RESEARCH ARTICLE

A Test of Clathrin Function in Protein Secretion and Cell Growth

Gregory S. Payne and Randy Schekman

Abstract. *Clathrin-coated membranes are intimately associated with a variety of protein transport processes in eukaryotic cells, yet no direct test of clathrin function has been possible. The data presented demonstrate that* Saccharomyces cerevisiae *does not require clathrin for either cell growth or protein secretion. Antiserum to the yeast clathrin heavy chain has been used to isolate a molecular clone of the heavy chain gene (CHC1) from a library of yeast DNA in λgt11. Clathrin-deficient mutant yeast have been obtained by replacing the single chromosomal CHC1 gene with a disrupted version of the cloned DNA. Cells harboring a nonfunctional chc1 allele produce no immunoreactive heavy chain polypeptide, and vesicles prepared from mutant cells are devoid of clathrin heavy and light chains. Although clathrin-deficient cells grow two to three times more slowly than normal, secretion of invertase occurs at a nearly normal rate. Therefore protein transport through the secretory pathway is not obligately coupled to the formation of clathrin-coated vesicles.*

Coated regions of membranes and coated vesicles have been implicated as intermediates in the vesicle traffic that mediates transport of proteins between membrane-enclosed subcellular compartments in eukaryotic cells (*1, 2*). These membranes were first characterized by electron microscopy (*3*) and derive their appearance (and name) from a proteinaceous structure displayed on the cytoplasmic surface of the lipid bilayer. The participation of coated membranes in the early stages of vesicular transport has been demonstrated by studies of receptor-mediated endocytosis in mammalian cells. During endocytosis, molecules bound to cell surface receptors cluster at indentations, termed coated pits, that invaginate and then bud to form vesicles enriched in receptor-bound ligands (*1, 4*). In accord with these observations, structural and biochemical investigations suggest that the proteins that comprise the coat are suited to function in transport vesicle biogenesis. Detailed inspection of coated membranes with the use of electron microscopic techniques reveals that the coat consists of a polyhedral protein lattice (*5*), the principal constituent of which is clathrin (*6*). Coats on purified vesicles can be disassembled and the clathrin can be extracted as a distinctive three-legged "triskelion" composed of three molecules of heavy chain (180 kD) and three noncovalently associated light chains (usually two species ranging in size from 30 to 40 kD) (*7*). Purified triskelions can reassemble to generate empty cages (*7*) or rebind to coated vesicles previously stripped of clathrin (*8*). These properties form the basis for a model in which the assembly of triskelions into a lattice cage in vivo drives the formation of a coated vesicle from a coated region of membrane (*2, 9*). This assembly process also represents a paradigm for the biogenesis of vesicles during other stages of intracellular transport. Indeed, coated vesicles have been implicated in transport between the endoplasmic reticulum and the Golgi body (*10*), through the Golgi body (*11*), and between the Golgi body and the lysosome (*12*).

Although these observations provide circumstantial evidence, there has been no direct demonstration that clathrin is required for membrane vesiculation and the formation of transport vesicles. Alternatively, clathrin may serve a more static role as a molecular filter that collects or excludes membrane-bound molecules with no active participation in vesicle morphogenesis (*4*).

In order to address directly the role of clathrin in protein transport, we have sought mutant cells unable to form coated vesicles. This became feasible when Mueller and Branton identified coated vesicles in the yeast *Saccharomyces cerevisiae* (*13*). In yeast, facile manipulation of gene structure in vivo is possible once a molecular clone of the gene has been obtained (*14, 15*). For this reason, we have cloned the yeast gene encoding clathrin heavy chain. Using the molecular clone, we have disrupted the chromosomal clathrin heavy chain gene in vivo to assess the phenotypic consequences of a clathrin deficiency.

Purification and characterization of yeast clathrin. Chromatography of yeast membranes through a Sephacryl S-1000 gel filtration column yields fractions enriched for coated vesicles (*13*). The Coomassie blue–stained protein profiles of fractions enriched in coated vesicles display a prominent 190-kD protein proposed to be clathrin heavy chain (*13*). Clathrin triskelions can be specifically extracted from mammalian coated vesicles by either 2*M* urea or 0.5*M* tris (*7*).The 190-kD yeast protein behaved similarly. Sephacryl S-1000 column fractions containing coated vesicles were pooled, concentrated by centrifugation, then exposed to 2*M* urea. Membrane vesicles were sedimented, and proteins found in the supernatant and vesicle pellet were examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (*16*) and staining with Coomassie blue (Fig. 1A). Most of the vesicle proteins were insensitive to urea treatment and remained with the vesicle pellet (compare the total vesicle proteins in lane 1 with the vesicle proteins after urea extraction in lane 2). Urea extraction specifically released 190-kD, 55-kD, and 36-kD proteins into the supernatant (lane 3). A similar spectrum of proteins with several additional minor species was extracted by 0.5*M* tris-HCl, *p*H 7.5 (*17*).

Sepharose CL-4B gel filtration of the urea-extracted proteins resolved an apparent complex containing the 190-kD and 36-kD proteins from the 55-kD protein (Fig. 1B). The elution volume of the complex was beyond the range of the standard curve established with marker proteins (Fig. 1C), suggesting a large Stokes radius or an oligomeric arrangement (or both) of the subunits. In addition, assuming that the two proteins bind Coomassie blue stain in proportion to their size, we can estimate by densitometry that the complex contains equimolar quantities of the two proteins. Mammalian clathrin displays the same chromato-

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graphic behavior and subunit stoichiometry (*7, 18*). When the yeast protein complex was visualized in the electron microscope with rotary shadowing, the fields showed a homogeneous population of triskelions that were essentially identical to mammalian triskelions (Fig. 1D). Lemmon *et al.* have succeeded in assembling polyhedral cages from yeast triskelions obtained by Sepharose CL-4B chromatography (*19*). Reassembled cages contain the same 190-kD and 36-kD polypeptides found in triskelions (*19*). These results suggest that the 190-kD protein is yeast clathrin heavy chain and that the 36-kD protein is clathrin light chain.

Table 1. Genetic characterization of meiotic products from the sporulation of diploid strains transformed with disrupted *CHC1* DNA. Linear fragments of DNA from pCHC1-Δ2a and pCHC1-Δ2b (*31*) were introduced into GPDY1004 (*46*) by lithium acetate–mediated transformation (*32*). In an analogous fashion, linear DNA from pCHC1-Δ8 (*31*) was introduced into GPDY1007 (*46*). *Leu*$^+$ transformants were isolated, sporulated, and the asci dissected into tetrads. Not all asci consisted of four viable spores. The first two columns indicate the distribution of "tetrads" arising from four, three, or two viable spores.

Ratio of viable to not viable spores	Tetrads of each type (No.)	Ratio of large to small colonies	Tetrads (No.)	Tetrads tested for leucine prototrophy* (No.)	Ratio of Leu$^+$ to Leu$^-$	
					Large colonies	Small colonies
4:0	25	2:2	25	21	0:42	42:0
3:1	11	2:1	10	6	0:12	6:0
		1:2	1	1	0:1	2:0
2:2	3	2:0	2	2	0:2	–
		1:1	1	1	0:1	1:0

*Some colonies were too small to assess their growth in the absence of leucine. Tetrads containing such colonies were not included in this analysis.

Antibodies specific for clathrin heavy chain. Yeast heavy chain was used to immunize rabbits (*20*). The elicited immune response yielded an antiserum that specifically recognized the heavy chain polypeptide (Fig. 2). Yeast clathrin was subjected to electrophoresis adjacent to proteins from a yeast homogenate in an SDS–polyacrylamide gel and stained either by Coomassie blue (Fig. 2, lanes 1 and 2) or transferred to nitrocellulose and then incubated with immune (lanes 3 and 4) or preimmune serum (lanes 5 and 6) (*21*). Bound antibodies were visualized with ^{125}I-labeled *Staphylococcus aureus* protein A (*21*). The 190-kD protein specifically recognized amid the total homogenate proteins (Fig. 2, lane 3) comigrated with clathrin heavy chain detected in the adjacent lane 4 (lane 4 contains one-tenth the amount of clathrin displayed in lane 2). Serum from nonimmunized animals (preimmune serum) did not react with any yeast proteins (lanes 5 and 6). Serum from immunized animals (antiserum or immune serum) also specifically precipitated the heavy chain present in total yeast proteins labeled with $^{35}SO_4^{2-}$ (lane 7).

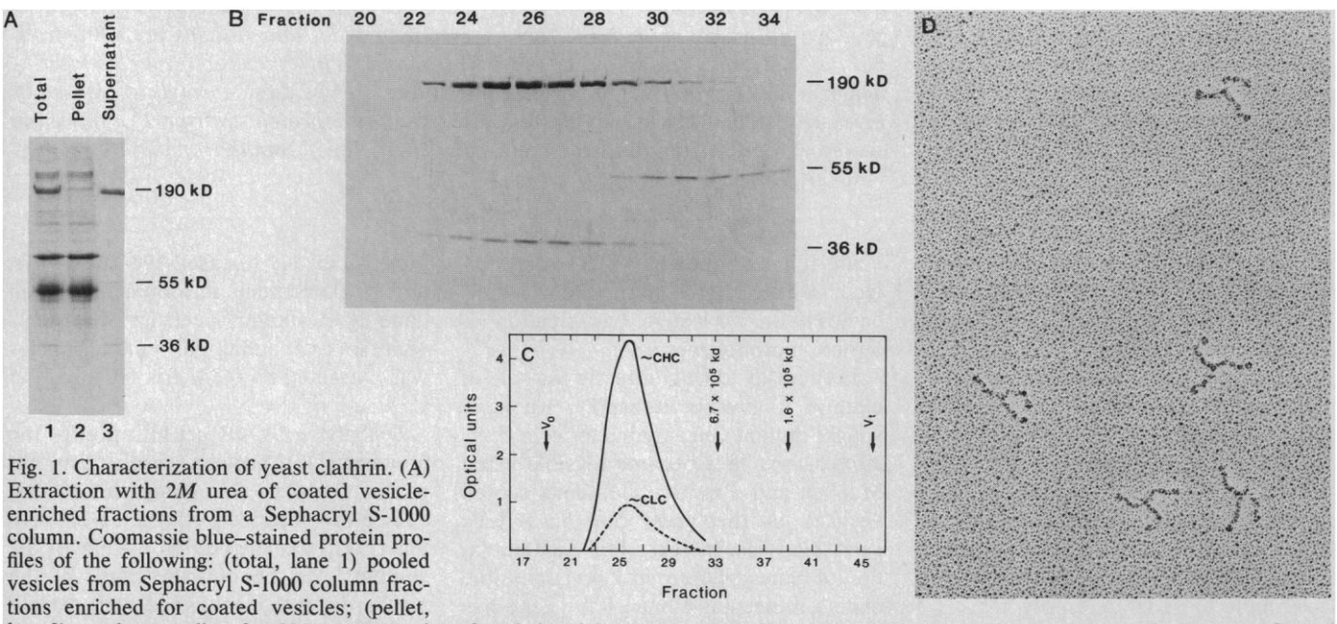

Fig. 1. Characterization of yeast clathrin. (A) Extraction with 2*M* urea of coated vesicle-enriched fractions from a Sephacryl S-1000 column. Coomassie blue–stained protein profiles of the following: (total, lane 1) pooled vesicles from Sephacryl S-1000 column fractions enriched for coated vesicles; (pellet, lane 2) membrane pellet after 2*M* urea extraction of pooled vesicles; (supernatant, lane 3) proteins extracted from the vesicles by 2*M* urea. Coated vesicles from *S. cerevisiae* strain X2180-1A were prepared as described (*13*) except that, after sedimenting, cells were resuspended in 100 m*M* MES · KOH, *p*H 6.5, 0.5 m*M* $MgCl_2$, 1 m*M* EGTA, 0.2 m*M* dithiothreitol (DTT), 0.02 percent NaN_3, and 0.6 m*M* PMSF (buffer A) and lysed by agitation with glass beads in a "bead-beater" (Biospec Products, Bartlesville, Oklahoma). Sephacryl S-1000 column fractions containing coated vesicles were pooled, and the membrane vesicles were sedimented at 100,000*g* for 1 hour and resuspended in 0.4 ml of buffer A (total). Buffer A (0.1 ml) containing 10*M* urea was added, and the solution was incubated for 1 hour at room temperature. Membranes were sedimented as described above and resuspended in buffer A (pellet). The urea supernatant was dialyzed overnight against a solution containing 20 m*M* tris-HCl, *p*H 7.5, 1 m*M* EDTA, 50 m*M* NaCl, 0.02 percent NaN_3, 0.2 m*M* DTT, and 0.2 m*M* PMSF (buffer C) (supernatant). An equal percentage of each sample was subjected to electrophoresis through an 8 percent SDS–polyacrylamide gel (SDS-PAGE) (*16*). (B) Gel filtration of urea-extracted proteins on Sepharose CL-4B. Urea-extracted proteins in buffer C were chromatographed at 4°C on a Sephrarose CL-4B column (0.9 by 57 cm) equilibrated with buffer C. Fractions (0.8 ml) were collected at a rate of 2.4 ml/hour. Proteins in each fraction were analyzed by SDS-PAGE. (C) Sepharose CL-4B elution profile of urea-extracted clathrin. The Coomassie blue–stained proteins displayed in (B) were quantified by densitometry (*25*). Blue-dextran 2000 (V_o), thyroglobulin, ferritin, catalase, aldolase, and cytochrome c (V_t) were used for column calibration. V_o, V_t, and the elution positions of thyroglobulin and aldolase are shown by the arrows. (D) Electron micrograph of rotary-shadowed clathrin triskelions in Sepharose CL-4B column fractions 24 to 27 (see Fig. 1B). Fractions were pooled, and the proteins were concentrated tenfold by ultrafiltration (*39*). Rotary shadowing and electron microscopy were performed as described in (*40*).

Again, preimmune serum was unreactive (lane 8). Identical one-dimensional peptide patterns were generated by partial proteolysis of the immune-precipitated protein and urea-extracted, gel-purified clathrin heavy chain (*17*). From the results of immunoblotting experiments with the immune serum, we estimate that clathrin heavy chain constitutes 0.1 percent of the total mass of yeast proteins. This quantity is similar to the amount of clathrin heavy chain measured in a variety of mammalian cells (*22*). Attempts to detect bovine brain clathrin heavy chain with the immune serum have been unsuccessful (*23*).

Isolation of a molecular clone of the clathrin heavy chain gene. We used the immune serum to screen a library of yeast DNA inserted into the expression vector λgt11 (*24*). From 300,000 phage, 16 were isolated which produced immunoreactive plaques. Restriction endonuclease and molecular hybridization analyses separated the isolates into nine nonoverlapping sets. DNA from only two insert classes annealed to a species of yeast polyadenylated RNA large enough (5.4 kilobases) to encode a 190-kD polypeptide (*17*). Lysates from cells infected with phage carrying these two insert types were affixed to nitrocellulose and tested for their ability to deplete antibodies to heavy chain from the immune serum. Only one class of phage (designated λ7) satisfied this criterion, implying that the protein produced from this yeast DNA insert displayed most, if not all, of the heavy chain antigenic determinants recognized by the immune serum antibodies. Finally, RNA homologous to the DNA insert was purified from a preparation of total yeast RNA by hybrid selection (*25*). Translation of the purified RNA (*25*) generated primarily a 190-kD polypeptide (Fig. 3, lane 1). This species comigrated with authentic clathrin heavy chain (Fig. 3, lane 2) obtained by immune-precipitation from translation products programmed by total yeast RNA. RNA encoding the 190-kD protein was selected by a plasmid vector carrying the yeast DNA but not by vector alone (Fig. 3, lanes 3 and 4). Antiserum specific to the clathrin heavy chain precipitated the 190-kD protein, whereas preimmune serum did not (Fig. 3, lanes 5 to 7). These observations offer convincing evidence that λ7 contains at least a fragment of the clathrin heavy chain gene (designated *CHC1* for *c*lathrin *h*eavy *c*hain). We have not further characterized the other eight insert classes.

A map of restriction endonuclease recognition sites in *CHC1* was prepared both by analyzing λ7 DNA and by using fragments of cloned DNA to probe restriction enzyme digests of genomic DNA (Fig. 4). Even under low stringency conditions of hybridization [30 percent formamide, 5× SSC (saline sodium citrate), 37°C; see legend to Fig. 4], only one copy of the *CHC1* sequence was detected in the yeast genome. The *CHC1* DNA carried by λ7 spanned the four boxed Eco RI sites shown in Fig. 4, commencing 300 base pairs (bp) upstream from the leftmost boxed Eco RI site and ending at, or just to the right of, the rightmost boxed Eco RI site. Hybridization of strand-specific probes (*26*) to polyadenylated RNA was used to demonstrate the direction of *CHC1* transcription shown in Fig. 4. Contrary to expectation, this result indicated that the *CHC1* sequence in λ7 was not oriented in the same transcriptional direction as *lacZ* (*27*). Hence, the immunoreactive polypeptide produced by the recombinant phage was not a *lacZ* fusion protein. A similar observation has been reported by Goto and Wang (*28*) who cloned the yeast topoisomerase I gene from this library. Boundaries for the *CHC1* tran-

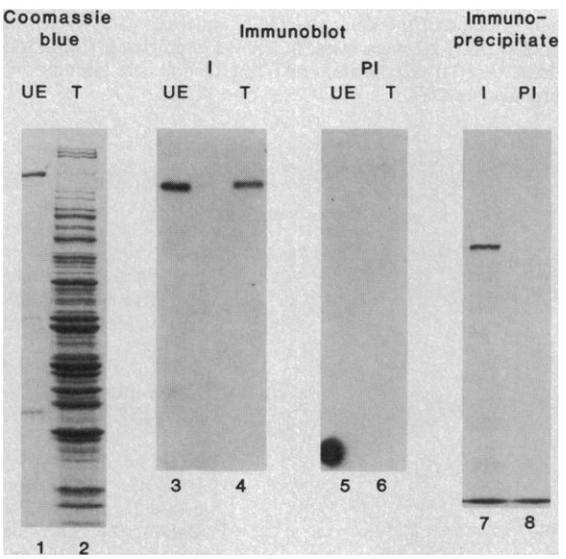

Fig. 2. Characterization of antiserum specific for clathrin heavy chain. (Coomassie blue panel) Urea-extracted coated vesicle proteins (UE, lane 1) or total yeast proteins (T, lane 2) prepared by glass bead lysis (*41*) of X2180-1A cells in 1 percent SDS were separated by SDS-PAGE and stained with Coomassie blue. The UE sample contained approximately 0.5 μg of clathrin heavy chain. The T lane represents proteins from 0.5 OD_{600} units of cells. (Immunoblot panels) UE sample (lanes 3 and 5), 0.05 μg of clathrin heavy chain, and T sample (lanes 4 and 6), same amount as Coomassie blue panel, were analyzed by immunoblotting (*21*). Filters were incubated with a 1/100 dilution of either immune (I, lanes 3 and 4) or preimmune (PI, lanes 5 and 6) serum. Bound antibody was decorated with ^{125}I-labeled *Staphylococcus aureus* protein A and visualized by autoradiography (*19*). (Immunoprecipitate panel) The $^{35}SO_4^{2-}$-labeled X2180-1A cell lysates (1 OD_{600} unit of cells) were incubated with 5 μl of immune (I, lane 7) or preimmune serum (PI, lane 8) (*41*). Immune complexes were harvested by addition of fixed *Staphylococcus aureus* cells (*42*) and precipitated proteins were analyzed by SDS-PAGE and autoradiography.

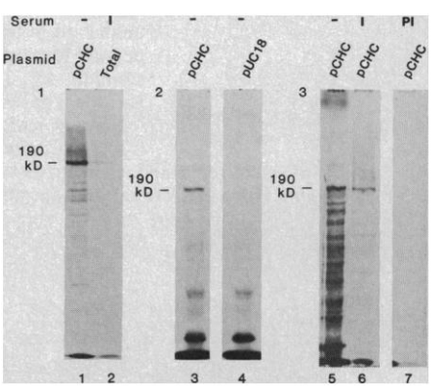

Fig. 3. Hybrid selection and in vitro translation of clathrin heavy chain. DNA from the λgt11 recombinant bacteriophage carrying the putative heavy chain gene (see text) was digested with Eco RI, and a 3.5-kbp fragment from the yeast DNA insert (see Fig. 4) was subcloned into pUC9 (*43*) to generate pCHC. A probe derived from the 3.5-kbp fragment anneals to a single 5.4-kb species of yeast polyadenylated RNA. pCHC and pUC18 [essentially identical to pUC9 except for a slightly longer "multiple cloning sites" sequence (*43*)] were separately adsorbed onto nitrocellulose and used to "hybrid-select" homologous RNA from total yeast RNA (*25*). (Panel 1, lane 1) ^{35}S-labeled proteins produced in a reticulocyte lysate translation system (*25*) programmed with RNA hybrid-selected by pCHC. (Lane 2) Clathrin heavy chain immune-precipitated (with 5 μl of immune serum) from products synthesized by in vitro translation of 10 μg of total yeast RNA. Proteins were analyzed by SDS-PAGE and detected by autoradiography. (Panel 2) RNA purified from total yeast RNA by hybridization to either pCHC (lane 3) or pUC18 (lane 4) was translated in vitro, and the products were separated by SDS-PAGE. (Panel 3) pCHC-selected RNA was translated in vitro then (lane 5) one-tenth of the products were analyzed directly by SDS-PAGE. The remainder was divided into two equal parts, and the proteins were precipitated by 5 μl of immune serum (I, lane 6) or by 5 μl of preimmune serum (PI, lane 7). Hybrid selection, in vitro translation, immune precipitation, and SDS-PAGE were performed as described (*25*) except that translation reactions were incubated for 2 hours at 30°C.

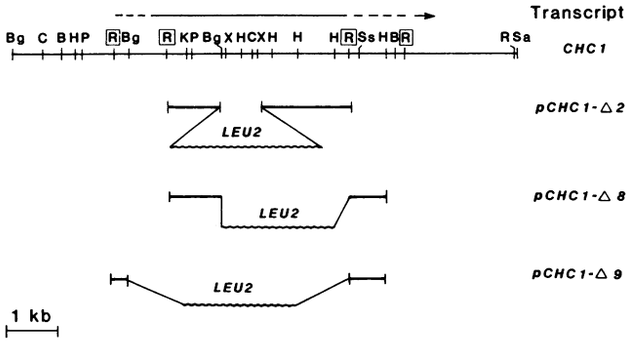

Fig. 4. Map of restriction endonuclease sites in *CHC1* DNA, position of the limits of *CHC1* transcription and perturbations of the cloned *CHC1* DNA used to disrupt *CHC1* in vivo. Abbreviations: B, Bam HI; Bg, Bgl II; C, Cla I; H, Hind III; K, Kpn I; P, Pst I; R, Eco RI; Sa, Sal I; Ss, Sst I; X, Xba I. Restriction endonuclease sites in *CHC1* DNA were positioned by analyzing cloned DNA and by Southern analysis (*44*) with the use of probes derived from cloned DNA. Three disruptions of *CHC1* have been constructed in vitro by standard manipulations (*44*). The solid lines represent regions of *CHC1* present in each plasmid. The gap in the solid lines is placed beneath segments of *CHC1* DNA removed from each construction. In each case, DNA encoding *LEU2* isolated from vector YEp13 (*45*) was inserted in place of the deleted *CHC1* sequences. The vector in each case was pUC9. Two versions of pCHC1-Δ2 were built: pCHC1-Δ2a carries *LEU2* in the same transcriptional direction as *CHC*1. pCHC1-Δ2b harbors *LEU2* in the opposite orientation. *LEU2* is transcribed in the same direction as *CHC1* in pCHC1-Δ8 and pCHC1-Δ9.

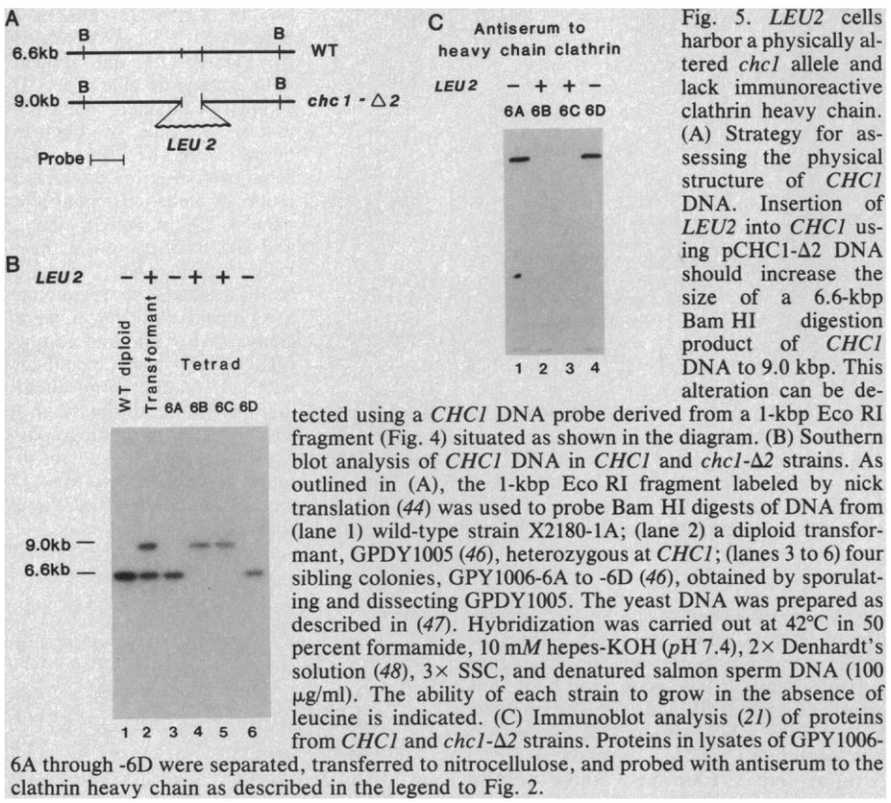

Fig. 5. *LEU2* cells harbor a physically altered *chc1* allele and lack immunoreactive clathrin heavy chain. (A) Strategy for assessing the physical structure of *CHC1* DNA. Insertion of *LEU2* into *CHC1* using pCHC1-Δ2 DNA should increase the size of a 6.6-kbp Bam HI digestion product of *CHC1* DNA to 9.0 kbp. This alteration can be detected using a *CHC1* DNA probe derived from a 1-kbp Eco RI fragment (Fig. 4) situated as shown in the diagram. (B) Southern blot analysis of *CHC1* DNA in *CHC1* and *chc1*-Δ2 strains. As outlined in (A), the 1-kbp Eco RI fragment labeled by nick translation (*44*) was used to probe Bam HI digests of DNA from (lane 1) wild-type strain X2180-1A; (lane 2) a diploid transformant, GPDY1005 (*46*), heterozygous at *CHC1*; (lanes 3 to 6) four sibling colonies, GPY1006-6A to -6D (*46*), obtained by sporulating and dissecting GPDY1005. The yeast DNA was prepared as described in (*47*). Hybridization was carried out at 42°C in 50 percent formamide, 10 m*M* hepes-KOH (*p*H 7.4), 2× Denhardt's solution (*48*), 3× SSC, and denatured salmon sperm DNA (100 μg/ml). The ability of each strain to grow in the absence of leucine is indicated. (C) Immunoblot analysis (*21*) of proteins from *CHC1* and *chc1*-Δ2 strains. Proteins in lysates of GPY1006-6A through -6D were separated, transferred to nitrocellulose, and probed with antiserum to the clathrin heavy chain as described in the legend to Fig. 2.

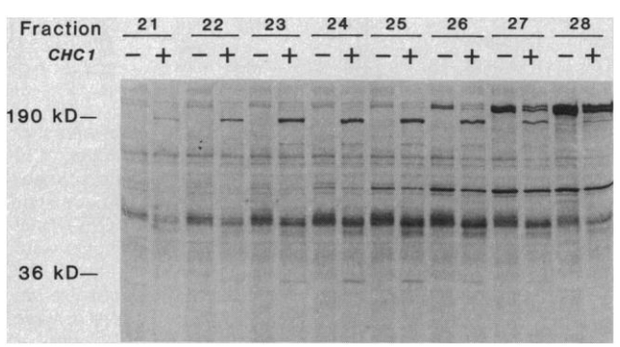

Fig. 6. Protein profiles of vesicles from *CHC1* and *chc1*-Δ2 strains. Vesicles were prepared by Sephacryl S-1000 column chromatography of membranes from *CHC1* strain GPY1006-6D and *chc1*-Δ2 strain GPY1006-6B (legend to Fig. 1). Vesicle proteins from column fractions (*chc1* and *CHC1*) were displayed in alternating lanes on an 8 percent SDS–polyacrylamide gel and stained with Coomassie blue.

script were roughly established by testing segments of cloned DNA for the ability to anneal to the 5.4 kilobase *CHC1* messenger RNA. Northern blot analysis revealed that a fragment extending 300-bp upstream from the leftmost Eco RI site boxed in Fig. 4 did not anneal to polyadenylated RNA. A 3.5-kilobase pair (kbp) and two 1-kbp DNA fragments produced by cleavage at the boxed Eco RI sites in Fig. 4 hybridized to a single species of 5.4-kb polyadenylated RNA. Coding sequences were identified by inserting these three DNA segments into a series of expression vectors designed to append peptides to the carboxyl terminus of the *Escherichia coli* trpE protein (*29*). Vectors carrying the central and downstream fragments encoded fusion proteins that were recognized by antiserum to heavy chain. The molecular weights of the two fusion proteins suggested that *CHC1* coding information proceeds uninterrupted through sequences defined by the three downstream, boxed Eco RI sites in Fig. 4. A fusion protein was not generated by insertion of the upstream 1-kbp Eco RI fragment into the expression vectors. Thus, the leftmost Eco RI site in Fig. 4 does not lie within *CHC1* coding sequences. Together, these experiments position the start of *CHC1* within the 1-kbp of DNA demarcated by the leftmost two Eco RI recognition sites (Fig. 4, above the *CHC1* map).

***CHC1* gene disruptions.** Our approach to disrupting the *CHC1* gene in vivo was influenced by studies of temperature-sensitive conditionally lethal *sec* mutations which block secretion in yeast (*30*). Analyses of *sec* mutant strains demonstrate that the secretory process is required for cell growth. The expectation that clathrin would prove necessary for intracellular transport implied that a *chc1* mutation would be lethal. Consequently, we arranged to disrupt only one of the two homologous *CHC1* alleles in a diploid cell. In order to mutate *CHC1* by a single-step gene transplacement, a 600-bp Bgl II–Xba I fragment located no more than 1.5-kb from the start of *CHC1* was replaced by DNA encoding *LEU2* (labeled *chc1*-Δ2 in Fig. 4). Linear DNA spanning the *chc1*-Δ2 disruption (*31*) was introduced into diploid *leu2* cells (*32*). Leu$^+$ transformants were isolated and sporulated, and the resulting asci were dissected into individual spores. Surprisingly, most diploid cells gave rise to four viable spores (Table 1). Each complete tetrad, however, consisted of two wild-type–sized colonies and two small colonies. Characterization of complete tetrads indicated that large colonies were

composed of *Leu*$^-$ cells and that small colonies were formed by *Leu*$^+$ cells (Table 1), providing genetic evidence that the slow-growing cells had suffered the *chc1-Δ2* disruption. Analysis of the physical structure of the heavy chain gene in sibling spores from two tetrads confirmed this interpretation. The strategy for this analysis and an example of the results obtained from one tetrad are in Fig. 5, A and B. A 6.6-kbp Bam HI fragment in *CHC1* DNA is expanded to 9.0-kbp in *chc1-Δ2* DNA (Fig. 5A). To expose this difference, the Eco RI DNA fragment indicated in Fig. 5A was used to probe Bam HI cleaved DNA from a wild-type strain (Fig. 5B, lane 1), a diploid transformant heterozygous at the *CHC1* locus (Fig. 5B, lane 2), and four members of a tetrad derived from that transformant (Fig. 5B, lanes 3 to 6). The observed size (or sizes) of the Bam HI digestion products conformed in every case to the structure predicted from genetic characterizations.

***chc1* cells fail to express clathrin heavy chain.** Deletion of a portion of *CHC1* and replacement with *LEU2* was expected to eliminate expression of clathrin heavy chain. To test this prediction, we searched cell extracts of the sibling strains (described in Fig. 5B) for proteins capable of reacting with antiserum to heavy chain. In contrast to *CHC1* strains (Fig. 5C, lanes 1 and 4), *chc1-Δ2* strains showed no immunoreactive clathrin heavy chain or heavy chain peptide fragments (Fig. 5C, lanes 2 and 3).

Two additional experiments support the argument that the *chc1-Δ2* mutation abolished production of clathrin heavy chain. First, protein profiles of wild-type and *chc1-Δ2* vesicles fractionated by Sephacryl S-1000 column chromatography were compared. Whereas clathrin subunits were clearly enriched in *CHC1* vesicle fractions 22 to 27, *chc1-Δ2* vesicles were devoid of both the heavy and light chains (Fig. 6). Immunoblotting analysis of the *chc1* vesicle fractions confirmed the absence of heavy chain (*17*). The lack of clathrin light chains in *chc1-Δ2* vesicle fractions was expected since light chains probably associate with vesicles by binding to heavy chain (*33*). At least four other proteins (205 kD, 135 kD, 50 kD, and 48 kD) are present only in *CHC1* vesicles. These species could represent constituents of coated vesicles. Second, two other gene disruptions have been constructed (Fig. 4). The more extensive alteration, *chc1-Δ9*, substituted 4.5 kb of *CHC1* DNA with *LEU2* DNA. We estimate that this deletion removed at least 70 percent of the *CHC1* gene. Cells carrying either of the two more extensive deletions exhibited the same growth properties as *chc1-Δ2* mutant cells.

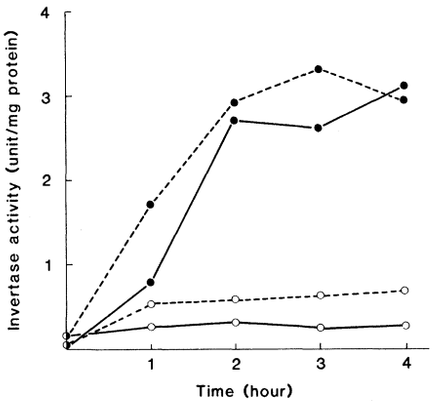

Fig. 7. Invertase secretion by *CHC1* and *chc1-Δ8* strains. GPY1100 (*CHC1*) and GPY1101 (*chc1-Δ8*) are isogenic except at *CHC1*. Cells from each strain were grown overnight at 30°C to early log phase (OD_{600} = 1 to 2) in YP medium (1 percent yeast extract, 2 percent peptone) plus 2 percent glucose. Cells were then shifted to YP medium plus 0.1 percent glucose. At the indicated times samples were harvested and internal (open circles) and external (closed circles) invertase activity was assayed (*42*). The solid lines represent invertase activity in GPY1100, and the dotted lines represent activity in GPY1101.

***chc1* cells secrete invertase.** Since cell surface growth and secretion are coupled (*30*), the growth of *chc1* cells implies that newly synthesized cell surface components are exported. Secretion was examined directly by monitoring the transport of newly synthesized invertase. A single gene, *SUC2*, encodes two forms of invertase; the cytoplasmic form is expressed constitutively while synthesis of the external form is derepressed by low concentrations of glucose (*34*). Passage of newly synthesized external invertase through the secretory pathway to the cell surface has been well documented (*30*). Secretion of invertase from wild-type and *chc1* cells was assessed by measuring the internal and external invertase enzyme activity after derepression. Newly synthesized invertase reached the cell surface at similar rates in *CHC1* and *chc1* strains (Fig. 7). Therefore, a clathrin heavy chain deficiency does not dramatically deter passage of invertase through the secretory pathway. Mutant cells did, however, accumulate some internal invertase (Fig. 7). A slight delay during secretion could cause this retention without noticeably affecting the external appearance of invertase. Possibly, this delay accounts for the slow growth of *chc1* cells, which have a generation time two- to threefold longer than the *CHC1* parent. On the other hand, misrouting of a fraction of the invertase to a stable intracellular compartment could also produce the internal pool of enzyme.

Reexamining the function of clathrin. The construction and characterization of yeast strains deficient in clathrin heavy chain has revealed that this protein plays an important but not essential role in cell growth. Our results challenge the model that postulates coupling between the formation of clathrin-coated vesicles and protein secretion. A protein capable of functionally substituting for clathrin heavy chain would reconcile our data with this model. However, sequences related to *CHC1* were not detected under reduced stringency DNA hybridization conditions, and polyclonal antiserum to the heavy chain failed to detect another protein in *chc1* extracts. Hence a putative substitute could display only limited primary sequence homology.

Evidence has emerged which suggests that other proteins may comprise vesicle coats. When cultured epithelial cells are incubated at 20°C, the secretory process is blocked within the Golgi apparatus. Golgi-like organelles with cytoplasmic coats are observed under these conditions, but the coat proteins fail to react with anticlathrin antibody (*35*). This and other work has shown that authentic clathrin-coating areas on the *trans* gene of the Golgi body are devoid of glycoproteins that are in transit to the plasma membrane (*36*). At present, there is no evidence in yeast for an alternative vesicle coat protein.

As mentioned above, studies of endocytosis present the most compelling support for the involvement of clathrin in membrane vesiculation. It is conceivable that exocytosis occurs in the absence of clathrin heavy chain, but endocytosis is eliminated. Endocytosis in yeast has been described by Riezman (*37*) who measured fluid-phase uptake using a fluorescent, membrane-impermeant dye which collects in the vacuole. By applying this assay to *chc1* cells, it should be possible to determine the requirement for clathrin during endocytosis.

Our results are equally compatible with the possibility that clathrin does not actively promote membrane vesiculation. The clathrin coat has been proposed to act as a molecular sorting agent which collects and concentrates molecules destined for transport while excluding others (*2, 4*). If this view is valid, proteins that normally reside in coated membranes will, in clathrin-deficient cells, be scrambled among the membranes that communicate by intracellular transport. On the other hand, clathrin

may function during events temporally and spatially distinct from transport vesicle biogenesis. For instance, the clathrin coat prevents calcium-mediated fusion of coated vesicles to lysosomes in vitro (*38*). In this capacity clathrin may act to shield vesicles from nonspecific fusion with other intracellular membranes encountered during transit to targeted destinations. Clathrin coats could also prevent fusion of distinct, stable organelles, such as the Golgi body, to other membrane compartments. The ability to assess the rate and fidelity of secreted, plasma membrane, and vacuolar protein transport (*30*) should provide tests of these hypotheses.

In conclusion, our experiments offer a foundation for further genetic and biochemical characterizations of clathrin-deficient mutants. Such studies may elucidate the precise role of clathrin coats during intracellular protein transport.

References and Notes

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31. Prior to transformation, pCHC1-Δ2 was cleaved by Pvu II. This restriction enzyme releases a fragment which includes the entire *chc1*::*LEU2* insert and extends 90 bp and 232 bp into pUC9 sequences at either end. pCHC1-Δ8 was cut by Hind III and Pst I before transformation. This produces a *chc1*::*LEU2* DNA fragment with *CHC1* DNA at the ends. Hind III and Pvu II digestion of pCHC1-Δ9 generates a *chc1*::*LEU2* DNA fragment with 90 bp of pUC9 at one end and *CHC1* DNA at the other end.
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    GPDY1004: *MATa/MATα leu2-3/leu2-3 leu2-112/leu2-112 ura3-52/ura3-52 his4/his4 trp1-289/TRP1 can1/CAN1 mnn4-1/MNN4 pep4-3/PEP4*.
    GPDY1005: Same as GPDY1004 plus *chc1-Δ2a*::*LEU2/CHC1*.
    GPDY1006: Same as GPDY1004 plus *chc1-Δ2b*::*LEU2/CHC1*.
    GPDY1007: *MATa/MATα leu2-3/leu2-3 leu2-112/leu2-112 ura3-52/ura3-52 ade6/ADE6 his4-519/HIS4 trp1-289/TRP1 can1/CAN1*.
    GPDY1008: Same as GPDY1007 plus *chc1-Δ8*::*LEU2*.
    GPY1006-6A: MAT*a leu2-3 leu2-112 ura3-52 his4* (*MNN4* not scored).
    GPY1006-6B: MATα *leu2-3 leu2-112 ura3-52 his4 trp1-289 pep4-3 chc1-Δ2b*::*LEU2* (*MNN4* not scored).
    GPY1006-6C: *MATa leu2-3 leu2-112 ura3-52 his4 chc1-Δ2b*::*LEU2* (*MNN4* not scored).
    GPY1006-6D: *MATα leu2-3 leu2-112 ura3-52 his4 trp1-289 can1 pep4-3* (*MNN4* not scored).
    GPY1100: *MATa leu2-3 leu2-112 ura3-52 his4-519 trp1-289 can1* (alias JRY19 from J. Rine).
    GPY1101: GPY1100 plus *chc1-Δ8*::*LEU2*.
47. Cells were grown to OD_{600} 2 to 8 in yeast-peptone medium plus 2 percent glucose. Cells (20 OD_{600} units) were converted to spheroplasts (*42*). Spheroplasts were sedimented and then lysed in 0.5 ml of 50 m*M* EDTA, *p*H 8.1, containing 0.2 percent SDS. Lysates were heated at 70°C for 15 minutes, and potassium acetate (5*M* stock solution) was added to 0.5*M*. Samples were placed on ice for 30 minutes. The precipitates were centrifuged (12,000*g*, 15 minutes, 4°C), the supernatants were removed to fresh tubes, and the DNA was precipitated by addition of two volumes of ethanol. Precipitates were collected by centrifugation for 15 seconds at 12,000*g*. The pellets were resuspended in 200 μl of RNase A (10 μg/ml) and incubated for 15 minutes at 37°C. The samples were then extracted three times with phenol and chloroform (1:1). DNA was precipitated with ethanol, sedimented, and then dissolved in TE. Approximately one-fourth of each sample was used for the analysis.
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