatant was further fractionated into a high speed supernatant at 26,000 × g for 30 min; HS, high speed (100,000 × g for 20 min); T, total. The low speed spin (1,500 × g for 5 min) was not included in the quantitation because it contained a significant level of unbroken cells as determined from the distribution of the cytosolic marker glucose-6-phosphate dehydrogenase. We note that a 3-fold increase in Chc1p was detected in the low speed pellet from ΔMC CHC1 extracts compared with WT extracts. The ability of Chc1p to polymerize in the absence of Clc1p was evaluated by SDS-PAGE and immunoblotted with polyclonal antibodies to the Chc1p, Clc1p, G6PD, and AP activity. Values for glucose-6-phosphate dehydrogenase and ALP represent the average from two experiments. Values for Chc1p were collected from three experiments, except those for WT and ΔMC CHC1/CLC1, which represent one experiment. Protein levels were determined by densitometry. Numbers represent the relative distribution of each protein in the supernatant (S) and pellet fractions (P) of each centrifugation step (MS, medium speed (26,000 × g for 30 min); HS, high speed (100,000 × g for 20 min); T, total).

### Table II

| Protein marker | Strain | WT | ΔMC CHC1 | WT | ΔMC CHC1 | WT | ΔMC CHC1 | WT | ΔMC CHC1 |
|---------------|--------|----|----------|----|----------|----|----------|----|----------|
| G6PD          |        | 10 | 90       | 20 | 80       | 28 | 72       |    |          |
| ALP           |        | 100| 0        | 0  | 0        | 100| 0        |    |          |
| Chc1p         |        | 5  | 95       | 9  | 91       | 14 | 86       |    |          |
| G6PD WT       |        | 10 | 90       | 20 | 80       | 28 | 72       |    |          |
| G6PD ΔMC CHC1 |       | 5  | 95       | 9  | 91       | 14 | 86       |    |          |
| ALP WT        |       | 100| 0        | 0  | 0        | 100| 0        |    |          |
| ALP ΔMC CHC1  |       | 100| 0        | 0  | 0        | 100| 0        |    |          |
| Chc1p WT      |       | 11 | 71       | 22 | 78       | 22 | 78       |    |          |
| Chc1p ΔMC CHC1|       | 54 | 45       | 5  | 45       | 4  | 45       | 78 | 22       |

**Chc1p Does Not Form Coated Vesicles in the Absence of Clc1p in Vivo**—The ability of Chc1p to polymerize in the absence of Clc1p raised the possibility that coated vesicles could form in the ΔMC CHC1 cells. We investigated this possibility by subjecting HSP fractions from WT and ΔMC CHC1 cells to chromatography through Sephacryl S-1000. This procedure has been the standard approach used to obtain enriched preparations of clathrin-coated vesicles from yeast (28, 29). Fractions obtained from Sephacryl S-1000 column chromatography of the WT HSP were probed with antibodies directed against Chc1p, Aps1p, and Kex2p. Aps1p is a component of the AP complex related to the mammalian Golgi-localized AP-1 complex (21).
As expected, Aps1 co-fractionated with clathrin-coated vesicles, which peaked in fractions 42–48 (Fig. 7A). A portion of Kex2p also coeluted with Chc1p and Aps1p, consistent with a role for clathrin coats in the localization of Kex2p to the TGN as reported previously (21). Electron microscopy of samples negatively stained with uranyl acetate confirmed the presence of clathrin-coated vesicles in these fractions (data not shown). When the HSP prepared from ΔMC CHC1 cells was subjected to Sephacryl S-1000 chromatography, a dramatic shift in the elution profile of Chc1p was observed (Fig. 7B). Chc1p was not enriched in fractions expected to contain coated vesicles; instead, it separated into two peaks, one at fractions 26–28, which may be either Chc1p associated with large membranes or very large assemblies of Chc1p, and a pool at fractions 48–52, which eluted after the position of WT coated vesicles. The Chc1p in fractions 48–52 could still be sedimented by centrifugation at 100,000 × g, indicating that it does not represent free Chc1p released during resuspension of the HSP or during chromatography. Thus, this population of Chc1p appears to be assembled in a form that elutes as a complex that is smaller than the clathrin coats surrounding vesicles in WT cells. The elution profiles of Aps1p and Kex2p from the ΔMC CHC1 HSP were also shifted with respect to the position of WT clathrin-coated vesicles (Fig. 7B). The perturbation of Aps1p and Kex2p fractionation properties in cells with aberrant clathrin provides further evidence that these proteins are normally associated with clathrin coats. Both Aps1p and Kex2p were enriched in fractions 26–28, most likely indicative of association with larger membrane structures. Kex2p also showed a smaller peak in fractions 48–52, and a slight increase in the abundance of Aps1p was also observed in these fractions. The coincident elution of Chc1p with Aps1p and Kex2p with Chc1p raised the possibility that these proteins are able to associate into coat structures. Electron microscopic examination of material from fractions 26, 42, 48, and 53 showed clear vesicular structures were present; however, it did not reveal the presence of lattice-like structures characteristic of conventional clathrin coats. Thus, in cells lacking Clc1p, vesicles with conventional clathrin coats were not detected.

**Fig. 7.** Coated vesicles do not form in the absence of Clc1p. 100,000 × g pellet fraction from WT or ΔMC CHC1 cells was resuspended in Buffer A and chromatographed through Sephacryl S-1000. A portion of each fraction was trichloroacetic acid-precipitated, analyzed by SDS-PAGE (6% acrylamide), and immunoblotted with antibodies to Chc1p, Aps1p, or Kex2p. Column fraction numbers are indicated at the top of the panels.

**Discussion**

Because of the instability of Chc1p in clc1Δ cells, phenotypes in a clc1Δ strain cannot be attributed solely to the loss of Clc1p. We have used a clc1Δ strain expressing elevated levels of Chc1p to investigate the effect of the absence of Clc1p on clathrin-mediated processes within the cell. Our results reveal that the clathrin-mediated transport processes of receptor-mediated endocytosis and TGN membrane protein localization are compromised by the absence of Clc1p. Biochemical analyses indicate that there are defects in Chc1p trimerization, coat assembly, and coated vesicle formation. However, elevated expression of Chc1p partly suppresses clc1Δ cell growth and α-factor maturation defects, arguing that Chc1p retains some degree of function in the absence of Clc1p. Consistent with the *in vivo* results, Chc1p in cell extracts from the ΔMC CHC1 cells is capable of forming trimers and associating into larger, Tris-sensitive structures, which could represent assembled clathrin coats.

The α-factor maturation defect in clc1Δ cells argues that Chc1p contributes to clathrin function in the TGN localization of Kex2p. The prevailing model for TGN protein localization in yeast suggests that proper localization is maintained by transport of TGN proteins to endosomes followed by retrieval to the Golgi complex (2, 30). Based on studies of chc1 mutants, clathrin has been proposed to act in the localization pathway by collecting proteins into clathrin-coated vesicles, which form from the TGN and are targeted to endosomes (2, 17). In the absence of Chc1p function, Kex2p is no longer directed to the endosomal pathway but instead is mislocalized to the cell surface, resulting in decreased maturation of α-factor precursor (17, 24). Our analyses of clc1Δ strains adds support for this model of clathrin function in Kex2p localization. First, even with elevated Chc1p levels, clc1Δ cells exhibit α-factor maturation defects diagnostic of Kex2p mislocalization. Second, the loss of clathrin-coated vesicles in ΔMC CHC1 cells detected by Sephacryl S-1000 column chromatography was accompanied by a dramatic shift in the fractionation pattern of Kex2p compared with wild-type cells. This shift argues that Kex2p is associated with clathrin-coated membranes in wild-type cells. However, the increased expression of Chc1p in ΔMC CHC1 cells partially ameliorates the α-factor maturation defect caused by clc1Δ, suggesting that Chc1p alone can function weakly in localization of Kex2p to the TGN. This localization could be due to a low level of Chc1p-coated vesicle formation. Compatible with this suggestion, coelution of Chc1p and Kex2p in fractions 48–52 from the Sephacryl S-1000 column could reflect aberrantly or partially coated vesicles. Alternatively, even without being able to form vesicles, Chc1p coats assembled onto TGN membranes could still interact with Kex2p, thereby retarding transport from the TGN to the cell surface and raising the level of TGN Kex2p available to cleave the α-factor precursor.

In contrast to growth and pheromone maturation, receptor-mediated endocytosis of α-factor is not affected by the increased levels of Chc1p in clc1Δ cells. This difference offers a potential insight into the suppressing activity of Chc1p in growth and TGN protein localization. The assay for α-factor internalization assesses the complete sequestration of surface-bound pheromone resulting from incorporation into endocytic vesicles. Thus, this assay presumably represents a direct measure of clathrin function in the genesis of a transport vesicle. The inability of high Chc1p levels to suppress the endocytosis defect in clc1Δ cells suggests that Chc1p cannot function in membrane vesiculation without Clc1p. This observation, combined with the absence of conventional clathrin-coated vesicles in ΔMC CHC1 cell extracts, leads us to favor the aforementioned possibility that the suppression of the pheromone maturation
defects by increased Chc1p expression is due to assembly of static or frozen Chc1p coat structures incapable of forming vesicles rather than to low levels of Chc1p-coated vesicle formation. The partial suppression of TGN protein mislocalization might then contribute to improved growth rates.

In vitro studies of mammalian clathrin suggest that Clc does not require Clc subunits to trimerize. Trimer stability is not affected by removal of Clc from preformed triskelions (31), and an N-terminally truncated Clc forms trimers when expressed in E. coli in the absence of Clc (32). Our analysis of ΔMC CHC1 cells also indicates that Chc1p is able to trimerize in the absence of Clc1p, but Clc-deficient triskelions appear to be unstable. The difference in Chc trimer stability likely reflects the different preparations of clathrin. The mammalian studies used preformed triskelions obtained from purified clathrin-coated vesicles or used Chc trimers formed by overexpression in bacteria. In contrast, our experiments analyzed Chc1p expressed in a native context, albeit at somewhat elevated levels. Taken together, the analyses of mammalian and yeast clathrin suggest that Clc is capable of trimerizing without Clc but that Clc facilitates the formation of stable trimers.

Clc-deficient mammalian triskelions will assemble into coat-like lattices in vitro, but assembly does not display the dependence on calcium or clathrin AP complexes that wild-type triskelion assembly exhibits (33, 34). The less stringent requirements for Clc-deficient triskelion assembly in vitro have fostered the proposal that Clc acts to prevent premature assembly of clathrin in vivo. The distribution of Chc1p in differential centrifugation fractions of ΔMC CHC1 extracts conforms to this model. Compared with wild-type clathrin, a much higher percentage of Chc1p is associated with the pellet fractions, a result expected if Clc-deficient Chc1p assembled in an unregulated manner (33, 34). We cannot completely exclude the possibility that the Chc1p in the pellets is nonspecifically aggregated rather than polymerized into cages. However, the sensitivity of the sedimented Chc1p to Tris extraction and the trimeric properties of the extracted Chc1p are characteristic of clathrin coats and support the possibility that a significant portion of Chc1p is polymerizing into coat-like structures. Even with this possible increase in polymerized clathrin, clathrin-coated vesicle formation in ΔMC CHC1 cells is severely defective. This defect could be the consequence of nonproductive Chc1p assembly without Clc1p, or it could reveal a role for Clc1p in the conversion of planar clathrin lattices into closed polyhedral cages. Further experiments will be needed to distinguish between these possibilities. In either case, our data provide evidence that Clc1p is necessary in vivo to maintain Chc1p in a form capable of participating in transport vesicle biogenesis.

The studies presented here represent the first characterization of Clc function in clathrin-dependent protein transport in vivo. Clc1p is important for clathrin triskelion stability and appears to be required for formation of clathrin-coated vesicles at the plasma membrane and Golgi complex. These phenotypes now provide a foundation for a genetic characterization of specific Clc properties such as phosphorylation, calcium binding, and calmodulin binding.

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