Suppressors of Clathrin Deficiency: Overexpression of Ubiquitin Rescues Lethal Strains of Clathrin-Deficient *Saccharomyces cerevisiae*

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Clathrin-mediated vesicular transport is important for normal growth of the yeast *Saccharomyces cerevisiae*. Previously, we identified a genetic locus (*SCD1*) that influences the ability of clathrin heavy-chain-deficient (Chc^-) yeast cells to survive. With the *scd1-v* allele, Chc^- yeast cells are viable but grow poorly; with the *scd1-i* allele, Chc^- cells are inviable. To identify the *SCD1* locus and other genes that can rescue *chc1Δ scd1-i* cells to viability, a multicopy suppressor selection strategy was developed. A strain of *scd1-i* genotype carrying the clathrin heavy-chain gene under *GAL1* control (*GAL1:CHC1*) was transformed with a YE_p24 yeast genomic library, and colonies that could grow on glucose were selected. Plasmids from six distinct genetic loci, none of which encoded *CHC1*, were recovered. One of the suppressor loci was shown to be *UBI4*, the polyubiquitin gene. *UBI4* rescues only in high copy number and is not allelic to *SCD1*. The conjugation of ubiquitin to intracellular proteins can mediate their selective degradation. Since *UBI4* is required for survival of yeast cells under stress and is induced during starvation, ubiquitin expression in *GAL1:CHC1* cells was examined. After a shift to growth on glucose to repress synthesis of clathrin heavy chains, *UBI4* mRNA levels were elevated >10-fold, whereas the quantity of free ubiquitin declined severalfold relative to that of Chc^+ cells. In addition, novel higher-molecular-weight ubiquitin conjugates appeared in clathrin-deficient cells. We suggest that higher levels of ubiquitin are required for turnover of mislocalized or improperly processed proteins that accumulate in the absence of clathrin and that ubiquitin may play a general role in turnover of proteins in the secretory or endocytic pathway.

Clathrin is the major protein forming the lattice-like coat of clathrin-coated membranes and coated vesicles (for reviews, see references 7 and 55). In animal cells, clathrin plays an important role in receptor-mediated endocytosis, the major mechanism by which cells take up a variety of ligands, required nutrients, viruses, and regulatory proteins from the extracellular environment. Clathrin enhances the efficiency of internalization and delivery to endosomes of certain cell surface receptors while excluding other proteins from coated pits. Non-clathrin-mediated endocytosis also occurs but is much less efficient (59). In the secretory pathway, clathrin is localized primarily to the *trans* Golgi network, which is the major location of protein sorting in the export pathway. It is involved in mannose-6-phosphate receptor-mediated transport of lysosomal enzymes from the Golgi to a prelysosomal compartment and in the formation of regulated secretory granules such as those found in hormone-secreting cells (8, 24, 73).

Most early information on clathrin structure and function was derived from biochemical analysis of purified clathrin and coated vesicles or studies of clathrin in animal cells. It became feasible to apply molecular genetic approaches to the study of clathrin and its role in protein transport when coated vesicles were identified in the yeast *Saccharomyces cerevisiae* (45). The clathrin heavy chains (HCs) and light chains from this yeast were subsequently purified, and the genes were cloned and sequenced (36-38, 53, 68). To examine the role of clathrin in yeast cells, gene disruptions were performed. In some strains, clathrin HC gene (*CHC1*) dele-

tions are lethal; in others, they are not (36, 46, 52, 53). In strains studied in this laboratory, this difference was found to result from an independently segregating gene, called suppressor of clathrin deficiency (*SCD1*) (36). In the presence of the *scd1-i* allele, cells lacking clathrin HCs are inviable; in the presence of the *scd1-v* allele, clathrin-deficient cells are viable but grow poorly.

Chc^- (*chc1Δ::LEU2 scd1-v*) strains, although viable, are not normal. They grow poorly, are genetically unstable, and display abnormal morphology, mating, and sporulation (35, 36, 53, 54). In addition, processing of pro- α -factor is defective, and a precursor form of the pheromone is secreted. This is caused by the mislocalization from the *trans* Golgi to the cell surface of the endoproteases which are involved in proteolytic maturation of α -factor (54, 65). These results suggest that clathrin is required for retention of proteins at the *trans* Golgi in *S. cerevisiae* yeast and presumably other organisms as well.

To identify additional gene products involved in cellular processes of interest in *S. cerevisiae*, there is a repertoire of additional molecular genetic tools at one's disposal, including second-site reversion analysis, synthetic lethal screens, and multicopy suppressor searches. Multicopy suppression is observed with some frequency during attempts to clone genes by complementation, and in many cases it is clear that the genes identified in this way encode proteins that serve a related function or interact with the originally mutated gene product (2, 4, 14, 47, 50, 57). In some cases, suppression analysis also uncovers relationships between gene products not previously predicted (13).

In this report, we describe our use of the multicopy suppressor approach to the clathrin system. The goal was to

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TABLE 1. *S. cerevisiae* strains

Strain	Genotype	Reference or source
SL107	<i>MATα CHC1 scd1-i leu2 ura3-52 trp1 his1 GAL2</i>	This study
SL214	<i>MATα GAL1:CHC1 scd1-i leu2 ura3-52 trp1 his1 GAL2</i>	This study
SL350	<i>MATα GAL1:CHC1 scd1-v leu2 ura3-52 trp1 ade6 GAL2</i>	This study
SL480	<i>MATα GAL1:CHC1 scd1-i SCD2:YIpSCD2(URA3) leu2 ura3-52 trp1 his1 GAL2</i>	This study
SL591	<i>MATα GAL1:CHC1 scd1-i leu2 ura3-52 trp1 his1 GAL2 YCp50-CHC1</i>	This study
DBY939	<i>MATα CHC1 suc2-215am ade2-101 gal2</i>	9
BJ3529	<i>MATα/MATα CHC1/chc1Δ::LEU2 scd1-i/scd1-i ura3-52/ura3-52 leu2/leu2 ADE6/ade6 his1/HIS1 gal2/gal2</i>	36
BJ4225	<i>MATα CHC1 GAL2 ura3-52 leu2</i>	(E. W. Jones)
BJ4319	<i>MATα/MATα CHC1/chc1Δ::LEU2 scd1-v/scd1-v gal2/gal2 leu2/leu2 ura3-52/ura3-52 TRP1/trp1 ADE6/ade6</i>	This study
BJ4591	<i>MATα/MATα CHC1/chc1Δ::LEU2 scd1-v/scd1-v GAL2/gal2 leu2/leu2 ura3-52/ura3-52 TRP1/trp1 ADE6/ade6</i>	This study
YAS352	<i>MATα CHC1 pab1Δ::HIS3 ade2 his3 leu2 trp1 ura3 pAS137(pGAL1:PAB1)</i>	56

identify genes whose overexpression could suppress the lethality of clathrin HC-deficient yeast strains carrying the *scd1-i* allele. We report here the isolation of six such multicopy suppressors and describe the characterization of one, *UBI4*, in detail.

MATERIALS AND METHODS

Strains, media, and genetic methods. Strains used in this study are listed in Table 1 and were derived from the X2180-1B (S288C) background (*MAT α gal2 SUC2*). The nomenclature for the two alleles of *SCD1* has been changed since the original discovery of the locus (36). The allele previously called *SCD1* is now referred to as the *scd1-i* allele, and the allele previously called *scd1* is now referred to as the *scd1-v* allele. The nomenclature has been revised because a dominant/recessive study of the two alleles has revealed that *chc1 Δ ::LEU2/chc1 Δ ::LEU2 scd1-i/scd1-v* heterozygous diploids with *GAL1:CHC1* on a plasmid are viable on glucose, although they do not grow as well as homozygous *scd1-v* strains (unpublished data). This finding indicates that the *SCD1* alleles are codominant and/or the dosage of the *scd1-v* protein is important. Until the *SCD1* locus is cloned and the true dominance relationship of the two alleles is established, the revised gene designations will be used.

To obtain optimal regulation of the *GAL1* promoter (see below), it was necessary to have strains that had a normal galactose permease, which is encoded by the *GAL2* gene and is mutant in strains of S288C genetic background (42). To obtain a collection of *GAL2* strains, YEp24 (6) and the 3.9-kb *HindIII* fragment from pTUG6 containing *GAL2* (74) were cotransformed into a diploid of genotype *MAT α /MAT α CHC1/chc1 Δ ::LEU2 scd1-v/scd1-v gal2/gal2 ura3-52/ura3-52 leu2/leu2* (BJ4319). Ura⁺ transformants were selected and screened for growth on galactose under anaerobic conditions. Plasmid (YEp24) loss was performed on Gal⁺ strains. The resulting diploid (BJ4591) was transformed with YCp50-*CHC1* (36) and sporulated. Progeny Gal⁺ haploids were backcrossed to a known *GAL2* strain (BJ4225), and segregational analysis confirmed that Gal⁺ strains were converted to *GAL2*, presumably by transplacement of *gal2*, and were not Gal⁺ because of an unlinked suppressor mutation. Other *GAL2* strains shown in Table 1 were derived from this parent by backcrossing.

YEPD and minimal synthetic media were prepared as described previously (17). YEP-Gal was identical to YEPD except that galactose was substituted for dextrose. Complete

medium was minimal synthetic medium (SD) (17) containing adenine, arginine, histidine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, uracil, and serine. c-Ura medium was complete medium without uracil. c-Ura+Gal medium was identical to c-Ura except galactose was substituted for dextrose. Plates containing 5-fluoroorotic acid (5-FOA) plus galactose (5-FOA+Gal) were complete plates (with galactose substituted for dextrose) containing 50 μ g of uracil per ml and 0.1% 5-FOA (3). Yeast mating, sporulation, and tetrad analyses were carried out as described previously (17). *Escherichia coli* DH5 α (26) was used for plasmid propagation. Yeast strains were transformed by either a lithium acetate method (29) or electroporation (1). Plasmids were isolated from yeast cells and transformed into *E. coli* as described previously (28).

Plasmid constructions. YCp50-*CHC1* (pSL6), which contains the complete *CHC1* gene from *S. cerevisiae*, has been described previously (36). pKC2 contains the 5' end of *CHC1* fused at a position 41 bases upstream of the translational start site to the *GAL1* promoter in pBM258 (32). pKC2 was constructed by ligating the 1.3-kb *XmnI* fragment of *CHC1* into pBM258 at the unique *BamHI* site, which had been blunted with the Klenow fragment of DNA polymerase. The integrating plasmid, pKN8, contains a truncated *CHC1* under *GAL1* control and was constructed in the following steps. First, the 2.5-kb *BamHI-SphI* fragment of *CHC1* containing 1.5 kb of 5' flanking sequences of *CHC1* and extending through codon 322 was ligated into YIp5 (6) to create pKN5. The *GAL1* partial *CHC1* fusion in pKC2 (see above) was then moved (as a 1.3-kb *EcoRI* fragment) into the *EcoRI* site of pKN5 to yield pKN8. This produced a plasmid with *CHC1* sequences flanking both sides of the *GAL1* promoter, which was necessary for integration at the *CHC1* locus (Fig. 1).

YEpSCD2, YEpSCD3, YEpSCD4, YEpSCD5, YEpSCD6, and YEpSCD7 were isolated from a YEp24 yeast genomic DNA library (9) by their ability to rescue SL214, a *MAT α GAL1:CHC1 scd1-i ura3-52* strain, from lethality on glucose. pKN25 contains the 5.2-kb *BamHI* fragment of YEpSCD2, which includes the complementing region of *SCD2* (see Fig. 4, line 3) in YEp24. pKN32 contains the smallest complementing region of *SCD2*, the 2.0-kb *BamHI-NruI* fragment of YEpSCD2 (see Fig. 4, line 7), in YEp24. YIpSCD2 was created by ligating the 5.2-kb *BamHI* fragment of pKN25 into the *BamHI* site of YIp5. pKN38 and pKN39 contain the 650-bp *XbaI-BglII* fragment from pKN32 ligated into Bluescript vectors pSK⁻ and pSK⁺ (Stratagene,

FIG. 1. Construction of a strain in which *GAL1:CHC1* has replaced *CHC1*. (A) SL107, a *CHC1 ura3-52 scd1-i* strain (strain a), was transformed with the YipGAL1:CHC1 plasmid (pKN8) which had been linearized at *MstII* to direct integration of the plasmid at the *CHC1* locus. This yielded strain b, which had a tandem repeat of *CHC1* truncated at *SphI* and a full-length *GAL1:CHC1*. Then strain b was streaked on 5-FOA+Gal to force the excision of plasmid sequences and the *URA3* gene, yielding SL214, a strain in which *GAL1:CHC1* had replaced *CHC1* (strain c). Endonuclease cleavage sites are indicated (B, *BamHI*; Bg, *BglII*; M, *MstII*; R, *EcoRI*; S, *SalI*; Sp, *SphI*; X, *XmnI*). Thin lines, genomic DNA; thick lines, plasmid DNA; open boxes, *CHC1* sequence; hatched boxes, *GAL1* promoter sequence. (B) Strains a, b, and c (corresponding to those in panel A) were streaked for single colonies on YEPD and YEP-Gal and incubated for 3 and 4 days, respectively, at 30°C. Note that all strains grew well on galactose. On glucose, only the *CHC1* recipient strain (strain a) formed colonies. The strains with the tandem repeat containing the truncated *CHC1* plus a full-length *GAL1:CHC1* (strain b) and the final *GAL1:CHC1* construct (strain c) did not grow on glucose even after prolonged incubation.

La Jolla, Calif.), respectively, cut with *XbaI* and *BamHI* in the multiple cloning site.

Replacement of *CHC1* by *GAL1:CHC1*. To construct SL214, a strain containing the clathrin HC gene under control of the *GAL1* promoter, pKN8 was linearized with *MstII* within the *CHC1* insert and transformed into a *CHC1*

scd1-i recipient strain, SL107 (Fig. 1, strain a). Transformants were recovered on c-Ura+Gal plates. This led to integration of pKN8 at the clathrin HC locus, generating a truncated *CHC1* gene under control of its endogenous promoter followed by plasmid sequences and then a full-length *CHC1* gene under *GAL1* control (Fig. 1A, strain b). Unlike the *CHC1* recipient strain a, these transformants did not grow on glucose (Fig. 1B, strain b). Plasmid sequences and one copy of the *CHC1* gene (either under *GAL1* control or not) were excised by plating the transformants of interest on 5-FOA+Gal plates. Ura⁻ recombinants were checked for loss of growth on glucose to identify cells retaining the final *GAL1:CHC1* construct (Fig. 1, strain c). Excision by 5-FOA was necessary to remove the *URA3* marker and the truncated *CHC1* gene in order to prevent further recombination that could reconstitute the wild-type gene instead of *GAL1:CHC1*. The advantage of integrating the *GAL1:CHC1* promoter fusion at *CHC1* rather than maintaining it on a plasmid in a strain of *chc1Δ::LEU2* genotype or integrating it at another locus, such as *URA3*, was that *URA3*, *LEU2*, or other commonly used genes were available as selectable markers and the promoter fusion was stably maintained within cells. Yeast genomic DNA was prepared from the transformants essentially as described by Struhl et al. (72) and DNA blot analysis (70) confirmed both the integration and the appropriate excision events (data not shown).

Integration of YIpSCD2. YIpSCD2, linearized within the *SCD2* insert with *HpaI* in order to direct integration of the *URA3* selectable marker on the plasmid to *SCD2* in the genome, was transformed into SL214, and transformants were recovered on c-Ura+Gal plates. DNA blot analysis (not shown) confirmed the integration at the *SCD2* locus.

Sequencing *SCD2*. pKN38 and pKN39 were transformed into XL1 Blue (Stratagene), and single-stranded DNA templates were made as described previously (58) with R408 helper phage from Stratagene. DNA sequencing was performed by the dideoxy-chain termination procedure (60), using the Sequenase sequencing kit (U.S. Biochemical Corp., Cleveland, Ohio) and [α -³²S]dATP (Amersham, Arlington Heights, Ill.). GenBank (release 67) was searched by using the MacVector sequence analysis software (International Biotechnologies, Inc., New Haven, Conn.).

RNA blot analysis of ubiquitin transcript levels. Cells were diluted from log-phase YEP-Gal cultures into YEPD. Dilutions (0.2×10^5 to 1×10^5 cells per ml) were made to prevent exit from log phase during the time course of the experiment. At various times, 2×10^8 cells, as determined by hemacytometer counting, were harvested. Total yeast RNA was prepared as described elsewhere (9). Electrophoresis of RNA (10 μ g per sample) in a 1% agarose formaldehyde gel and transfer to Nytran (Schleicher & Schuell, Keene, N.H.) were carried out as described previously (58). Blots were probed with the 1,000-bp *BglII-NruI* fragment of pKN25, which was labeled by using the Stratagene random primer labeling kit and [α -³²P]dCTP (New England Nuclear, Boston, Mass.). The blot was prehybridized, hybridized, and washed as described by Church and Gilbert (11). To check for equal loading of RNA in each lane, blots were stained with methylene blue (58). Densitometry was done on autoradiograms by using a Sci Scan 5000 (U.S. Biochemical). Since sorbitol was included in the growth medium for protein extractions (see below), control experiments were performed to confirm that the induction of ubiquitin mRNA was the same with or without sorbitol (not shown).

Clathrin HC turnover. SL214 and SL107 were diluted to 5×10^5 cells per ml into either YEP-Gal or YEPD+1 M

sorbitol from log-phase YEP-Gal cultures. At various times after the shift, 5×10^8 cells, as determined by hemacytometer counting, were harvested. The sorbitol allowed more consistent protein recovery from *GAL1:CHC1* cells at later times after the shift to glucose. The cultures were diluted back into fresh, prewarmed medium two times during the course of the experiment to prevent exit from log-phase growth. The cell pellet was resuspended in 400 μ l of 2% sodium dodecyl sulfate (SDS) and ~ 0.5 ml of glass beads (0.45 μ m) was added. Cells were lysed by vortexing on the highest setting of a Fisher Vortex Genie for 90 s. The samples were heated to 100°C for 5 min, and 10- μ l aliquots were removed for protein determination. Then 100 μ l of 5 $\times$ SDS sample buffer (33) was added, and the liquid was transferred to a new microcentrifuge tube. The sample was spun in a microcentrifuge for 10 min at 12,300 $\times$ g, and the supernatant was aliquoted and frozen at -90°C until it was loaded on the gel. Protein was determined by the bicinchoninic acid protein assay (Pierce, Rockford, Ill.). Equal amounts of protein (100 μ g) were loaded in each lane of an SDS-7.5% polyacrylamide gel. The protein was transferred to an Immobilon P membrane (Millipore, Bedford, Mass.) for 1 h 20 min at ~ 0.9 A, using a Genie electrophoretic blotter (Idea Scientific Co., Corvallis, Ore.). Uniform loading was checked by staining the blots with amido black. Blots were incubated with a pool of six purified yeast anti-clathrin HC monoclonal antibodies (~ 2 μ g/ml) (37), washed, and incubated for 1 h with rabbit anti-mouse immunoglobulin G (10 μ g/ml; Cooper Biomedical, Malvern, Pa.) prior to incubation with ^{125}I -protein A (1 $\mu\text{Ci/ml}$; Amersham). To quantify the clathrin HC present in each sample, the 186-kDa band was cut out of the membrane and counted with a Gamma 5500B (Beckman).

Detection of free ubiquitin protein and ubiquitin-conjugated proteins. Cells were diluted from log-phase YEP-Gal cultures into either YEP-Gal or YEPD+1 M sorbitol, and at various times protein was extracted as described above for the clathrin turnover experiment. Dilutions (0.2×10^5 to 1×10^5 cells per ml) were made so that cultures did not exit from log phase during the experiment. For detection of free ubiquitin, equal amounts of protein (100 μ g) were loaded in each lane of an SDS-18% polyacrylamide gel made according to Laemmli (33), with modifications as described elsewhere (20). After electrophoresis of the gel at 100 V for 3 h, proteins were transferred to an Immobilon P membrane for 20 min at ~ 0.8 A using the Genie electrophoretic blotter. Free ubiquitin and ubiquitin conjugates were detected with rabbit antiubiquitin polyclonal antibodies (Sigma, St. Louis, Mo.) at 40 μ g/ml followed by ^{125}I -protein A (1 $\mu\text{Ci/ml}$; Amersham). Quantification of autoradiograms was identical to that for Northern (RNA) blots. Inviability cells were estimated over the course of the experiment by methylene blue staining (35). Percentages for SL214 were comparable to those of SL591 and never exceeded 0.05%. There was 100% plasmid retention for SL591 (SL214 with YCp50-*CHC1*) on nonselective medium.

Ubiquitin-conjugated proteins were detected as described above for free ubiquitin except that equal amounts of protein (100 μ g) were loaded in each lane of an SDS-5 to 15% polyacrylamide gradient gel, and protein was transferred to Immobilon P for 1 h 20 min at ~ 0.9 A.

RESULTS

Characterization of strains with the HC gene under *GAL1* control. The identification of high-copy-number suppressors

FIG. 2. Time course of growth arrest and clathrin HC turnover in the *GAL1:CHC1* strain after a shift to glucose. (A) *GAL1:CHC1* (SL214) and *CHC1* (SL107) strains grown to log phase in YEP-Gal were diluted directly into YEPD, and at the times indicated, cell number was determined by hemacytometer counting. (B) SL214 and SL107 were grown to log phase in YEP-Gal and diluted in glucose medium as described in Materials and Methods. Cell extracts were prepared for immunoblot analysis at the times (hours) indicated after the shift to glucose. Clathrin HC was detected as indicated in Materials and Methods.

of clathrin deficiency made use of a transformation recipient (SL214) that had its chromosomal *CHC1* sequences replaced with a *GAL1:CHC1* promoter fusion (Fig. 1A). This *GAL1:CHC1 scd1-i* strain (SL214) grew well on galactose but was inviable on glucose (Fig. 1B). Further characterization of SL214 was performed to examine the time course of growth arrest and clathrin turnover after a shift from galactose to glucose medium. Immunoblot analysis showed that *CHC1* expression from *GAL1:CHC1* was approximately two- to fourfold greater than in strains carrying *CHC1* under control of its own promoter (Fig. 2B). This overexpression of *CHC1* apparently had no adverse effects on yeast cells, consistent with previous studies using *CHC1* on a high-copy-number 2- μ m vector (34a, 52). After the shift from galactose to glucose, clathrin HC levels declined gradually and by 13 to 15 h were $<1\%$ of the level in cells grown on galactose (Fig. 2B). After 10 h on glucose, growth began to slow, and by 15 h, cell numbers no longer increased (Fig. 2A). The amount of clathrin HC present in SL214 cultures grown in galactose and *CHC1* strains grown on galactose or glucose remained unchanged over the course of the experiment (not shown). Cell morphology of SL214 also changed after the shift from galactose to glucose and was similar to that described earlier for *chc1 Δ ::LEU2 scd1-v* strains. Cells increased in size and became rounded and granulated in appearance, and small aggregates of cells or cells with multiple buds began to appear. This finding accounts for the observation that in liquid culture, A_{600} (but not cell number) continued to

TABLE 2. Isolation of *SCD* plasmids

Plasmid class ^a	Gene locus	No. of independent transformants	% Rescue after reshuffling ^b
1	<i>SCD2</i>	18	38
2	<i>SCD3</i>	4	37
3	<i>SCD4</i>	2	53
4	<i>SCD5</i>	1	103
5	<i>SCD6</i>	1	132
6	<i>SCD7</i>	1	90
7		1	2
8		1	2
9		1	0.2
10		1	0

^a In the initial selection for high-copy-number plasmids that could rescue SL214 (*GAL1:CHC1 scd1-i*) to viability on glucose, 10 different plasmid classes (based on restriction enzyme mapping) were identified from the original 31 independent colonies that grew on c-Ura medium. Upon transformation (reshuffling) of one plasmid from each class back into the original yeast recipient strain (SL214), only classes 1 to 6 (*SCD2* to *SCD7*) retained rescuing ability on glucose.

^b Determined as (number of colonies on glucose/number of colonies on galactose) and measured by inoculating cultures from c-Ura+Gal plates into YEPD and growing the cells for 16 h before plating equal numbers on YEPD and YEP-Gal plates. YEPD plates were incubated for 6 days, and YEP-Gal plates were incubated for 4 days, at 30°C. Data are from a typical experiment. Plasmid loss after growth for 16 h on nonselective medium (YEPD) was determined by replica plating from the YEP-Gal plates onto c-Ura+Gal plates. Plasmid loss was never greater than 15% and therefore cannot account for differences in rescuing ability.

increase slowly between 15 and 20 h after the shift to glucose before completely leveling off (not shown). Overall, these results indicate that the *GAL1* promoter conferred excellent control of clathrin HC synthesis, leading to growth arrest when clathrin expression was repressed on glucose.

Cloning high-copy-number suppressors of clathrin deficiency. To identify plasmids encoding suppressors of clathrin deficiency, SL214 (*GAL1:CHC1 scd1-i*) was transformed with a yeast genomic DNA library constructed in YEp24 (9), a 2- μ m *URA3* vector, which is typically maintained at 10 to 40 copies per yeast cell (17), and cells were plated directly on c-Ura+Glu medium to select transformants that could grow in the absence of genomically encoded clathrin HC. By this approach, since *chc1 Δ scd1-i* cells are inviable, selection of a *GAL1:CHC1 scd1-i* strain (SL214) on glucose medium allowed identification of transformants that carried a plasmid permitting growth of normally inviable *Chc⁻* yeast. Direct selection, rather than first plating on galactose-containing medium and then replica plating to glucose, was used because of the slow turnover of clathrin. Transformation frequency was determined independently for a given experiment by plating cells on c-Ura plus Gal. From approximately 30,000 Ura⁺ transformants, 31 independent colonies grew on c-Ura+Glu medium (Table 2). Plasmids, isolated from candidate yeast transformants, were transformed into *E. coli*, and restriction maps were generated. This analysis indicated that 10 distinct genetic loci were represented among the original yeast transformants. The number of isolates from each plasmid class is indicated in Table 2.

To confirm that the viability of the original yeast transformants on glucose was due to a gene on the plasmid and not a secondary chromosomal mutations, one plasmid from each class was transformed back into the original yeast recipient strain, SL214 (*GAL1:CHC1 scd1-i*), and Ura⁺ transformants were selected on galactose. The ability of plasmids to rescue SL214 to viability on glucose was measured as indicated in Table 2, footnote b. Six of the plasmids still allowed growth

of SL214 on glucose; these were designated YEp*SCD2* to YEp*SCD7* (Table 2). Several of the rescuing plasmids were recovered more than once in this selection, but those that were unable to rescue after retransforming yeast strains only had one representative each. In addition, YEp*SCD5* to YEp*SCD7* consistently yielded a higher efficiency of rescue on glucose than did YEp*SCD2* to YEp*SCD4*. *CHC1* (as assessed by restriction mapping) was not represented in any of the *SCD* plasmid classes. This was not unexpected, since previous attempts to clone the complete *CHC1* gene from this particular library were unsuccessful (34a). In addition, none of the plasmids appeared to affect *GAL1* regulation, thereby allowing leak through expression of *CHC1* on glucose, since transformation of the YEp*SCD* plasmids into a strain carrying a *GAL1:lacZ* fusion showed no expression of β -galactosidase on glucose (data not shown).

Colonies from a typical rescue assay are shown in Fig. 3. SL214 (Fig. 3A) and SL214(YEp24) (not shown) were inviable on glucose, but after transformation with the rescuing plasmids, for example YEp*SCD2* (Fig. 3B) or YEp*SCD7* (Fig. 3C), colonies grew on glucose medium. However, growth of SL214 carrying any of the *SCD* plasmids was much slower on glucose than on galactose medium and much slower than that of the control strain SL591 [SL214(YCp50-*CHC1*)] grown on glucose (Fig. 3E). Rather, the phenotype of SL214 transformed with the YEp*SCD* plasmids and grown on glucose was comparable to that of *chc1 Δ scd1-v* strains (not shown) or SL350 (*GAL1:CHC1 scd1-v*) grown on glucose (Fig. 3D). Other phenotypes typical of *Chc⁻* strains, such as temperature sensitivity and the defect in secretion of biologically active α -factor, also were not rescued by the *SCD* plasmids. *SCD* plasmids did not enhance the growth of *scd1-v* strains (not shown).

Suppressor plasmids can rescue *chc1 Δ scd1-i* yeast strains to viability. Although repression of the *GAL1:CHC1* promoter seemed complete on glucose, it was possible that the plasmids rescue only in the presence of small residual amounts of clathrin, which might still be expressed in cells grown on glucose. To test this possibility, the YEp*SCD* plasmids were transformed individually into a *chc1 Δ :LEU2/CHC1 scd1-i* diploid (BJ3529), and tetrad analysis was performed (Table 3). All of the YEp*SCD*(*URA3*) plasmids were able to rescue *chc1 Δ :LEU2 scd1-i* spores from inviability, as noted by the recovery of Leu⁺ Ura⁺ spores, while no Leu⁺ spores were recovered in the parent diploid or in BJ3529(YEp24). This finding indicates that clathrin HCs are not required for rescue by the suppressor genes.

Characterization of YEp*SCD2*. Since *SCD2* was recovered from 18 of the 31 colonies that grew on c-Ura+Glu medium in the original selection scheme, it was chosen for characterization in more detail. To more narrowly define the complementing region of YEp*SCD2*, DNA fragments from the insert were subcloned into various vectors and tested for the ability to rescue SL214, the original *GAL1:CHC1 scd1-i* transformation recipient strain, from lethality on glucose (Fig. 4). The smallest complementing region lies in the 2.0-kb *Bam*HI-*Nru*I fragment (Fig. 4, line 7).

One gene that could have been recovered from the screen for high-copy-number suppressors was the *scd1-v* allele of the *SCD1* locus. Dominant/recessive analysis (see Materials and Methods) indicated that *scd1-v* should be able to rescue a *GAL1:CHC1 scd1-i* strain from lethality, and segregational analysis with the parental strain (DBY939) used to construct the YEp24 library indicated that the library would contain *scd1-v* (data not shown). The first indication that *SCD2* is not allelic to *SCD1* was that *SCD2* rescued only in high copy

FIG. 3. Plating assay for rescue of *GAL1:CHC1* strains with and without *SCD* plasmids. Strains were inoculated from YEP-Gal (A and D) or c-Ura+Gal (B, C, and E) plates into YEPD broth, grown for 16 h, and then plated on YEPD (Glucose) and YEP-Gal (Galactose) plates. For visualization in this experiment, serial dilutions were made to obtain approximately equal numbers of colonies on each plate. YEP-Gal plates and the YEPD plate shown in panel E were incubated for 4 days; YEPD plates in panels A to D were

number. It did not rescue when present on a *CEN* plasmid (Fig. 4, line 4) or when *YIpSCD2* was integrated at the *SCD2* locus in the genome (Fig. 4, line 5), as would be expected of *scd1-v*. A more rigorous proof that *SCD2* and *SCD1* are not allelic was obtained by segregational analysis (Table 4). SL480 [*MAT α GAL1:CHC1 ura3-52 scd1-i SCD2:YIpSCD2 (URA3)*], which contained the integrated *YIpSCD2* marking the *SCD2* locus with the *URA3* gene, was mated to a haploid (SL350) of *MAT α GAL1:CHC1 ura3-52 scd1-v* genotype. Tetrads from this cross were dissected, and spores were grown up on YEP-Gal plates. If *SCD2* was *scd1-v*, then integration should have occurred at the *scd1-i* locus in SL480 and *scd1-i* and *SCD2:YIpSCD2(URA3)* would always cosegregate; that is, all tetrads would contain two spores that were Ura⁺ and inviable on glucose and two spores that were Ura⁻ and viable on glucose. If, on the other hand, *SCD2* was not allelic with *SCD1*, some Ura⁺ spores that were viable on glucose would be obtained, since *SCD2:YIpSCD2(URA3)* would segregate independently from the *scd1-i* allele. Results were indicative of two-gene segregation with a parental ditype/nonparental ditype/tetratype ratio near 1:1:4 (Table 4). Therefore, *SCD2* is not allelic to the *SCD1* locus.

SCD2 is the polyubiquitin gene, *UBI4*. The sequence of the 650-bp *XbaI-BglII* fragment of *SCD2* (Fig. 4) was determined. A search of GenBank showed a perfect match to *UBI4*, which encodes a polyubiquitin precursor protein containing five ubiquitin-coding elements (51). The complete *UBI4* gene is contained within the *BamHI-NruI* fragment and spans virtually the complete segment of DNA, leaving no flanking sequence for other complementing genes. In addition, the *UBI4* minigene constructed by Finley et al. (21), which contains one ubiquitin-coding element, also rescued SL214 on glucose (data not shown), providing further proof that it is the *UBI4* gene on this DNA fragment that conferred viability to *Chc⁻ scd1-i* strains.

Examination of ubiquitin transcript and protein levels in clathrin-deficient yeast strains. Enzymatic conjugation of ubiquitin to proteins can target those proteins for selective degradation. In *S. cerevisiae*, four distinct ubiquitin-coding loci have been described. In logarithmically growing cells, most ubiquitin is generated from *UBI1*, *UBI2*, and *UBI3*, which encode hybrid proteins of ubiquitin fused to ribosomal proteins (18). *UBI4* is dispensable during vegetative growth, but during stress and in stationary phase, its expression is induced and it becomes an essential gene (21). It has been shown that the stress-specific and stationary-phase loss of viability of *ubi4* cells is caused by their inability to maintain required levels of free ubiquitin (21). Since overexpression of ubiquitin rescued clathrin-deficient *scd1-i* yeast strains, we examined the levels of ubiquitin transcript and protein in *Chc⁻* cells.

First, expression of *UBI4* mRNA was analyzed during a shift of *GAL1:CHC1* cells from galactose to glucose (Fig. 5). On galactose medium, the amount of the 1.5-kb stress-inducible *UBI4* transcript was identical in strains of *GAL1:CHC1 scd1-i* genotype with and without *YCp50-CHC1* (SL591 and SL214, respectively). Upon the shift to glucose medium, the *UBI4* transcript declined approximately 5- to 10-fold in SL591 in response to the shift to the more favorable carbon source. In SL214, in contrast, the *UBI4*

incubated for 6 days at 30°C. (A) SL214 (*GAL1:CHC1 scd1-i*); (B) SL214(*YEpSCD2*); (C) SL214(*YEpSCD7*); (D) SL350 (*GAL1:CHC1 scd1-v*); (E) SL591 [*SL214(YCp50-CHC1)*].

TABLE 3. Rescue of *chc1Δ::LEU2* strains with *SCD* plasmids

BJ3529 transformed with:	No. of tetrads	No. of spores with phenotype ^a :				No. dead
		Leu ⁻ Ura ⁺	Leu ⁻ Ura ⁻	Leu ⁺ Ura ⁺	Leu ⁺ Ura ^{-b}	
YEpSCD2 ^c	14	3	25	4	0	24
YEpSCD3	16	6	26	5	0	27
YEpSCD4	16	16	16	10	0	22
YEpSCD5	16	17	15	14	0	18
YEpSCD6	22	16	28	20	1	23
YEpSCD7	16	19	12	16	0	17
YCpCHC1	28	22	34	30	0	26
YEp24	16	22	9	0	0	33
No plasmid	23	0	46	0	0	46

^a Note the Leu⁺ Ura⁺ spores were recovered less frequently from the YEpSCD2 and YEpSCD3 transformants but Leu⁻ Ura⁺ spores (*CHC1* spores with the YEpSCD plasmid) were also less frequent, suggesting that these plasmids were less stable during meiosis or germination. There is no evidence that overexpression of the genes on these plasmids was detrimental during vegetative growth, since Leu⁻ Ura⁺ strains grew like wild-type cells and all *GAL1:CHC1* YEpSCD transformants grew well on galactose.

^b In over 1,000 tetrads dissected from the BJ3529 parent strain, Leu⁺ Ura⁻ segregants, as observed in the YEpSCD6 transformant, were extremely rare (only two to three spores).

^c In this transformation, pKN25 (Fig. 4, line 3) was used in place of YEpSCD2.

transcript increased 2- to 3-fold as clathrin was depleted after 15 h on glucose, yielding at least a 10-fold increase in *UBI4* mRNA in *Chc*⁻ cells compared with its expression in the *Chc*⁺ strain grown in glucose.

Immunoblot analysis was performed to examine free ubiquitin pools. A typical result is shown in Fig. 6. After a shift of SL214 from galactose to glucose medium, the level of free ubiquitin remained relatively constant; in contrast, a fivefold increase was detected in wild-type control cells (SL591) (also see Fig. 8E for SL107, a comparable *CHC1* control strain). Thus, the increased *UBI4* transcript did not result in a correspondingly large increase in free ubiquitin in *Chc*⁻

strains, and in comparison with the *CHC1* strain grown on glucose, free ubiquitin was actually depleted in *Chc*⁻ cells (compare Fig. 6A and B at 15 h).

When *UBI4* transcript and free ubiquitin levels were examined in SL350, a *GAL1:CHC1 scd1-v* strain, the results were similar to those obtained with SL214 (Fig. 7A and B and Fig. 8A and B). Therefore, *Chc*⁻ strains of both *scd1-i* and *scd1-v* genotypes require higher levels of ubiquitin.

Analysis of *UBI4* mRNA was also performed on strains carrying the rescuing plasmid, pKN32, which contains *UBI4* on YEp24. *UBI4* transcript was further increased 10- to 20-fold in SL214 containing pKN32 (Fig. 7C) compared with SL214 lacking the plasmid (Fig. 7A) or transformed with the parental plasmid not containing *UBI4* (not shown). This additional transcript, synthesized as a result of the extra copies of *UBI4* on YEp24, was sufficient to rescue the *GAL1:CHC1 scd1-i* (SL214) strain to viability on glucose medium.

***Chc*⁻ strains have a unique pattern of ubiquitin-conjugated proteins.** Immunoblots of whole lysates were probed with antiubiquitin antibodies to examine the ubiquitin-conjugated proteins in cells depleted of clathrin (Fig. 8). On galactose medium, all strains gave comparable protein patterns. Interestingly, the pattern of ubiquitin-conjugated proteins was different between *Chc*⁻ and wild-type strains. More specifically, new conjugates appeared in the ranges of ~25 to 40 and ~55 to 70 kDa when *GAL1:CHC1* cells were shifted to glucose medium for 15 h. These bands were absent from the wild-type control cells, SL107 (Fig. 8) or SL591 (not shown). Since these conjugates were present in strains of both *scd1-i* and *scd1-v* genotypes, they were specific to clathrin deficiency and not associated with the state of the *SCD1* locus.

To determine whether this new pattern was dependent upon clathrin depletion or was a property common to all sick or dying cells, we examined the ubiquitin conjugates in YAS352, a strain that has a deletion of the gene encoding the poly(A)-binding protein (*PAB1*) but carries *GAL1:PAB1* on a plasmid (56). YAS352 has a growth pattern similar to that of SL214, arresting cell division ~15 h after a shift from galactose to glucose medium (56). The profile of ubiquitin conjugates for YAS352 (Fig. 8C) more closely resembled that of the *CHC1* strain than that of SL214 or SL350, although the free ubiquitin pool also appeared to be lower and some of the lower-molecular-weight conjugates that

FIG. 4. Complementing region of YEpSCD2. DNA fragments from YEpSCD2 were subcloned into the indicated vectors and tested for the ability to rescue growth of SL214 on glucose as described in Table 2, footnote b. The smallest complementing region lies in the 2.0-kb *Bam*HI-*Nru*I fragment (line 7). *SCD2* rescues only in high copy number, since it did not rescue when present on a *CEN* plasmid (line 4) or when integrated into the genome (line 5). Sequence analysis of the 650-bp *Xba*I-*Bgl*II fragment of *SCD2* showed that *SCD2* is identical to *UBI4*, the polyubiquitin gene. Restriction endonuclease sites: B, *Bam*HI; G, *Bgl*II; H, *Hpa*I; N, *Nru*I; P, *Pvu*II; S, *Sal*I; V, *Eco*RV; X, *Xba*I. Open boxes, ubiquitin coding elements; triangles, heat shock response element in the promoter of *UBI4*.

TABLE 4. Evidence that *SCD2* is not allelic to *SCD1*

Growth condition or determination	No. of the following tetrad types ^a		
	Parental ditype	Tetratype	Nonparental ditype
Growth on:			
Glu	2 D, 2 V	2 D, 2 V	2 D, 2 V
Gal	2 Ura ⁺ , 2 Ura ⁻	1 Ura ⁺ , 1 Ura ⁺ 1 Ura ⁻ , 1 Ura ⁻	2 Ura ⁻ , 2 Ura ⁺
Determination			
Expected no. (<i>n</i> = 20 tetrads)			
Integration at <i>scd1-i</i>	20	0	0
Unlinked to <i>SCD1</i> locus	3	14	3
Observed no.	2	15	3

^a Tetrads from the cross SL480 × SL350 (for genotypes, see Table 1) were dissected, and spores were grown on YEP-Gal plates to score for nutritional markers. Then spore clones were grown overnight in YEPD, and equal numbers of cells were plated on galactose and glucose plates to score for growth on glucose. Note that SL480 with the integrated YIpSCD2 was not viable on glucose (Fig. 4, line 5). D, dead (spore clones that gave <1% colonies on Glu compared with Gal); V, viable (virtually all viable colonies gave >90% colonies on Glu compared with Gal, typical of *scd1-v* strains).

became more prominent in wild-type strains after the shift to glucose were depleted in YAS352 as well as in SL214 or SL350. RNA blot analysis confirmed that YAS352 also contained increased levels of *UBI4* transcript after the shift to glucose (not shown). It is possible that the novel ubiquitinated proteins found in *Chc*⁻ cells are normally conjugated in *CHC1* cells, but in the *Chc*⁻ cells they display a more rapid mobility on SDS-polyacrylamide gel electrophoresis because of the presence of shorter multiubiquitinated side chains. This could occur in circumstances under which ubiquitin is being depleted, but again our results with *Pab*⁻ cells, which contained lowered levels of free ubiquitin, argue against this explanation. In addition, overexpression of *UBI4* from YEp24 in clathrin-deficient strains grown on glucose did not completely restore the wild-type pattern of ubiquitin conjugates (for example, compare the conjugated protein in the region of 55 to 70 kDa at 15 h on glucose in Fig. 8A, B, and D with those in Fig. 8E). Overall, these results show that the novel high-molecular-weight conjugates accumulated only in cells depleted of clathrin HCs and were not generally indicative of stressed or dying cells.

DISCUSSION

In previous studies, we identified a genetic locus, termed *SCD1*, that influences the ability of *Chc*⁻ yeast cells to

survive (36). Taking advantage of this finding, we developed a selection for the isolation of high-copy-number suppressors of clathrin deficiency. The cloning strategy depended upon use of a *GAL1:CHC1 scd1-i* strain, which is inviable when grown in glucose medium. We have identified six genetic loci whose overexpression abrogates the lethality of *chc1Δ scd1-i* and *GAL1:CHC1 scd1-i* strains grown on glucose. Lethality was abrogated by these genes, but other aspects of the clathrin-deficient phenotype were not. At present, we cannot determine whether overexpression of the *SCD* genes allows for suppression of the *scd1-i* allele, partial suppression of the clathrin deficiency, or both. We have observed partial suppression of *Chc*⁻ phenotypes by mutant clathrin HCs that contain COOH-terminal truncations (38). For some deletions, the mutant HC can rescue *chc1Δ scd1-i* strains to viability without rescue of any of the other defects associated with clathrin deficiency (34a).

The one suppressor gene that was characterized in detail, *SCD2*, is *UBI4*, the polyubiquitin gene. Ubiquitin is an abundant, highly conserved protein found associated with a number of intracellular compartments, including the cytosol, the nucleus, and the cell surface (for reviews on the ubiquitin conjugation system, see references 19, 27, and 30). Ubiquitin functions by its covalent attachment to cellular acceptor proteins in a multistep process involving ATP and the enzymes of the ubiquitin conjugation pathway. The primary role of conjugation of ubiquitin to cellular substrates is to target proteins for selective degradation. The ubiquitin con-

FIG. 5. Induction of *UBI4* transcript after a shift of SL214 and SL591 from galactose to glucose medium. Precultures were grown to log phase in YEP-Gal and diluted directly into YEPD as described in Materials and Methods. At the times (hours) indicated after the shift to glucose, total yeast RNA was isolated and fractionated on a formaldehyde gel for Northern blot analysis. *UBI4* mRNA was detected as indicated in Materials and Methods. (A) SL214 (*GAL1:CHC1 scd1-i*); (B) SL591 [SL214(*YCp50-CHC1*)]. The minor non-stress-inducible 2.6-kb *UBI4* transcript described previously (21) remained the same for all strains with a single copy of *UBI4* even after the shift to glucose (not shown).

FIG. 6. Change in free ubiquitin protein levels after a shift from galactose to glucose medium. Yeast cells grown to log phase in YEP-Gal were diluted directly into glucose medium as described in Materials and Methods. At the times (hours) indicated after the shift to glucose, cell extracts were prepared for immunoblot analysis and ubiquitin was detected as indicated in Materials and Methods. The arrow indicates the position of free ubiquitin. (A) SL214 (*GAL1:CHC1 scd1-i*); (B) SL591 [SL214(*YCp50-CHC1*)].

FIG. 7. RNA blot analysis of *UBI4* transcript in *scd1-v* and *UBI4* overexpression strains. Yeast cells were grown as described in the legend to Fig. 5; at the times (hours) after the shift to glucose, total yeast RNA was isolated and fractionated on a formaldehyde gel for Northern blot analysis. *UBI4* mRNA was detected as indicated in Materials and Methods. (A) SL214 (*GAL1:CHC1 scd1-i*); (B) SL350 (*GAL1:CHC1 scd1-v*); (C) SL214(pKN32) (pKN32 contains the smallest complementing region of YEpSCD2 [see Fig. 4, line 7]).

jugation system provides the major nonlysosomal proteolytic pathway in the cell, is important for turnover of short-lived and abnormal proteins, and plays a role in regulation of diverse cellular processes, such as the cell cycle (20, 22), DNA repair (31), and the stress response (21, 66). Generally, the ubiquitin conjugates that serve as substrates for degradation contain branched multiubiquitin chains and are hydrolyzed by the large ATP-dependent proteasome, which resides in both the nucleus and the cytosol (23, 49).

During vegetative growth of yeast cells, ubiquitin is primarily provided by *UBI1*, *UBI2*, and *UBI3* (21), which encode ubiquitin-ribosomal protein hybrids (18). *UBI4* becomes essential during stress and starvation (21). Transcript levels for *SSA4*, a major HSP70 stress response gene of *S.*

FIG. 8. Accumulation of novel high-molecular-weight ubiquitin conjugates in clathrin-deficient yeast cells. Strains were grown as described in the legend to Fig. 6. At the times (hours) after the shift to glucose, cell extracts were prepared for immunoblot analysis and fractionated on an SDS-5 to 15% acrylamide gradient gel. Ubiquitin and ubiquitin conjugates were detected as indicated in Materials and Methods. (A) SL214 (*GAL1:CHC1 scd1-i*); (B) SL350 (*GAL1:CHC1 scd1-v*); (C) YAS352 [*CHC1 PAB1::HIS3* plus *pAS137(pGAL1:PAB1)*]; (D) SL214(pKN32); (E) SL107 (*CHC1*). The pattern of high-molecular-weight ubiquitin conjugates in SL214(YEp24) was identical to that of SL214 (not shown). Migration of molecular weight standards (in kilodaltons) is indicated on the right. Brackets indicate the regions of the gel where new ubiquitin conjugates appeared when *GAL1:CHC1* cells were shifted to glucose medium for 15 h. The arrow indicates the position of free ubiquitin.

cerevisiae (5), are elevated in *GAL1:CHC1* cells grown in glucose medium to approximately the same level as in heat-shocked *CHC1* cells (data not shown), indicating that *Chc*⁻ cells are in a generally stressed state. Interestingly, overexpression of *UBI3* was also able to rescue the *GAL1:CHC1 scd1-i* strain on glucose medium (not shown), suggesting that overexpression of the non-stress-inducible *UBI* genes provides sufficient ubiquitin to rescue clathrin-deficient cells of *scd1-i* genotype. However, none of the other *SCD* plasmids appear to encode *UBI1*, *UBI2*, or *UBI3*. Additionally, *UBI4* is not a generalized suppressor of all cells undergoing cell death. To our knowledge, there is only one other documented case of suppression by overexpression of *UBI4*. In that case, *UBI4* was recovered in a screen for high-copy-number suppressors of a temperature-sensitive mutant of the *RPC31* gene (71), which encodes a subunit of yeast RNA polymerase III (44). *UBI4* overexpression also did not suppress the lethality of *GAL1:PAB1* cells (YAS352) on glucose, although these cells also had elevated *UBI4* transcript levels (not shown) and free ubiquitin levels were depleted relative to those in wild-type strains.

Recently, Munn et al. mutagenized a *GAL1:CHC1 chc1Δ* strain that was viable on glucose medium in order to search for mutants that were unable to grow in the absence of clathrin HCs (46). Although their *GAL1:CHC1* construct did not provide complete repression of clathrin expression, they were able to identify mutations at numerous genetic loci that led to poorer growth or, in a few cases, lethality on glucose medium. It was argued that the lethality of *chc1Δ* strains in some genetic backgrounds, e.g., *scd1-i* or *cdl1* (clathrin-deficient lethal), is due to perturbation of processes not directly related to clathrin function. Our discovery that overexpression of ubiquitin rescues a *GAL1:CHC1 scd1-i* strain from inviability on glucose may lend support to this hypothesis, although we cannot exclude the possibility that lack of clathrin directly affects protein functions or turnover involving ubiquitination of proteins. The large number of complementation groups obtained by Munn et al. (46) could be explained if many mutations in the stress response or protein turnover systems of the cell resulted in lethality of the already impaired *Chc*⁻ *scd1-v* cells. However, in the absence of data concerning the biochemical function of any of the genes identified in their study, other possibilities, such as the existence of pathways that can partially substitute for clathrin-mediated vesicular transport or that provide functions directly perturbed in clathrin-deficient cells, still cannot be eliminated. Characterization of the other suppressor plasmids we have identified and cloning of *SCD1* should help to resolve these issues.

The mechanism by which overexpression of ubiquitin rescues clathrin-deficient cells carrying the *scd1-i* allele remains to be determined. Clathrin deficiency results in global effects on yeast cells, including poor growth, genetic instability, and defective mating and sporulation (35, 36, 53, 54). *Chc*⁻ cells also have abnormal morphology, including a larger and more granulated appearance (35, 36), aggregated growth (35), an accumulation of aberrant organelles, such as Berkeley bodies (52), which are thought to be an abnormal form of the Golgi complex that arise in certain *sec* mutants (48), fragmented and multivesiculated vacuoles (52), and a pronounced thickening of the cell wall (47a). These phenotypes presumably arise from the mislocalization and misprocessing of proteins of the secretory (and possibly the endocytic) pathway, which has been shown to occur in clathrin-deficient yeast cells. For example, DPAPa and Kex2p, Golgi enzymes involved in processing of pro-α-

factor, are mislocalized to the cell surface in clathrin-deficient cells (54, 65). This defect results in the secretion of an uncleaved precursor form of α -factor (54). In addition, a transient defect in sorting of the vacuolar protease, CPY, has been observed in a temperature-sensitive *chc1* mutant at the nonpermissive temperature (64).

Our results suggest that the ubiquitin conjugation system, which is involved in degradation of aberrant proteins, may be important for turnover of mislocalized and misprocessed proteins that arise in clathrin-deficient cells. It is clear that clathrin-deficient cells have a higher demand for ubiquitin. *UBI4* transcription is induced, but even so, *Chc*⁻ cells cannot maintain the levels of free ubiquitin that *CHC1* strains do. It has also been shown that *Chc*⁻ cells are supersensitive to the arginine analog canavanine (35, 52). This finding indicates that the protein turnover machinery either is directly impaired or cannot keep pace with the need to remove additional aberrant or mislocalized proteins.

An important question that arises from these studies concerns the compartment for degradation of these mislocalized or misprocessed proteins. The ubiquitin-conjugating enzymes and the proteasome have been localized to the cytoplasm and nucleus and are not found at the luminal side of secretory/endocytic organelles (23, 75). The sorting and misprocessing defects of clathrin-deficient yeast cells could result in loss of integrity of organelles of the secretory pathway, which might lead to the leakage of potentially toxic proteins into the cytoplasm. Enhancement of *UBI4* expression in response to this cellular stress could facilitate degradation of these proteins. If the leakage of secretory proteins into the cytoplasm was more severe in *Chc*⁻ cells of *scd1-i* genotype, this could explain how overexpression of ubiquitin allows survival of these cells.

Alternatively, modification of membrane proteins at the cytoplasmic face of secretory/endocytic organelles could target them for turnover. A number of cell surface proteins have been shown to be ubiquitinated, and there is evidence for a direct role of the ubiquitin conjugation system in the function or turnover of membrane proteins (10, 40, 41, 67). In addition, one of the 10 known yeast ubiquitin-conjugating enzymes (E2), encoded by *UBC6*, has recently been localized to the endoplasmic reticulum, with the active domain of the enzyme facing the cytoplasm (30). Other membrane-bound E2 enzymes could exist in other compartments and might be important for turnover of misfolded, missorted, or resident membrane proteins. Our studies identified a novel pattern of ubiquitinated proteins in clathrin-deficient cells. It will be interesting to determine whether any of these proteins are membrane proteins associated with the secretory/endocytic machinery or are directly misprocessed or mis-sorted as a result of lack of clathrin.

Several studies have also linked the ubiquitin conjugation system directly to lysosomal turnover of proteins. Both free ubiquitin and ubiquitin-protein conjugates have been found within the endosomal/lysosomal vacuolar compartments and in autophagic vacuoles of animal cells (15, 34, 62). Autophagy is a process by which endogenous cellular proteins are degraded by the lysosomal system (16, 43). Two of its major functions are degradation of normal proteins in response to nutrient deprivation and removal of abnormal cellular components that accumulate during stress, cell toxicity, or death. Recently, the ubiquitin-activating enzyme (E1) was localized to the cytoplasmic face of autolysosomes (63), and mammalian cells harboring a temperature-sensitive mutation in E1 were found to be defective in the autophagic response upon exposure to high temperatures (12, 25, 61). Late-stage

degradative vacuoles containing acid hydrolases accumulated, but conversion to residual bodies was blocked and luminal ubiquitin conjugates, which are normally found in autolysosomes, were absent (39). In *S. cerevisiae*, ubiquitin conjugates have also been localized to the lysosome-like vacuole and accumulate in strains which lack the key vacuolar peptidases, proteinases A and B (69). As indicated above, multivesicular vacuoles and aberrant membrane-bound organelles accumulate in clathrin-deficient yeast cells (35, 36, 52). This might occur if an autophagic response system in the cells became overwhelmed by the large mass of aberrant organelles that need to be turned over. Alternatively, but not mutually exclusive of this possibility, such an autophagic process itself could be directly perturbed by clathrin deficiency. In *Chc*⁻ *scd1-i* strains, this defect could be more severe, resulting in lethality. Overexpression of ubiquitin might serve to drive the ubiquitination process, allowing for more efficient turnover of ubiquitinated proteins in the vacuolar lumen, or increased expression of ubiquitin could allow enhanced efficiency of sequestration of the aberrant or mislocalized proteins destined for turnover in the vacuole.

Overall, our studies lend support to the idea that ubiquitin plays a general role in turnover of proteins of the secretory and endocytic pathways. Further studies, such as localization of ubiquitin and characterization of the ubiquitin conjugates that accumulate in *Chc*⁻ yeast cells, may lead to the identification of other proteins that are missorted as a result of lack of clathrin and should provide new information concerning the connection between the vesicular transport machinery and ubiquitin conjugation system.

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