Novel functions of clathrin light chains: clathrin heavy chain trimerization is defective in light chain-deficient yeast

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SUMMARY

Clathrin is a major coat protein involved in sorting and retention of proteins at the late Golgi and in endocytosis from the cell surface. The clathrin triskelion contains three heavy chains, which provide the structural backbone of the clathrin lattice and three light chains, which are thought to regulate the formation or disassembly of clathrin coats. To better understand the function of the clathrin light chain, we characterized yeast strains carrying a disruption of the clathrin light chain gene (*CLC1*). Light chain-deficient cells showed phenotypes similar to those displayed by yeast that have a disruption in the clathrin heavy chain gene (*CHC1*). In *clc1-Δ* cells, the steady state level of the clathrin heavy chain was reduced to 20%-25% of wild-type levels and most of the heavy chain was not trimerized. If *CHC1* was overexpressed in *clc1-Δ* cells, heavy chain trimers were detected and several *clc1-Δ* phenotypes were partially rescued. These results indicate that the light chain is important for heavy chain trimerization and the heavy chain still has some function in the absence of the light chain.

In yeast, deletion of *CHC1* is lethal in strains carrying the *scd1-i* allele, while strains carrying the *scd1-v* allele can survive without the heavy chain. In previous studies we isolated several multicopy suppressors of inviability of *chc1-Δ scd1-i* cells. Surprisingly, one of these suppressors, *SCD4*, is identical to *CLC1*. Overexpression of *CLC1* in viable *chc1-Δ scd1-v* strains rescued some but not all of the phenotypes displayed by these cells. In the absence of the heavy chain, the light chain was not found in a high molecular mass complex, but still associated with membranes. These results suggest that the light chain can function independently of the clathrin heavy chain in yeast.

Key words: Clathrin, *Saccharomyces cerevisiae*, Triskelion, Golgi retention, Endocytosis

INTRODUCTION

Clathrin is the major component of the lattice-like coat of clathrin-coated vesicles and membranes. This lattice is assembled from a pool of soluble clathrin triskelions. Packaging material into clathrin-coated vesicles begins with the recruitment of the clathrin associated proteins (APs) to the cytoplasmic face of the membrane. The APs are thought to mediate receptor capture and are required for subsequent assembly of clathrin (reviewed by Robinson, 1994; Schmid and Damke, 1995). Soluble clathrin triskelions bind to the AP complexes and as the lattice extends, it converts from a planar to a curved configuration which eventually leads to pinching off of the coated vesicle (Kirchhausen, 1993). Shortly after the clathrin-coated vesicle is formed, the coats disassemble from the vesicle and become available in further rounds of vesicle budding.

Each clathrin triskelion is composed of three heavy chains (HC; 180 kDa) and three light chains (LC; 30-40 kDa). Clathrin heavy chains are trimerized at their C-termini with their bent arms extending radially from the vertex (Pearse and Crowther, 1987; Ungewickell and Branton, 1981). One clathrin LC is non-covalently bound along each HC arm, proximal to the vertex (Kirchhausen and Toyoda, 1993; Liu et al., 1995; Nathke et al., 1992; Ungewickell, 1983). Clathrin HCs and LCs are thought to interact in a coiled-coil fashion, mediated by heptad repeats contained within both proteins (Nathke et al., 1992; Scarmato and Kirchhausen, 1990). In mammalian cells, one major clathrin HC gene and two clathrin LC genes (encoding LCa and LCb) have been identified (Jackson et al., 1987; Kirchhausen et al., 1987a,b).

While the HC provides the structural backbone of the clathrin lattice, the role of the LC is still not clear. In triskelions, the LC is exposed to the cytoplasmic face of coated vesicles and pits, potentially facilitating its interaction with cytoplasmic regulatory elements (De Luca-Flaherty et al., 1990; Kirchhausen et al., 1983; Lisanti et al., 1982). In addition, clathrin LC binds Ca^{2+} (Mooibroek et al., 1987; Nathke et al., 1990) and calmodulin (Lisanti et al., 1982; Pley et al., 1995; Silveira et al., 1990) and mammalian LCb can be phosphorylated in vitro by casein kinase II (Bar-Zvi and Branton, 1986), implicating LCs as mediators of cytoplasmic

signals. The LCs have been proposed to regulate clathrin lattice assembly or disassembly from membranes. Hsc70 was originally thought to mediate the uncoating of coated vesicles (Rothman and Schmid, 1986), presumably through its interaction with a glycine- and proline-rich region on LCa (De Luca-Flaherty et al., 1990). However, more recent studies indicate that the LC is not necessary for this uncoating reaction (Ungewickell et al., 1995). Other studies have shown that the LC is not required for triskelions to assemble into cages in vitro (Winkler and Stanley, 1983), but they may have a role in preventing the inadvertent assembly of clathrin lattices (Ungewickell and Ungewickell, 1991). Furthermore, work using purified HC fragments suggests that the LC is not necessary for HC trimerization, even though the LC binding site on the HC is just upstream of the region required for trimerization (Liu et al., 1995).

Most of the studies on LC have been carried out in vitro using animal clathrin. The cloning of clathrin HC and LC genes in yeast have offered the opportunity to begin to address the role of the LC in an in vivo system. *Saccharomyces cerevisiae* contains one clathrin heavy chain gene, *CHC1* (Lemmon and Jones, 1987; Payne and Schekman, 1985). *CHC1* encodes a protein of 1,653 amino acids that is 50% identical to the clathrin HC from rat (Lemmon et al., 1991). The importance of the HC in yeast was first demonstrated when the *CHC1* gene was disrupted and it was found that HC-deficient yeast grow poorly or are inviable depending on their genetic background (Lemmon and Jones, 1987; Munn et al., 1991; Payne and Schekman, 1985). In our lab, the difference in viability of the *chc1-Δ* cells was shown to be the result of an independently segregating gene called *SCD1* (suppressor of clathrin deficiency 1; Lemmon and Jones, 1987; Nelson and Lemmon, 1993). In the presence of the *scd1-i* allele cells that lack clathrin HC are inviable; in the presence of the *scd1-v* allele clathrin HC-deficient cells are viable but grow poorly. In addition to their slow growth, *chc1-Δ scd1-v* cells display several phenotypes. These cells are genetically unstable and become polyploid at a high frequency (Lemmon et al., 1990). Viable HC-deficient cells also mislocalize Golgi-resident proteins to the plasma membrane and display slowed endocytosis (Payne et al., 1988; Payne and Schekman, 1989). Similar results have been obtained with a temperature sensitive allele of the clathrin HC gene (Seeger and Payne, 1992; Tan et al., 1993).

S. cerevisiae contains one clathrin light chain gene, *CLC1* (Silveira et al., 1990), which encodes a 233 amino acid protein with a predicted molecular mass of 26.5 kDa. The yeast LC is only 18% identical to the mammalian LCb; nevertheless, conserved regions in mammalian LCs, such as the calcium binding site, the calmodulin binding site and a heptad repeat region are also found in yeast LC, suggesting that yeast and mammalian LCs share similar functions (reviewed by Brodsky et al., 1991). Strains carrying a disruption in *CLC1* are viable but grow slowly. This is similar to the phenotype of *chc1-Δ scd1-v* cells, although further phenotypic analysis has not been reported. Interestingly, unlike clathrin HC-deficient strains, *clc1-Δ* strains are viable in the *scd1-i* background (Silveira et al., 1990). This finding indicates that clathrin HCs have some function in the absence of the LC.

In this paper we report the further characterization of clathrin LC-deficient yeast. We find the phenotypes of *clc1-Δ*

cells mimic those displayed by yeast lacking the clathrin HC. Our studies also provide evidence that the LC is important for the efficient formation of HC trimers in vivo. Overexpression of the HC results in partial rescue of the trimerization defect, as well as some of the phenotypes associated with the loss of clathrin function. In addition, we have uncovered a new role for clathrin LC by identifying the *CLC1* gene as a multicopy suppressor of inviable HC-deficient yeast. This surprising result indicates that the LC can function independent of the HC in yeast.

MATERIALS AND METHODS

Strains, media and genetic methods

Strains used in this study are listed in Table 1 and were derived from the S288C background (Mortimer and Johnston, 1986). YEP-dextrose (YEPD), minimal synthetic medium (MV) and dropout media were prepared as described (Nelson and Lemmon, 1993). Canavanine medium was arginine omission synthetic medium containing 60 mg/liter canavanine sulfate. Yeast mating, sporulation and tetrad analysis were carried out essentially as described by Guthrie and Fink (1991). *Escherichia coli* DH5α was used for plasmid propagation.

The canavanine resistance (Can^r) test used to monitor increased genome copy numbers was performed as described previously (Lemmon et al., 1991).

Plasmid construction and molecular methods

Plasmid pLS10, kindly provided by G. Payne, contains *CLC1* cloned into pUC119 (Silveira et al., 1990). The 2,300 bp *Xba*I to *Bam*HI *CLC1* fragment from pLS10 was subcloned into the polylinkers of: (1) pRS424 to create pJT1 (*CLC1, TRP1, 2μ*); (2) pRS426 to create pKH2 (*CLC1, URA3, 2μ*); and (3) pRS316 to create pKH4 (*CLC1, URA3, CEN*). The yeast pRS shuttle vectors have been described (Christianson et al., 1992; Sikorski and Hieter, 1989). Plasmid pKH7 is a *TRP1, 2μ* plasmid with a deletion of all but the first 69 codons of *CLC1*. This plasmid was constructed by cloning the 1,800 bp *Eco*RI to *Eco*RV fragment from pJT1 into the *Eco*RI/*Sma*I sites of pRS426. The clathrin HC plasmid pSL6 (*CHC1, URA3, CEN*) has been described (Lemmon et al., 1991) and pSL25 (*CHC1, URA3, 2μ*) was made by cloning the *Pvu*II to *Sal*I fragment of *CHC1* into YEp24. Throughout this paper, YCp*CLC1* refers to pKH4, YEp*CLC1* refers to either pJT1 or pKH2, YCp*CHC1* refers to pSL6 and YEp*CHC1* refers to pSL25.

A null allele of *CLC1*, marked by the *HIS3* gene, was generated using the deletion construct p*clc1-Δ2* described by Silveira et al. (1990). The 4 kb disruption fragment was excised from p*clc1-Δ2* using *Eco*RV and *Pvu*II and transformed into diploid cells SL1509 (*scd1-i/scd1-i*) and SL1528 (*scd1-v/scd1-v*) to generate *clc1-Δ::HIS3/CLC1* heterozygous diploids SL1522 and SL1590. Gene disruptions were verified by Southern blot hybridization.

The high copy suppressor of clathrin deficient lethality, *SCD4*, was isolated by Nelson and Lemmon (1993). The rescuing region on YEp*SCD4* was determined by subcloning portions of YEp*SCD4* and testing for the ability to rescue growth of a *GAL1:CHC1 scd1-i* strain (SL214) on glucose medium as described (Nelson and Lemmon, 1993).

A single myc epitope tag was appended to 3′ end of the *CHC1* open reading frame by the dut⁻ ung⁻ oligonucleotide mutagenesis method (Kunkel et al., 1987). The template was a M13 clone containing a 1.8 kb *Hin*dIII fragment that encodes the carboxy terminus of Chc1p. The oligonucleotide was (5′ to 3′) AACCCACAGGATTTCAA-CAAAAGTTGATCTCTGAAGAAGACTTGAACTAAGACGTG-GATAAA. Following mutagenesis, a 1.6 kb *Bam*HI to *Hin*dIII

Table 1. *Saccharomyces cerevisiae* strains

Number	Genotype	Reference or source*
BJ2649	*MATa lys2 trp5 can1^r*	(Lemmon et al., 1987)
BJ2651	*MATα lys2 trp5 can1^r*	(Lemmon et al., 1987)
BJ3255	*MATα chc1-Δ::LEU2 ura3-52 ade6*	
BJ3529	*MATa/MATα chc1-Δ::LEU2/ CHC1 leu2/leu2 ura3-52/ ura3-52 his1/HIS1 ade6/ ADE6 scd1-i/scd1-i*	(Lemmon et al., 1991)
BJ4319	*MATa/MATα chc1-Δ::LEU2/ CHC1 leu2/leu2 ura3-52/ ura3-52 ade6/ADE6 scd1-v/ scd1-v*	
RH266-1D	*MATa end3-1 bar1-1 leu2 ura3 his4*	(Riezman, 1993)
SL214	*MATa GAL1:CHC1 leu2 ura3-52 trp1 his1 GAL2 scd1-i*	(Nelson and Lemmon, 1993)
SL1463	*MATα leu2 ura3-53 trp1 his3- Δ200 GAL2 scd1-v*	
SL1509	*MATa/MATα CLC1/CLC1 GAL2/ GAL2 leu2/leu2 ura3-52/ ura3-52 trp1/trp1 his3-Δ 200/ his3-Δ200 scd1-i/scd1-i*	
SL1522	*MATa/MATα clc1-Δ::HIS3/CLC1 GAL2/GAL2 leu2/leu2 ura3-52/ ura3-52 trp1/trp1 his3-Δ200/ his3-Δ200 scd1-i/scd1-i*	
SL1528	*MATa/MATα CLC1/CLC1 GAL2/ GAL2 1eu2/leu2 ura3-52/ ura3-52 trp1/trp1 his3-Δ200/ his3-Δ200 scd1-v/scd1-v*	
SL1590	*MATa/MATα clc1-Δ::HIS3/CLC1 GAL2/GAL2 leu2/leu2 ura3-52/ ura3-52 trp1/trp1 his3-Δ200/ his3-Δ200 scd1-v/scd1-v*	
SL1620	*MATα clc1-Δ::HIS3 leu2 ura3-52 trp1 his3-Δ200 GAL2 scd1-v*	
SL1638	*MATa leu2 ura3-52 trp1 his3-Δ200 GAL2 scd1-v pJT1 (YEpCLC1)*	
SL1929	*MATa clc1-Δ::HIS3 bar1-1 leu2 ura3-52 trp1 scd1-v*	
SL2092	*MATa chc1-Δ::LEU2 leu2 ura3-52 trp1 scd1-v (YCpCHC1-myc)*	
SL2094	*MATa chc1-Δ::LEU2 clc1-Δ::HIS3 leu2 ura3-52 trp1 scd1-v (YCpCHC1-myc)*	

*All SL strains were generated for this study or are from this laboratory except where indicated.

fragment (containing the mutagenized sequence) was subcloned into YCp50 to create pAP14. The 5′ end of the gene was then reconstructed by subcloning the 6.2 kb *Bam*HI *CHC1* fragment into the *Bam*HI site of pAP14 to create YCp*CHC1-myc*. On SDS gels, the Chc1p-myc migrates slightly slower than the untagged HC, consistent with its increased molecular mass. YCp*CHC1-myc* complements the *chc1-Δ* mutation and rescues all Chc− phenotypes fully (data not shown).

DNA sequencing was performed by the dideoxy-chain termination procedure. GenBank searches were performed using the BLAST Server at NCBI (Altschul et al., 1990).

Biochemical and cellular procedures

Radiolabeling and immunoprecipitation of α-factor from the medium were preformed as described previously (Stepp et al., 1995). Autoradiograms were quantitated using a Sci-Scan 5000 (US Biochemical, Cleveland, OH). For endocytosis studies, ^{35}S-labeled α-factor was prepared as detailed previously (Blumer et al., 1988) and the uptake of radiolabeled α-factor was monitored essentially as described by Dulic et al. (1991).

For quantitative analysis of steady state levels of Chc1p, yeast protein extracts were prepared by glass bead homogenization (Stepp et al., 1995) and analyzed by SDS gel electrophoresis and immunoblotting. Chc1p was detected using anti-yeast clathrin HC monoclonal antibodies (Lemmon et al., 1988) followed by incubation with rabbit anti-mouse IgG (Zymed Labs, San Francisco, CA). Blots were developed using ^{125}I-Protein A (Amersham, Arlington Hts, IL) and autoradiograms were analyzed by densitometry as described above.

For size fractionation of native HC and LC, cells (3×10^9) were lysed with glass beads in a Braun homogenizer for 3 minutes. In experiments analyzing HC, cells were lysed in 1 ml Tris:Buffer A which contains a 1:1 volume ratio of 1.0 M Tris HCl, pH 7.0:buffer A (0.1 M MES, pH 6.5, 0.5 mM MgCl$_2$, 1.0 mM EGTA, 0.2 mM DTT, 0.02% NaN$_3$) in the presence of protease inhibitors (100 μM TPCK, 500 μM E64, 1 mM benzamidine HCl, 25 μM pepstatin A, 4 μM leupeptin). To analyze LCs by gel filtration, cells were lysed in TBS (50 mM Tris, 150 mM NaCl), pH 8.0. Extracts were centrifuged for 30 minutes at 29,000 **g** and for 1 hour at 100,000 **g**, and 250 μl of the supernatants (S100) were analyzed by gel filtration on 1 cm × 24 cm Superose 6 or Superose 12 columns (Pharmacia, Sweden). Extracts were separated on the Superose 12 column at a flow rate of 0.45 ml/minute, collecting 0.3 ml fractions starting 10 minutes after sample injection. For separation on Superose 6, the flow rate was 0.35 ml/minute and 0.25 ml fractions were collected beginning 10 minutes after sample injection. Fractions were analyzed by SDS gel electrophoresis and immunoblotting using anti-Chc1p or anti-myc 9E10 monoclonal antibodies or anti-Clc1p polyclonal antibodies (gift of G. Payne), followed by incubation in horseradish peroxidase-conjugated goat anti-mouse (Kirkegaard and Perry Labs, Gaithersburg, MD) or goat anti-rabbit (Zymed Labs) antibodies. Blots were developed using ECL (Amersham).

To analyze free clathrin light chain, a *CLC1* overexpression strain (SL1638) was lysed in Tris:Buffer A and centrifuged as above. The S100 fraction was then boiled for 10 minutes, centrifuged for 10 minutes at 14,000 rpm and the resulting supernatant was analyzed on the Superose 12 column.

For cross-linking experiments, cells (4×10^9) grown to mid-log phase were lysed in Tris:Buffer A containing protease inhibitors and subjected to gel filtration analysis on Superose 6 as described above. Column fractions were pooled and dialyzed for 3 hours against 3×2 liters of 50 mM sodium phosphate, pH 7.6, containing protease inhibitors. The reversible, homobifunctional cross-linking reagent DSP [dithiobis(succinimidyl propionate), Pierce] was dissolved in DMSO to 4 mg/ml and added to 200 μl of pooled column fractions at a final concentrations of 0 to 200 μg/ml DSP. Control samples without cross-linker received DMSO alone. After 30 minutes at 25°C the reactions were quenched with 0.5 M Tris base, 0.5 M glycine added to a final concentration of 10 mM. Samples (50 μl) were analyzed under non-reducing conditions on 3% acrylamide/SDS gels (acrylamide:bis-acrylamide 12:1) containing a 3% acrylamide/SDS stacker (acrylamide:bis-acrylamide 80:1). Gels were blotted onto Immobilon P (Millipore) in 25 mM Tris base, 192 mM glycine and developed with anti-Chc1p antibodies and ECL reagents. In all experiments, cross-linking was reversible by treating samples with β-mercaptoethanol (not shown).

Cell fractionation by differential centrifugation was performed as described previously (Nelson et al., 1996) to generate 10,000 **g** and 100,000 **g** pellets and supernatants (P10, S10, P100 and S100).

Sucrose step gradients were performed as described previously (Nelson et al., 1996) using only P10 fractions or combined P10 and P100 fractions. Fractions were analyzed by SDS gel electrophoresis and immunoblotting using anti-Chc1p, anti-Clc1p or anti-Sec12p antibodies (gift of A. Nakano). Blots were developed with ECL.

RESULTS

Steady state levels of the yeast HC are decreased in the absence of the LC

In previous studies, Silveira et al. (1990) showed that clathrin LC-deficient yeast display a slow-growth phenotype similar to clathrin HC-deficient yeast. The major difference observed between *clc1-Δ* and *chc1-Δ* strains was that HC-deficient yeast are inviable in the presence of the *scd1-i* allele, while *clc1-Δ* cells survive. This suggested that there is residual clathrin function without the LC. One possible explanation for the *clc1-Δ* phenotype is that the HC is functional in the absence of the LC, but the amount of HC in these cells is decreased. To investigate this, the steady state levels of the HC in cell extracts from an isogenic pair of *CLC1* and *clc1-Δ* strains were analyzed by immunoblotting. We found that the amount of HC in *clc1-Δ* cells was 20-25% of that in their wild-type counterparts (Fig. 1, lanes 1-3). This suggests that the LC is important for the stability of the HC.

CHC1 overexpression rescues phenotypes of *clc1-Δ* cells

If the phenotype of *clc1-Δ* cells was merely caused by the reduced levels of the HC, LC-deficient yeast expressing wild-type levels of the HC might be expected to grow normally and have rescued phenotypes. To test this hypothesis, we generated isogenic *clc1-Δ scd1-v* strains expressing increased levels of the HC by transforming with *CHC1* on low copy centromeric (YCp*CHC1*) and high copy 2 micron (YEp*CHC1*) plasmids. In LC-deficient cells carrying YCp*CHC1*, the steady state level of the HC was identical to wild-type cells (Fig. 1, lane 4), while expression of *CHC1* on a high copy plasmid resulted in a greater than 10-fold increase in the level of HC compared to wild-type cells (lane 5). A similar increase was also seen in *CLC1* cells carrying a high copy *CHC1* plasmid (lane 6).

Next, we compared the phenotypes of *clc1-Δ* cells to their isogenic counterparts carrying additional copies of *CHC1*. We examined *clc1-Δ* cells for temperature sensitivity, ploidy, α-factor processing and endocytosis, all of which are affected in *chc1-Δ* yeast. Consistent with results previously reported (Silveira et al., 1990), cells lacking the LC grew slower than

Fig. 1. Steady state levels of Chc1p are reduced in *clc1-Δ* strains. Cells were lysed and extract proteins (100 μg) were analyzed by SDS gel electrophoresis and immunoblotting using anti-yeast Chc1p monoclonal antibodies. Strains examined are SL1463 (*CLC1;* lane 1) and SL1620 (*clc1-Δ scd1-v*) transformed with: no plasmid (lane 2); YCp*CLC1*, pKH4 (lane 3); YCp*CHC1,* pSL6 (lane 4); or YEp*CHC1*, pSL25 (lane 5). Lane 6 is SL1463 transformed with YEp*CHC1*, pSL25.
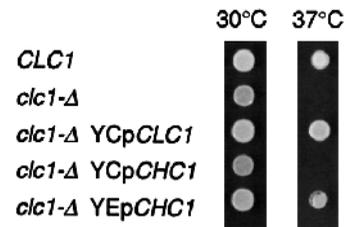

Fig. 2. Overexpression of *CHC1* partially rescues the growth defect of *clc1-Δ* cells. Cells were grown in selective medium to mid-log phase, diluted to 10^7 cells/ml in YEPD, spotted onto YEPD plates using a multi-prong inoculator and grown one day at 30°C or 37°C. Strains examined are SL1463 (*CLC1*) and SL1620 (*clc1-Δ scd1-v*) transformed with no plasmid, YCp*CLC1*, YCp*CHC1* or YEp*CHC1*. Note that the growth characteristics of SL1620 transfromed with empty vector were identical to untransformed cells.

CLC1 cells at 30°C and were inviable at 37°C (Fig. 2). A *clc1-Δ* strain transformed with YCp*CHC1*, which had normal levels of the HC, still displayed *clc1-Δ* growth characteristics. Doubling times at 30°C for *clc1-Δ* cells with and without YCp*CHC1* were 4-5 hours, as compared with 2 hours for *CLC1* cells. This indicates that LC-deficient phenotypes cannot be attributed solely to reduced HC levels. Surprisingly, *clc1-Δ* cells overexpressing *CHC1* grew nearly as well as wild-type cells at 30°C (doubling time of 2.5 hours at 30°C) and temperature sensitivity was rescued (Fig. 2). Therefore, overexpression of the HC can partially compensate for the loss of the LC. Similar degrees of rescue of growth phenotypes were observed for LC-deficient strains of the *scd1-i* genotype (not shown).

It has been shown that *chc1-Δ scd1-v* spore segregants increase their genome copy numbers upon propagation (Lemmon et al., 1990). We used a canavanine resistance test to examine the ploidy of *clc1-Δ* spore clones derived from the *clc1-Δ/CLC1* heterozygous diploid, SL1590, transformed with

Table 2. Can^r test for genome copy numbers in clathrin-deficient strains*

Relevant genotype of Can^s spore segregant†	Total zygotes scored	% papillating to Can^r	Inferred genome copy number
A.			
CLC1 scd1-v	128	100%	1n
clc1-Δ scd1-v	109	11%	>2n
clc1-Δ YCp*CHC1*	128	100%	1n
clc1-Δ YEp*CHC1*	128	100%	1n
B.			
CHC1 scd1-v	128	100%	1n
chc1-Δ scd1-v	64	13%	>2n
chc1-Δ YEp*CLC1*	128	100%	1n

*Two to four independent Can^s spore segregants of the indicated genotype were mass mated to haploid Can^r tester strains of the opposite mating type (BJ2649 or BJ2651). Fifteen to 32 prototrophic zygotes from each cross were then patched onto canavanine plates to test for papillation to Can^r. Since *CAN1^s* is dominant to *can1^r*, mating a haploid Can^s cell to a haploid tester Can^r cell results in zygotes that papillate extensively to Can^r. In contrast, mating a Can^s cell of higher ploidy (≥2n) to a haploid Can^r cell does not result in significant papillation. The data for spore segregants of a given genotype were pooled and represent the percentage of all zygotes that papillated to Can^r relative to the total scored.

†Spore segregants were derived from diploid SL1590 (A) or BJ4319 (B) carrying the indicated plasmids. Note that diploids transformed with empty parent plasmids gave results identical to untransformed diploids.
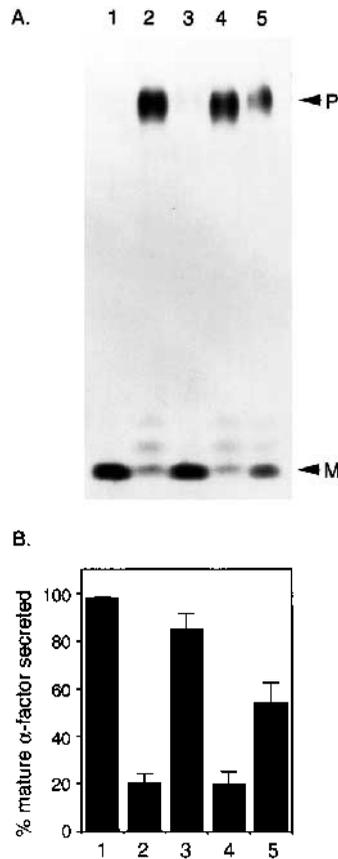
A.

B.

Fig. 3. Overexpression of *CHC1* partially rescues the α-factor processing defect in *clc1-Δ* cells. Cells were labeled with [^{35}S]Met, [^{35}S]Cys for 30 minutes, medium was collected and α-factor was immunoprecipitated and analyzed by SDS-PAGE and autoradiography. A ladder of three bands migrating close to mature α-factor appeared in all samples containing α-factor precursor and probably represents precursor degradation products or partially processed products. The smallest of these bands did not resolve from mature α-factor and was therefore included in the quantitation of mature α-factor. (A) Autoradiogram of a representative α-factor processing experiment. Positions of precursor (P) and mature (M) forms of α-factor are indicated. (B) Combined results of 5 independent α-factor processing experiments. Results are expressed as % mature α-factor secreted ± the s.e.m. Strains: SL1463 (*CLC1*, lane 1) and SL1620 (*clc1-Δ scd1-v*) transformed with no plasmid (lane 2); YCp*CLC1* (lane 3); YCp*CHC1* (lane 4), or YEp*CHC1* (lane 5).

no plasmid, YEp*CHC1* or YCp*CHC1*. We found that, similar to *chc1-Δ* cells, LC-deficient spore segregants became polyploid at a high frequency (Table 2A). This ploidy defect was eliminated in *clc1-Δ* spore segregants carrying either low or high copy *CHC1* plasmids. The finding that both low and high copy expression of *CHC1* rescued the ploidy defect of *clc1-Δ* cells was unexpected, since the low copy plasmid did not remedy the *clc1-Δ* growth phenotype (see above).

The form of α-factor secreted by *clc1-Δ* cells was also examined. Pro-α-factor (19 kDa) is core-glycosylated in the ER and the N-linked glycans are elongated within the Golgi complex. This high molecular mass precursor (~125 kDa) is then processed to mature α-factor by the late-Golgi localized proteases Kex2p, Kex1p and DPAP-A (Fuller et al., 1988). In *chc1-Δ* cells, these membrane-bound proteases are mislocal-
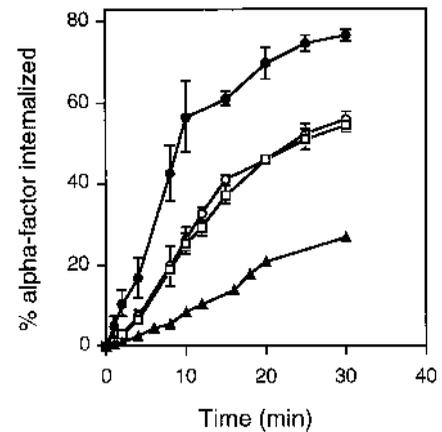

Fig. 4. Overexpression of *CHC1* does not rescue the internalization defect of cells lacking clathrin LCs. Alpha-factor uptake at 25°C was measured at the indicated times. The data shown are the average percentage of pre-bound α-factor internalized from three to five independent experiments ± the s.e.m., except in the case of *end3*, where the data are from one experiment. Strains shown: SL1929 (*MAT*a *clc1-Δ scd1-v*) transformed with YCp*CLC1* (●), YEp24 (○) or YEp*CHC1* (□); RH266-1D (▲, *end3*).

ized to the plasma membrane (Payne and Schekman, 1989; Seeger and Payne, 1992); as a result, HC-deficient cells secrete the highly glycosylated precursor form of α-factor. Alpha factor forms secreted by *MAT*α *clc1-Δ* cells and isogenic YCp*CHC1* and YEp*CHC1* transformants is shown in Fig. 3. In *CLC1* cells (lane 1), or *clc1-Δ* cells complemented with a *CLC1* plasmid (lane 3), almost all of the secreted α-factor was in the mature form. In contrast, most of the α-factor secreted by *clc1-Δ* cells (lane 2) was the high molecular mass precursor, indicating that Kex2p and other late-Golgi processing enzymes are mislocalized or inactive in LC-deficient yeast. Overexpression of *CHC1* partially corrected the α-factor processing defect in *clc1-Δ* strains (lane 5), with an average of 50% mature α-factor secreted by these cells (Fig. 3B). Transformation with YCp*CHC1* did not alter the α-factor processing defect of cells lacking the LC (lane 4).

Endocytosis in LC-deficient yeast was assessed by monitoring the uptake of ^{35}S-labeled α-factor. Alpha factor binds to the Ste2p receptor on *MAT*a cells. This binding initiates the mating signal transduction cascade and stimulates the internalization and subsequent degradation of the receptor-pheromone complex (Kurjan, 1992; Sprague and Thorner, 1992). HC-deficient yeast show a decreased rate of α-factor internalization, although the defect is not complete (Payne et al., 1988; Tan et al., 1993). As shown in Fig. 4, the uptake of radiolabeled α-factor was also significantly slowed in *clc1-Δ* cells relative to *CLC1* strains. Neither low (not shown) nor high copy expression of *CHC1* (Fig. 4) corrected this endocytosis defect. The rate of endocytosis in *clc1-Δ* strains was half that of *CLC1* cells. This endocytosis defect was not as severe as that of *end3* cells, which, consistent with previous results (Riezman, 1993), internalized α-factor seven times slower than wild-type cells.

The decreased rate of endocytosis of *clc1-Δ* cells was not just the result of their slowed growth rate because *clc1-Δ* cells overexpressing *CHC1* were equally slowed in endocytosis even though their growth rate was significantly faster than untrans-

formed *clc1-Δ* strains. Thus, the endocytosis defect of yeast lacking the LC was similar to that of *chc1-ts* and *chc1-Δ* cells (Payne et al., 1988; Tan et al., 1993), and this defect could not be corrected by providing additional copies of *CHC1*. Overall, the phenotypic studies indicate that some of the phenotypes of LC-deficient yeast can be rescued or partially rescued by increasing the levels of the HC; however, the LC is still required for full clathrin function.

Trimerization of the HC is defective in the absence of the LC

In vitro studies have indicated that mammalian HC can remain trimerized and assemble into lattices even after LC removal (Ungewickell and Ungewickell, 1991; Winkler and Stanley, 1983). Also, bacterial expression studies have shown that the HC may be able to trimerize in the absence of the LC (Liu et al., 1995). However, part of the LC binding site on the HC has been mapped to a segment near the C terminus, which is very close to the region required for triskelion formation (Liu et al., 1995). Therefore, we investigated whether the LC could influence HC trimer formation in vivo by examining the trimerization state of HCs isolated from LC-deficient yeast. Clathrin triskelions were separated from clathrin monomers by gel filtration chromatography on a Superose 6 column. Based on the elution pattern of protein standards, the peak of trimerized clathrin, which has a Stokes radius of 160 Å (Keen et al., 1979), would elute in fractions 12-14. We estimated monomeric HC to have a Stokes radius of ~80 Å by predicting that the radius of an individual monomer would be approximately half that of a triskelion. Therefore, HC monomers would elute much later than triskelions on this column. As expected, most of the HC extracted from *CHC1* cells eluted in fractions 12-14 and then trailed into later fractions, indicating that the majority of the HC in wild-type cells is assembled in triskelions (Fig. 5). In contrast, very little HC from *clc1-Δ* extracts eluted in triskelion fractions. Instead, most of the HC appeared later, peaking in fractions 22-28, corresponding to Stokes radii between 85 to 105 Å, near the predicted size of HC monomers.

It is possible that few HC trimers were detected in *clc1-Δ* cells because, in the absence of the LC, the HC is more susceptible to proteolysis at the C terminus, near the trimerization domain. To determine if the C terminus of the HC was intact in *clc1-Δ* cells, we took advantage of a modified *CHC1* which encodes the HC with a C-terminal myc epitope tag. This tagged version of the HC fully complements *chc1-Δ* cells. When gel filtration analysis was performed on extracts from *CLC1* cells carrying YCp*CHC1-myc* (Fig. 5B), the elution profile of Chc1p-myc, as detected using Ab 9E10, was identical to normal Chc1p indicating that this tagged version trimerized efficiently. Full length Chc1p-myc was also detected in extracts from *clc1-Δ* cells, yet the myc-tagged HC still eluted largely in monomer fractions (Fig. 5B). Therefore, even though HCs are not trimerized in *clc1-Δ* cells, this is not due to proteolysis of the HC at the vertex.

Since overexpression of *CHC1* in *clc1-Δ* cells partially rescued clathrin phenotypes, the elution pattern of the Chc1p in cells expressing additional copies of *CHC1* (Fig. 5C) was examined. In SL1620 (*clc1-Δ*) transformed with YCp*CHC1*, the HC elution pattern was very similar to *clc1-Δ* cells, indicating that the HC remained monomeric. In *clc1-Δ* cells carrying YEp*CHC1*, the distribution of HC was shifted to a
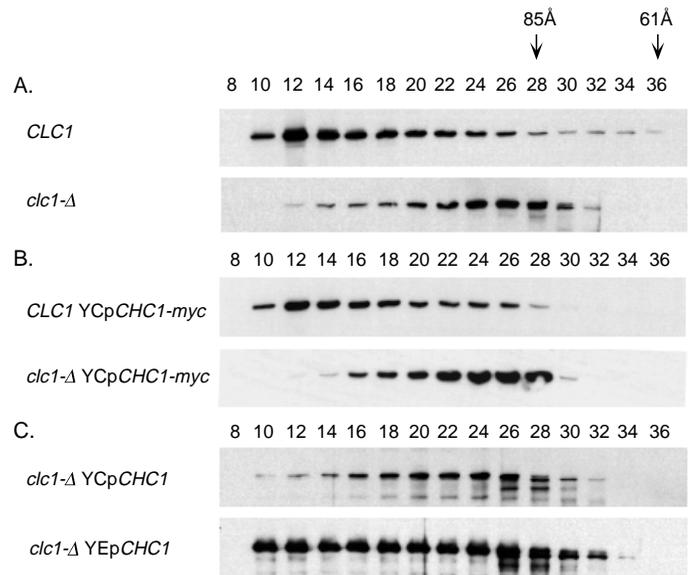

Fig. 5. LC-deficient cells contain few HC trimers. Cells were lysed in Tris:Buffer A and the 100,000 *g* supernatant was chromatographed on a 1 cm × 24 cm Superose 6 column. Fractions were collected and analyzed by SDS gel electrophoresis and immunoblotting with anti-Chc1p mAbs (A,C) or anti-myc mAb 9E10 (B). The void volume was at fraction 6 and the elution positions of thyroglobulin (85 Å) and ferritin (61 Å) are indicated. Strains analyzed are: (A) SL1463 (*CLC1*) and SL1620 (*clc1-Δ scd1-v*); (B) SL2092 (*chc1-Δ CLC1* YCp*CHC1-myc*) and SL2094 (*chc1-Δ clc1-Δ* YCp*CHC1-myc*); (C) SL1620 (*clc1-Δ scd1-v*) transformed with YCp*CHC1* or YEp*CHC1*.

higher molecular mass region, with more HC present in the triskelion fractions. To determine whether the HC eluting in these early fractions (10 through 19) was trimerized or aggregated material, we performed chemical cross-linking on pooled Superose 6 column fractions from *CLC1* or *clc1-Δ* YEp*CHC1* strains. Immunoblotting of the products separated by SDS-PAGE showed that in the absence of DSP, all of the HC migrated as a monomer of ~190 kDa (Fig. 6, lanes 1, 4 and 7). The addition of cross-linker to pooled fractions 10-14 from wild-type cells resulted in the appearance of several distinct higher molecular mass species. These multimeric HC species were identified based on their gel mobility, their immunoreactivity with anti-Clc1p antibodies (not shown) and their presence or absence in samples derived from LC-deficient cells. With 10 μg/ml cross-linker, HC/LC heterodimers, and HC homodimers (2HC) appeared (lane 2). With 50 μg/ml DSP, HC dimers containing one (2HC/LC) or two LCs (2HC/2LC) and HC heterotrimers with three LCs (3HC/3LC) were detected (lane 3). The HC from wild-type fractions 15-19 and 20-24 also cross-linked into triskelions (not shown), suggesting that the trailing on Superose 6 (see Fig. 5) may be due to the general elution properties of clathrin in crude extracts.

In samples from *clc1-Δ* cells overexpressing *CHC1*, treatment with 10 μg/ml DSP resulted in the appearance of HC homodimers as well as HC homotrimers (3HC, lane 5). No HC/LC species were detected in extracts from these strains, as expected. With 50 μg/ml DSP, the HC homotrimer band was still present, although much of the HC cross-linked into species migrating near the top of the gel, presumably representing
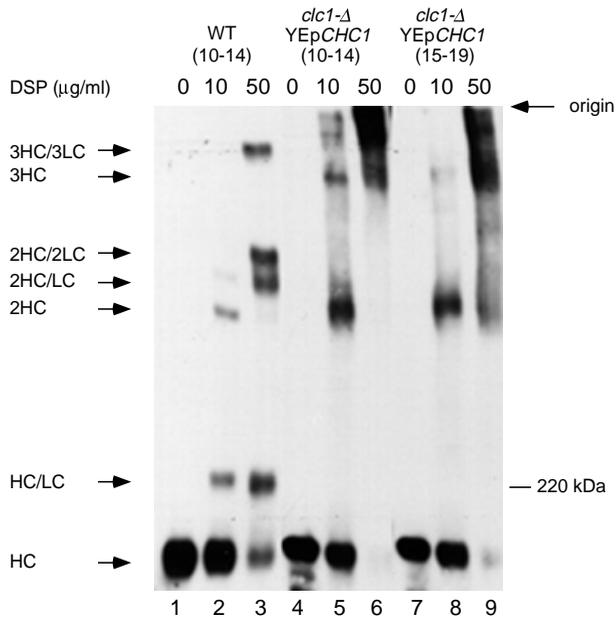
Fig. 6. Cross-linking indicates that *CHC1* overexpression restores HC trimerization in *clc1-Δ* cells. Indicated Superose 6 column fractions (see Fig. 5) were pooled, dialyzed and incubated with 0, 10 or 50 μg/ml DSP as described in Materials and Methods. Samples were analyzed by SDS-PAGE (3% polyacrylamide) and immunoblotting using anti-Chc1p mAbs. The top of the resolving gel (origin) and the migration position of a 220 kDa myosin molecular mass standard are marked. The HC/LC cross-linked species were identified as described in the text.
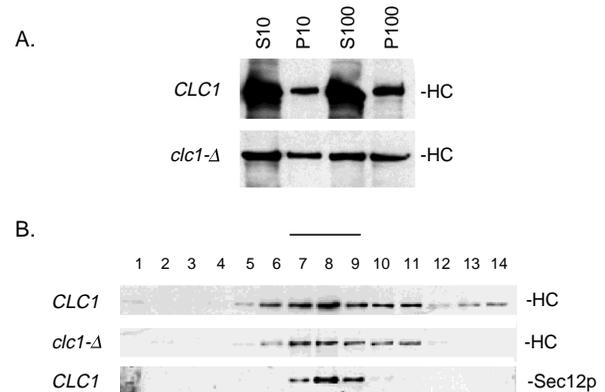

Fig. 7. Subcellular fractionation of Chc1p in *CLC1* and *clc1-Δ* strains. (A) Lysates from strains SL1463 (*CLC1*) and SL1620 (*clc1-Δ scd1-v*) were fractionated by differential centrifugation. Equivalent fraction volumes of the 10,000 *g* supernatants (S10) and pellets (P10) and the 100,000 *g* supernatant (S100) and pellet (P100) were analyzed by SDS gel electrophoresis and immunoblotting using anti-Chc1p antibodies. Note that Sec12p, an ER membrane protein, was found exclusively in the P10 fraction, as expected (not shown). (B) Sucrose float gradients of P10 fractions from *CLC1* and *clc1-Δ* strains. Extracts of SL1463 and SL1620 were fractionated as in A and the P10 fractions were resuspended in 60% sucrose, overlaid with 35% sucrose and centrifuged to equilibrium. Fractions from the gradient were collected from the top (35% sucrose, lane 1) to the bottom (60% sucrose, lane 14) and analyzed by gel electrophoresis and immunoblotting using anti-Chc1p or anti-Sec12p antisera. The relevant strain genotype is indicated on the left and the protein detected is shown on the right. The bar denotes the position of the 35%/60% gradient interface where membranes accumulate. Note that fractionation patterns of Sec12p were identical between *CLC1* and *clc1-Δ* cells.

aggregated clathrin. A similar pattern of cross-linked products was found in fractions 15-19 (lanes 8, 9). When *clc1-Δ* YEp*CHC1* samples were treated with higher concentrations of cross-linker (up to 200 μg/ml), HC homotrimers were still detected and the distribution of trimers to multimers did not change (not shown). Therefore, it is unlikely that these trimeric species were simply aggregated intermediates. In contrast, we were unable to detect HC dimers, trimers or other high molecular mass forms upon cross-linking any column fractions (including 10-19) from *clc1-Δ* or *clc1-Δ* YCp*CHC1* cells (not shown). Overall, the gel filtration and cross-linking experiments indicate that the LC is important for HC trimerization in vivo. In addition, overexpression of *CHC1* in the absence of the LC partially rescues the trimerization defect and this correlates with the observed phenotypic rescue.

Clathrin HC assembly in the absence of the LC

In animal cells, cytoplasmic clathrin triskelions are recruited to membranes via their interaction with the membrane-bound clathrin adaptor complexes, AP-1 and AP-2. In vitro studies have shown that the LC is not necessary for AP complexes to bind clathrin HC (Keen et al., 1991; Lindner and Ungewickell, 1991; Murphy and Keen, 1992), suggesting that the LC is not required for the recruitment of clathrin HC to membranes. To examine this question in vivo, *clc1-Δ* cells were subjected to differential centrifugation (Fig. 7; Walworth et al., 1989) and the distribution of HC was analyzed in the 10,000 *g* pellet (P10) containing large membranes and organelles (e.g. mitochondria, ER, plasma membrane and vacuole), 100,000 *g* pellet (P100)

containing smaller membranes and vesicles (e.g. Golgi, secretory vesicles and coated vesicles) and 100,000 *g* supernatants (S100). In *CLC1* cells, 78% of the total HC was found in the S100 and the remainder appeared in P10 and P100 fractions. In LC-deficient cells, only 46% of total cellular HC was in the S100 fraction and the rest appeared equally distributed between the P10 and P100, indicating that a smaller fraction of the cellular HC was cytosolic in the absence of the LC.

To determine if HC sedimenting at 10,000 *g* was membrane associated, the P10 pellets from *CLC1* and *clc1-Δ* extracts were subjected to equilibrium sucrose density flotation analysis. P10 was resuspended in 60% sucrose, overlaid with 35% sucrose and then centrifuged to equilibrium and fractionated. Due to their intrinsic buoyant densities, membranes float to the interface between 35% and 60% sucrose. Aggregated proteins that are not membrane-associated but that pellet remain in the 60% sucrose layer (Horazdovsky and Emr, 1993). The HC in both *CLC1* (56%) and *clc1-Δ* (69%) cells peaked in fractions 7-9, at the turbid 35%/60% interface. This coincided with the peak of Sec12p, an ER integral membrane protein (Nakano et al., 1988), although the peak of P10 HC was broader than that of Sec12p. This could be due to some disassembly of coat proteins during fractionation. Nevertheless, in both *CLC1* and *clc1-Δ* cells, a large percentage of the HC that pelleted at 10,000 *g* was membrane-associated.

The material sedimenting at 100,000 *g* was expected to be

enriched in clathrin coated vesicles. Since HC was present in P100 fractions from both *CLC1* and *clc1-Δ* cells, we attempted to isolate clathrin coated vesicles from *clc1-Δ* cells using Sephacryl S1000 chromatography as described previously for wild-type yeast (Lemmon et al., 1988). However, no clathrin HC was found in fractions that normally contain coated vesicles, even when these cells overexpressed *CHC1* (not shown). Instead, the HC from LC-deficient extracts eluted as unassembled HC. It is possible that the material in P100 from *clc1-Δ* cells was aggregated HC. Alternatively, the P100 could contain coated vesicles or HC assembled onto small membrane organelles which became unstable and fell apart during gel filtration. Sucrose flotation analysis of P100 did not distinguish between these possibilities because authentic coated vesicles sediment to equilibrium at 50 to 55% sucrose (Pearse, 1975).

Overexpression of *CLC1* rescues inviable strains of clathrin-deficient yeast

Clathrin HC-deficient yeast are viable or inviable depending on their genetic background (Lemmon and Jones, 1987; Munn et al., 1991; Payne and Schekman, 1985). In strains from our lab, the difference in viability is due to an independently segregating gene, *SCD1* (Lemmon and Jones, 1987). In previous work, we developed a strategy for isolating multicopy suppressors that rescue clathrin HC-deficient yeast of the *scd1-i* genotype to viability (Nelson and Lemmon, 1993). An *scd1-i* strain with *CHC1* under the control of the *GAL1* promoter (strain SL214) was transformed with a multicopy plasmid library, and colonies were selected for their ability to grow on glucose. Five multicopy suppressors of clathrin deficiency were isolated (*SCD2-SCD6*). By deletion mapping of the original YEp*SCD4* clone and subsequent sequencing of the rescuing region of the insert, we were surprised to discover that *SCD4* is identical to *CLC1*.

To confirm the rescue of HC-deficient lethality by overexpression of the clathrin LC, a *chc1-Δ /CHC1 scd1-i/scd1-i* heterozygous diploid (BJ3529) was transformed with a YEp*CLC1* plasmid (pKH4) and tetrads were dissected. Sporulation of the untransformed, parent diploid yielded tetrads with only two viable *CHC1* spores. In addition, sporulation of BJ3529 transformed with a high copy plasmid containing a large deletion within the *CLC1* open reading frame (pKH7) also resulted in two viable and two dead spores. In contrast, 54% of the tetrads (28 total) from BJ3529 transformed with YEp*CLC1* yielded 3 or 4 viable spores: two wild-type *CHC1* spores and one or two slow growing *chc1-Δ scd1-i* spore segregants carrying the *CLC1* multi-copy plasmid (data not shown). These results are similar to those previously reported for YEp*SCD4* (Nelson and Lemmon, 1993).

It was possible that *CLC1* overexpression rescued *chc1-Δ scd1-i* spore segregants by stabilizing the HC in germinating spores, enabling the cells to stay alive longer after HC depletion. This might allow more time for second site suppressor mutations to arise whereby the LC would no longer be required. To rule out this possibility, spore clones carrying YEp*CLC1* plasmids were grown non-selectively in liquid medium and cells were plated on YEPD. The resultant colonies (>2,500 examined) were then tested for the presence of the plasmid by replica-plating to selective medium. While greater than 90% of *CHC1 scd1-i* YEp*CLC1* spores lost the plasmid, no plasmid loss was detected in *chc1-Δ scd1-i* YEp*CLC1* cells,
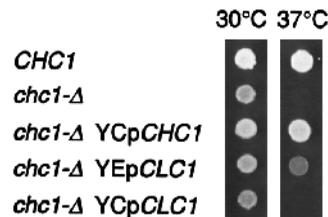

Fig. 8. Overexpression of *CLC1* partially rescues the growth defect of *chc1-Δ* cells. An isogenic set of *chc1-Δ scd1-v* strains was derived by transforming BJ3255 (*chc1-Δ scd1-v*) with no plasmid, YCp*CHC1* (pSL6), YEp*CLC1* (pKH2) or YCp*CLC1* (pKH4). Cells were grown in selective media to mid-log phase, diluted to 10^7 cells/ml in YEPD, spotted onto YEPD plates using a multi-prong inoculator and grown one day at 30°C or 37°C.

indicating that *CLC1* overexpression was responsible for rescuing HC-deficient strains. In fact, immunoblot analysis showed that *chc1-Δ* cells with YEp*CLC1* not only overexpressed *CLC1* but contained 2- to 3-fold more LC than *CHC1* cells with YEp*CHC1* (not shown). This suggests that there may be selection for *chc1-Δ* cells to overexpress the LC.

To test if *CLC1* overexpression could remedy other phenotypes of viable *chc1-Δ* cells, a set of isogenic *chc1-Δ scd1-v* strains was generated by transforming strain BJ3255 with various *CLC1* or *CHC1* plasmids. Indeed, overexpression of *CLC1* partially rescued the slow growth phenotype of *chc1-Δ scd1-v* cells at 30°C, as well as the temperature sensitivity at 37°C (Fig. 8). When grown in synthetic medium at 30°C, the doubling time of BJ3255 was 5.5 hours as compared to 3.5 hours for BJ3255 carrying YEp*CLC1*. Expression of *CLC1* on a low copy plasmid had no effect on the growth rate or temperature sensitivity of *chc1-Δ* strains.

The ploidy of *chc1-Δ* spore segregants overexpressing *CLC1* was determined by monitoring the degree of papillation to Can[r] after mating to haploid Can[r] strains (see Materials and Methods). As shown previously (Lemmon et al., 1990), Chc⁻ strains readily increase their genome copy numbers (Table 2B). In contrast, *chc1-Δ* spores that segregated with the YEp*CLC1* plasmid no longer displayed increases in ploidy. Similar results were observed with *chc1-Δ scd1-i* segregants (not shown). In contrast, sorting phenotypes of *chc1-Δ* strains did not appear to be rescued by *CLC1* overexpression. For example, both *chc1-Δ* and *chc1-Δ* YEp*CLC1* isogenic strains secreted mostly the high molecular mass α-factor precursor (not shown), indicating that late Golgi protein retention was still completely defective.

Fractionation of the LC in cells lacking the HC

The rescue of Chc⁻ lethality by LC overexpression suggests that, in the absence of the HC, the LC can carry out some partial clathrin function or that LC alone has a function independent of the HC. Perhaps, when the HC is not available, the LC associates with another HC-like molecule or vesicle coat protein forming a large molecular mass complex which rescues HC deficiency. To investigate this, Superose 12 gel filtration chromatography was performed on soluble extracts from *CHC1* and *chc1-Δ* cells overexpressing *CLC1*. For these experiments, cells were lysed in TBS, pH 8.0. This buffer, which removes clathrin LC from membranes (not shown), is of lower ionic strength than Tris:Buffer A extraction buffer and was

Fig. 9. Gel filtration chromatography of overexpressed LC from *CHC1* and *chc1-Δ* cells. Strains SL1638 (*CHC1* + YEp*CLC1*) or BJ3255 (*chc1-Δ scd1-v*) transformed with YEp*CLC1*, were lysed in TBS, pH 8.0 and the 100,000 *g* supernatant was chromatographed on a Superose 12 column. For the boiled extract sample, the 100,000 *g* supernatant from SL1638 was boiled for 10 minutes and centrifuged prior to chromatography. Fractions were analyzed by SDS gel electrophoresis and immunoblotting with anti-Clc1p antibodies. The void volume was at fraction 6 and the elution positions of ferritin (61Å), aldolase (48 Å), and BSA (35.5Å) are indicated.
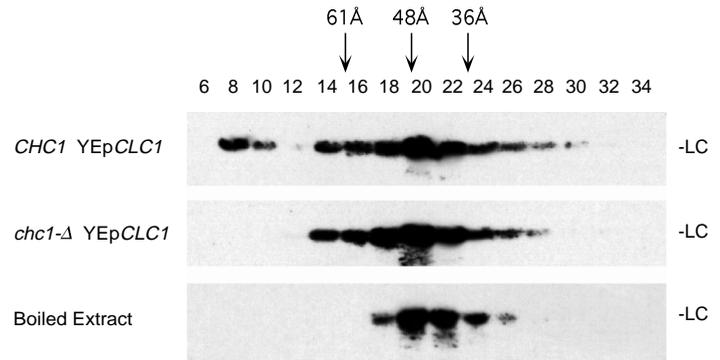
used in an attempt to prevent the disruption of weak interactions between the LC and other proteins. In *CHC1* cells overexpressing *CLC1*, a portion of the LC was associated with a high molecular mass complex (fractions 8-10), representing LC bound to clathrin HCs (Fig. 9). Most of the overexpressed LC eluted later, peaking at fraction 20. In other experiments using *CHC1* strains that did not overexpress *CLC1*, the LC was exclusively in triskelions, indicating that normally there is not a significant pool of free LC (data not shown). In *chc1-Δ* cells overexpressing *CLC1*, all of the LC eluted in later fractions, also peaking at fraction 20; no LC was detected in the high molecular mass region of the column. Similar profiles were obtained for *chc1-Δ* cells that did not overexpress the LC (data not shown). These results indicate that, in the absence of the HC, the LC is not associated with a high molecular mass coat complex.

From the Superose 12 elution profile, the calculated Stokes radius of the peak of LC eluting in the later fractions was 47 Å, which is larger than the 33 Å Stokes radius reported for free mammalian LC (Ungewickell, 1983). Therefore, to determine whether the LC eluting in the later fractions represented free yeast LC, we took advantage of the property of the LC to remain soluble after boiling, while HCs and most other proteins denature and precipitate (Lisanti et al., 1982). As shown in Fig. 9, the elution peak of LC from a boiled extract was identical to the peak of later eluting LC from overexpression strains. We note that the LC from boiled extracts had a much tighter elution profile. Boiling could result in a more

structurally homogenous population of LC molecules; however, we cannot rule out the possibility that some of the overexpressed LC from untreated extracts associated with other proteins. Nevertheless, most of the overexpressed LC in both *CHC1* and *chc1-Δ* cells appeared to exist as free, monomeric LC.

To examine whether the LC is associated with a membrane component when the HC is absent, we performed subcellular fractionation on *CHC1* and *chc1-Δ* strains overexpressing *CLC1* (Fig. 10A). In both strains, the majority of the overexpressed LC was found in the soluble fractions. However, about 10% of the overexpressed LC fractionated in P10 and P100, even in strains that did not express the HC. To determine if the LC that appeared in the pellet fractions was membrane-associated, we analyzed this material on a 35%/60% sucrose flotation gradient. In both the presence and the absence of the HC, nearly all of the overexpressed, pelletable LC appeared in the 35%/60% gradient interface, co-fractionating with the ER membrane protein Sec12p (Fig. 10B). These results suggest that the LC can bind to membranes even in the absence of the HC.

DISCUSSION

LC is important for HC trimerization

The characterization of clathrin LC-deficient yeast has uncovered a novel role for the LC in HC trimerization. We

Fig. 10. The LC can still associate with membranes in the absence of the HC. (A) Subcellular fractionation of Clc1p in strains overexpressing the LC. Cell extracts from SL1638 (*CHC1* + YEp*CLC1*) and BJ3255 (*chc1-Δ scd1-v*) transformed with YEp*CLC1* were fractionated by differential centrifugation. Equivalent fraction volumes were analyzed by SDS gel electrophoresis and immunoblotting using anti-Clc1p antibodies. The ER membrane protein, Sec12p, was found exclusively in the P10 fraction, as expected (not shown). (B) Sucrose float gradients (35%/60%) of combined P10/P100 fractions from the same strains as shown in A. The relevant strain genotype is indicated on the left and the protein detected is shown on the right. Gradients were fractionated from top (35% sucrose, lane 1) to bottom (60% sucrose, lane 13) and the bar denotes the position of the 35%/60% gradient interface, where membranes accumulated. The fractionation pattern of Sec12p was identical in *CHC1* cells and *chc1-Δ* cells (not shown).
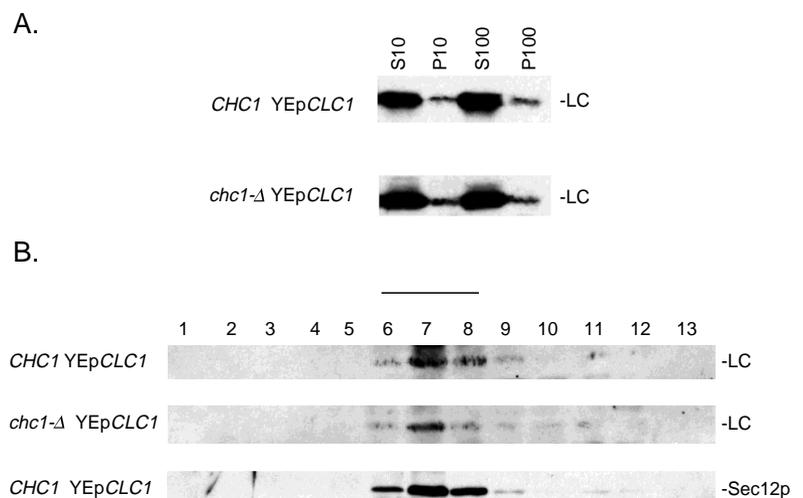
found that *clc1-Δ* cells contain very few HC trimers. There are at least three explanations for why we detect primarily HC monomers in *clc1-Δ* cells. First, the HC may trimerize in the absence of the LC, but such trimers could be unstable and fall apart during our extraction procedures. We consider this unlikely because a number of biochemical studies have shown that the LC can be removed from triskelions and trimers remain intact, even in buffers identical to those used in our studies (Ungewickell and Ungewickell, 1991; Winkler and Stanley, 1983). Second, it is possible that in the absence of the LC, the HC is more susceptible to proteolysis near the vertex and as a consequence, few HC trimers are detected. However, we found that Chc1p from *clc1-Δ* cells appeared full length on SDS gels (see Fig. 1) and Chc1p molecules containing a C-terminal myc epitope (Chc1p-myc) retained their myc epitopes, even in the absence of the LC. Since both normal and myc-epitope HC were monomeric in *clc1-Δ* cells, we favor a third model in which the HCs are unable to trimerize in the absence of the LC.

Recently, Liu et al. (1995) generated bacterially-expressed deletion mutants of bovine clathrin HC hubs and mapped the LC binding site to residues 1,213-1,522 and the trimerization domain to residues 1,550-1,615. The middle of the LC contains a region of extended α-helical structure which is thought to mediate binding to the HC via a coiled-coil interaction. Given the proximity of the LC-binding and trimerization domains, it is possible that LC binding to HC induces a conformational change within the neighboring trimerization domain which promotes efficient triskelion formation. The N or C terminus of the LC, which have been proposed to orient towards the vertex (Nathke et al., 1992), could themselves facilitate the folding of the HCs to form trimers. Alternatively, the LC could be required to assemble onto the HC proximal arm during synthesis in order to maintain the HC arm and vertex in an extended configuration which could be necessary for trimerization. In these models, LCs would not be required to maintain trimerization at the hub once folding was complete. Without LCs, HC molecules that did not trimerize efficiently might be turned over more rapidly, thereby explaining the lower HC steady state levels in *clc1-Δ* cells.

It is interesting to note that Liu et al. (1995) expressed bovine HC C-terminal hub fragments (containing amino acids 1,074-1,675 and lacking the entire HC distal domain) in bacteria and found that these fragments can form trimers even in the absence of the LC. The reason for the discrepancy between their expression system data and our in vivo studies using full length clathrin HC is not clear. Perhaps the removal of the HC distal arm could allow HC trimerization to become LC-independent. Alternatively, in the previous study the concentration of HC hub fragments could have been high enough to overcome a requirement for the LC. In support of this explanation, we found that *clc1-Δ* cells overexpressing *CHC1* contain a pool of trimeric HC.

Our studies also showed that *CHC1* overexpression partially rescued the growth and late sorting defects in *clc1-Δ* cells. These results suggest that the restoration of clathrin function, in vivo, is a direct consequence of increasing the pool of trimerized HC. However, we cannot rule out the possibility that the phenotypic rescue is the result of a general increase in the total pool of clathrin HC. Nevertheless, since it was not possible to completely restore clathrin function in the absence of the LC,

it is likely that the LC is required at additional steps in the clathrin coated vesicle cycle.

Role of the LC in vesicle budding

Our studies indicate that the LC is not necessary for one of the first steps in the formation of clathrin coated vesicles, the recruitment of clathrin to membranes. In animal cells, clathrin triskelions bind to membranes via their association with the β subunits of AP complexes (Ahle and Ungewickell, 1989; Gallusser and Kirchhausen, 1993; Shih et al., 1995; Wilde and Brodsky, 1996). A number of homologs of mammalian AP subunits, including the β chain, have been identified in yeast (Kirchhausen, 1990; Phan et al., 1994; Rad et al., 1995; Stepp et al., 1995). Our cell fractionation experiments indicate that in the absence of the LC, some HC still binds to membranes. This finding is consistent with in vitro studies showing that the interaction between clathrin and APs does not require the LC (Keen et al., 1991; Lindner and Ungewickell, 1991; Murphy and Keen, 1992). Additionally, since most of the HC in *clc1-Δ* cells is not trimerized, the β subunits of AP complexes might recognize monomeric HC.

It is intriguing that overexpression of *CHC1* in *clc1-Δ* cells partially rescued α-factor processing, and thus protein retention in the Golgi, but not endocytosis. It is not completely understood how clathrin mediates retention of Golgi membrane proteins, such as the α-factor processing enzyme Kex2p. This process could involve vesicle budding and recycling of escaped proteins. Alternatively, retention could be achieved by clustering and sequestering membrane proteins from the general secretory flow. Such a tethering mechanism would not require a vesicle budding event. If the LC is required for vesicle budding, LC-deficient cells overexpressing *CHC1* might be able to partially perform Golgi retention, but not endocytosis, which would require coated vesicle formation. Perhaps the LC is important for the rearrangement of planar clathrin lattices to allow vesicle budding and this can explain our inability to isolate coated vesicles from LC-deficient yeast even when *CHC1* is overexpressed.

Partial clathrin function can rescue clathrin-deficient phenotypes

Our results again reveal that the clathrin deficient phenotype is complex and the associated defects are differentially dependent on the degree of clathrin function (Lemmon et al., 1990; Lemmon and Jones, 1987; Payne et al., 1988; Payne and Schekman, 1989). For example, we found that low copy expression of *CHC1* in *clc1-Δ* cells was sufficient to rescue the ploidy phenotype, yet the growth and protein sorting defects were unchanged. Similarly, no clathrin dependent sorting events were rescued in *chc1-Δ* cells overexpressing *CLC1*; however, these cells did not become polyploid. Overexpression of *CHC1* in *clc1-Δ* cells raised phenotypic rescue to another level with partial restoration of growth and late Gogi retention defects, but without any effect on endocytosis. This kind of gradation in rescue of clathrin function has been observed in previous studies from our lab. Yeast expressing a HC truncation of 57 amino acids grow well at 30°C and do not become polyploid; although the mutant HCs in these cells are trimerized, these cells are still temperature sensitive and defective in α-factor processing (Lemmon et al., 1991). Overall, these findings indicate that clathrin plays a role in multiple

processes, either directly or indirectly, and these processes have different sensitivities to varying levels of clathrin function.

Clathrin LC can function independent of the HC

One of the most compelling findings of this study is that overexpression of *CLC1* can alleviate some clathrin HC-deficient phenotypes. There appears to be a selection for *chc1-Δ scd1-i* YEp*CLC1* cells that express high levels of the LC, which we suspect is the result of plasmid amplification. It seems unlikely that the LC could perform budding or tethering functions on its own, raising the possibility that the LC interacts with another coat protein. We did not detect overexpressed LC in high molecular mass complexes; however, our gel filtration studies leave open the possibility that the LC is associated with lower molecular mass constituents. Interestingly, some of the overexpressed LC was associated with membranes even in the absence of the HC. This is surprising because the LC is thought to bind to membranes indirectly, through its association with the clathrin HC. Although the membrane association of overexpressed LC could be non-specific, it is more intriguing to speculate that this membrane association is essential for the rescue of HC-deficient yeast. It is also possible that, in the absence of the HC, the overexpressed LC rescues by performing some regulatory function that would normally occur during clathrin-mediated vesicle transport. There could be a factor that interacts with the LC, and this association could be promoted by presentation in the context of the HC. Without the HC, this interaction might be reestablished by overexpression of the LC. Alternatively, our data support the novel idea that the LC has a function independent of the HC. The identification of other proteins that interact with the LC in yeast should help to distinguish these possibilities.

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Note added in proof

After this paper was submitted D. S. Chu, B. Pishavee and G. S. Payne reported similar results concerning the importance of the clathrin LC for clathrin HC trimerization in yeast: The light chain subunit is required for clathrin function in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **271**, 33123-22130 (1996).