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Topical Review

Genetic Analysis of Clathrin Function in Yeast

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Introduction

Eukaryotic cells are organized into functionally and structurally discrete, membrane-bounded organelles. Since each organelle contains a unique set of protein components, this elaborate organization poses fundamental traffic problems: how are proteins accurately directed to distinct compartments and, once in residence, how are they retained? The extent of the traffic problems can be appreciated by considering protein transport to and from the cell surface. Most newly synthesized proteins are transported from the cytoplasm to the cell surface via the organelles of the secretory pathway (*reviewed in* Palade, 1975; Schekman, 1985; Pfeffer & Rothman, 1987). These proteins gain access to the secretory pathway by translocation into the endoplasmic reticulum (ER). In the ER, proteins with two basic itinernaries must be distinguished, those which will take up permanent residence in the ER, and those bound for destinations beyond the ER. The proteins that exit the ER in transport vesicles and are delivered to the Golgi complex are confronted by more complex routes. Some will remain in the Golgi complex, some will be diverted from the secretory pathway and directed to hydrolytic organelles (lysosomes in mammalian cells and vacuoles in yeast), and another set will be packaged into secretory vesicles and shuttled to the cell surface. In some endocrine and neuroendocrine cell types, the route from the Golgi complex to the cell surface also branches into constitutive and regulated pathways (*reviewed in* Burgess & Kelly, 1987). An analogous system of protein sorting and inter-organelle vesicular traffic exists in the endocytic pathway (*reviewed in* Goldstein et al., 1985; Kornfeld & Mellman, 1989). Plasma membrane receptors for some extracellular ligands are internalized by specific incorporation into endocytic vesicles at the exclusion of other resident plasma membrane proteins. Vesicular transport delivers the receptors to a network of endosomal compartments where the receptors can be directed to lysosomes, the Golgi complex or back to the plasma membrane. Thus, both the exocytic and endocytic pathways exhibit multiple stages where proteins are sorted and distributed by vesicular transport to distinct compartments and where resident proteins are distinguished from proteins in transit to later stages.

Clathrin-coated membranes and vesicles have been implicated as part of the machinery responsible for mediating the traffic flow in the exocytic and endocytic pathways. The study of clathrin-coated membranes and their role in selective intercompartmental protein transport was launched by Roth and Porter (1964) who noted that adsorptive pinocytosis of yolk protein into mosquito oocytes involved plasma membrane pits and small vesicles that displayed a cytoplasmic bristle coat. Pearse (1975, 1976) purified the bristle-coated vesicles from a variety of cell types, characterized the structure and protein composition of the coat, and named the predominant coat protein *clathrin*. Numerous biochemical and microscopy studies have subsequently characterized the coat components in more detail and sought to determine the role of clathrin coats in various intracellular protein transport pathways. These approaches have been the subject of excellent reviews published elsewhere (Pearse & Bretscher, 1981; Goldstein et al., 1985; Pearse & Crowther, 1987; Brodsky, 1988). The purpose of this article is to review recent genetic approaches designed to examine the function of clathrin in the yeast *Saccharomyces cerevisiae*.

Key Words clathrin · genetics · *Saccharomyces cerevisiae* · exocytosis · endocytosis · prohormone maturation

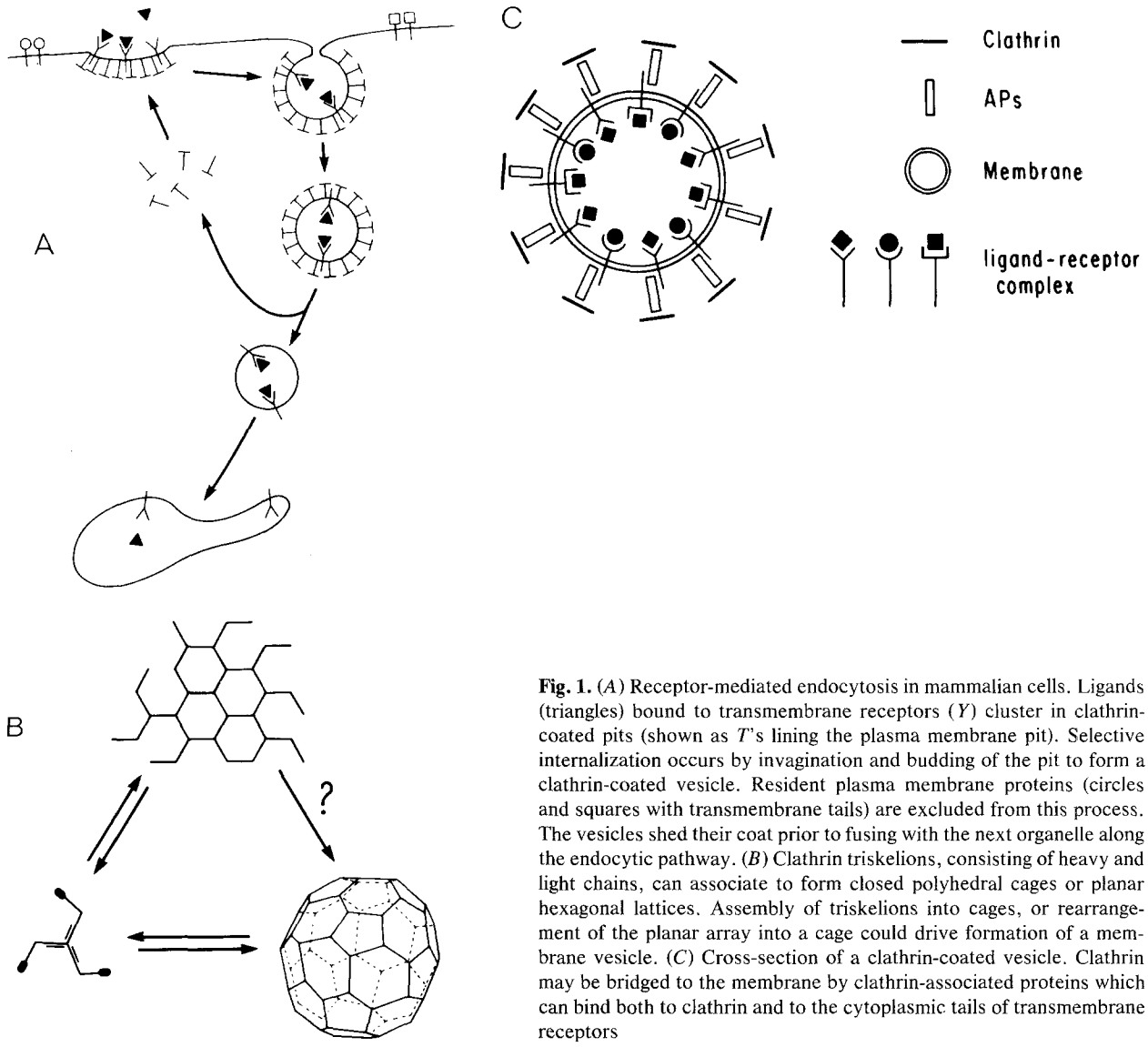

Fig. 1. (*A*) Receptor-mediated endocytosis in mammalian cells. Ligands (triangles) bound to transmembrane receptors (*Y*) cluster in clathrin-coated pits (shown as *T*'s lining the plasma membrane pit). Selective internalization occurs by invagination and budding of the pit to form a clathrin-coated vesicle. Resident plasma membrane proteins (circles and squares with transmembrane tails) are excluded from this process. The vesicles shed their coat prior to fusing with the next organelle along the endocytic pathway. (*B*) Clathrin triskelions, consisting of heavy and light chains, can associate to form closed polyhedral cages or planar hexagonal lattices. Assembly of triskelions into cages, or rearrangement of the planar array into a cage could drive formation of a membrane vesicle. (*C*) Cross-section of a clathrin-coated vesicle. Clathrin may be bridged to the membrane by clathrin-associated proteins which can bind both to clathrin and to the cytoplasmic tails of transmembrane receptors

Clathrin is Associated with Endocytosis and Exocytosis in Mammalian Cells

Receptor-mediated internalization of extracellular ligands by mammalian cells has received much attention as an example of selective intercompartmental protein transfer and has served as a paradigm for secretory pathway protein sorting and transport. Figure 1 presents a current picture of the structural features of clathrin coats and the involvement of the coats in receptor-mediated endocytosis. Uptake of an extracellular ligand initiates when the ligand binds to a receptor on the plasma membrane (Fig. 1*A*). The receptors are either clustered in, or migrate upon binding to, clathrin-coated pits. The pits invaginate and then fuse at the neck to yield clathrin-coated vesicles bearing the receptor-bound ligand. Prior to fusing with the next compartment along the endocytic pathway, the coat is disassembled, perhaps by an ATP-dependent mechanism. The soluble clathrin is then free to reassemble into coated pits at the plasma membrane. Clathrin can undergo a similar cycle of assembly and disassembly in vitro (Fig. 1*B*). The structural unit of assembly is a triskelion formed from three molecules of clathrin heavy chain (about 180 kD) noncovalently joined at their carboxy-termini, and three molecules of noncovalently bound clathrin light chains (30–40 kD). Associated triskelions can either form planar hexagonal arrays or polyhedral cages. In clathrin-coated vesicles, protein complexes, each with members of 100–110, 50 and 15–20 kD, apparently

lie between the clathrin lattice and the membrane (Fig. 1*C*). These complexes (referred to here as clathrin-associated proteins or APs) augment assembly of triskelions into cages in vitro. Recent experiments from Pearse's group (Pearse, 1988; Glickman, Conibar & Pearse, 1989) suggest that APs recognize the cytoplasmic tails of transmembrane receptors, and it has been proposed that APs mediate the assembly of clathrin coats onto membranes in vivo by bridging triskelions to the cytoplasmic domains of transmembrane proteins.

Taken together, the in vivo and in vitro properties of clathrin have led to the proposal that polymerization of clathrin triskelions into polyhedral cages, either directly or by rearrangement of hexagonal arrays (Fig. 1*B*), provides the driving force to generate a transport vesicle from a coated pit (Pearse & Bretscher, 1981; Harrison & Kirchhausen, 1983). Also, it has been suggested that interactions between clathrin coats and receptors carrying ligands could confer specificity to endocytosis by collecting the cargo at membrane sites undergoing vesiculation (Pearse & Bretscher, 1981; Pastan & Willingham, 1983; Goldstein et al., 1985). In this view, clathrin plays a pivotal role in the biogenesis and selectivity of endocytic vesicles. Clathrin coats have also been observed by electron microscopy on Golgi membranes, vesicles containing lysosomal enzymes and their receptors and, in endocrine cells, nascent secretory granules (*reviewed in* Griffiths & Simons, 1986; von Figura & Hasilik, 1986; Burgess & Kelly, 1987; Brodsky, 1988). Biochemical evidence supports participation of clathrin in protein transport from the ER to the Golgi complex, through the Golgi complex or from the Golgi complex to the plasma membrane, and from the Golgi complex to lysosomes (*reviewed in* Brodsky, 1988). Thus, the paradigm of clathrin's role in selective protein transport during endocytosis can be extended to stages of the exocytic process.

Clathrin Heavy Chain-Deficient Yeast Mutants are Viable

Tests of clathrin function in vivo in mammalian cells have proven difficult. For example, attempts to inhibit clathrin function by introduction of anti-clathrin antibodies into living cells have yielded different results. Wehland et al. (1981, 1982) observed no effects of anti-clathrin antibodies on either endocytosis or exocytosis. Doxsey et al. (1987) noted a 50% inhibition of endocytosis but no change in exocytosis after introduction of antibodies against clathrin heavy chain. The apparent discrepancy could reflect the differences in antibodies, cells, techniques to introduce the antibodies or assays for endocytosis that were used by each group.

An alternative strategy for characterization of clathrin function in vivo is to identify cells harboring mutations that prevent formation of clathrin coats. Analyses of intracellular transport pathways in mutant cells can then reveal stages of transport affected by the absence of clathrin coats. Such perturbations provide evidence for the functional involvement of clathrin in the affected step in vivo. *Saccharomyces cerevisiae* is particularly well-suited for this type of genetic physiology. Classical and molecular genetic techniques can be used to isolate mutations in unidentified genes or in genes coding for known proteins (Botstein & Fink, 1988). Furthermore, *S. cerevisiae* cells exhibit most of the attributes associated with secretion and membrane organelle assembly in mammalian cells (Schekman, 1985). However, early electron microscope attempts to visualize coats along the cytoplasmic surfaces of membranes in thin sections of *S. cerevisiae* cells were hampered by the granular appearance of the cytoplasm. Mueller and Branton (1984) circumvented this problem by resolving coated vesicles from a yeast membrane fraction by gel filtration chromatography. As with bovine brain coated vesicles, the coated vesicles from yeast disassembled into naked vesicles and triskelions when examined by rotary shadowing. A protein of approximately 190 kD cofractionated by gel filtration with the coated vesicles, leading Mueller and Branton to suggest that this protein was clathrin heavy chain. Proteins with molecular weights similar to mammalian APs also coeluted with the coated vesicles. Further analyses (Payne & Schekman, 1985; Lemmon et al., 1988) demonstrated that triskelions can be extracted and purified from yeast coated vesicles using procedures previously applied to mammalian clathrin-coated vesicles. Yeast triskelions contain 190-kD heavy chain and 36-kD light chain subunits (Payne & Schekman, 1985; Lemmon et al., 1988), and, like their mammalian counterparts, the triskelions spontaneously reassemble into empty cages (Lemmon et al., 1988). The 190-kD protein constitutes about 0.1% of the total protein mass in a yeast cell, a level commensurate with the abundance of clathrin heavy chain in mammalian cells (Payne & Schekman, 1985). Polyclonal antiserum specific for yeast clathin heavy chain was used to isolate the structural gene (*CHC1*) from a library of yeast DNA carried by the expression vector λgt11 (Payne & Schekman, 1985). Subsequent isolation of the rat clathrin heavy chain gene (Kirchhausen et al., 1987) provided a basis for sequence comparison that indicated 50% amino acid identity between the yeast and rat proteins (S.K. Lemmon, *personal commun-*

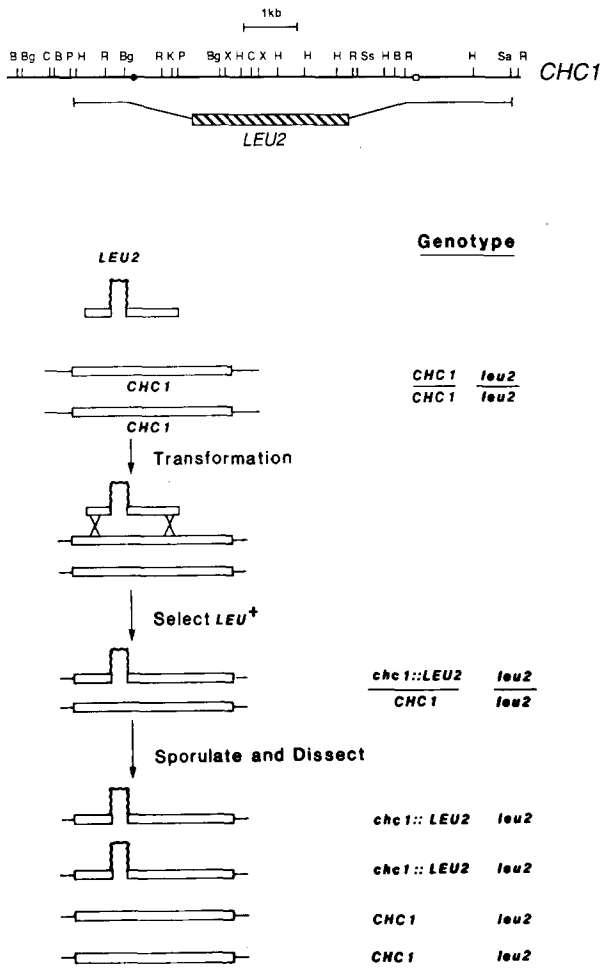

Fig. 2. Strategy for disruption of *CHC1* in yeast. The top part of the figure diagrams the structure of the *CHC1* gene and one gene disruption constructed in vitro. The likely translation initiation site (filled circle) and termination site (open square) for *CHC1* is shown. *B*, *Bam*H1; *Bg*, *Bgl*II; *C*, *Cla*I; *H*, *Hind*III; *K*, *Kpn*I; *P*, *Pst*I; *R*, *Eco*RI; *S*, *Sal*I; *Ss*, *Sst*I; *X*, *Xba*I. The bottom part illustrates the strategy for single-step gene disruption in diploid cells. The structure of the chromosomal *CHC1* genes and the introduced disruption is on the left and the genotype of the cells is on the right.

ication). Together, these observations provide clear evidence for the existence of clathrin in yeast.

Elimination of the *CHC1* gene in yeast allowed a genetic test of clathrin function in vivo. The strategy for disrupting *CHC1* is described below and outlined in Fig. 2. Exogenous DNA, when introduced into *S. cerevisiae* cells, efficiently and stably integrates into chromosomal DNA by homologous recombination (Rothstein, 1983). Given this propensity for homologous recombination, a cloned gene can be engineered in vitro so that integration results in the replacement of the wild-type gene with a nonfunctional copy. Typically, the nonfunctional allele is generated by replacing part, or all, of the wild-type coding sequences with a selectable yeast gene (Fig. 2) (*reviewed in* Bostein & Fink, 1988). This allows easy identification of cells that acquire the disrupted gene. The strategy adopted for *CHC1* gene disruption (Fig. 2) was based on the following logic. Clathrin-coated membranes were proposed to generate transport vesicles in the secretory process of mammalian cells (Pearse & Bretscher, 1981). In yeast, studies of temperature-sensitive, conditionally lethal mutations that block the secretory pathway (*sec* mutations) indicated that secretion is required for cell growth (*reviewed in* Schekman, 1985). Thus, the elimination of clathrin heavy chain might prevent formation of transport vesicles which, in turn, would block secretion and cell growth. In order to circumvent potential lethal effects, integration of the mutant clathrin heavy chain was carried out in diploid cells to generate strains heterozygous for the gene disruption. Heterozygous diploids were induced to undergo meiosis, resulting in four haploid spores, two of which carried the mutant allele (and the selectable marker) and two of which carried the wild-type allele. Growth properties of cells derived from the spores were assesed by placing individual members of each four-spore set at defined positions on solid medium. A set of four spores represents a tetrad. An example of the tetrads is shown in Fig. 3*A*. Each tetrad consisted of two segregants that grew slowly, producing small colonies, and two that grew at wild-type rates. The cells in the small colonies carried the *LEU2* gene and, by inference, the clathrin heavy chain gene disruption. Further analysis of the slow-growing cells showed that they harbored the mutant allele, did not express clathrin heavy chain and apparently did not assemble clathrin coats (Payne & Schekman, 1985). It was concluded from these results that, contrary to expectation, clathrin heavy chain is important but not essential for cell growth.

The growth of clathrin-deficient cells implies that the secretory pathway can function in the absence of clathrin-coated membranes. This was directly demonstrated by monitoring the export of invertase, which is a periplasmic glycoprotein (Payne et al., 1987). In order to eliminate potential genetic variability at loci other than *CHC1*, haploid strains were used as recipients of the *chc1* disruption. Following gene disruption, the only genetic difference between the recipient strain and the derivative *chc1* cells should be at the *CHC1* locus. Any phenotypic differences between such congenic strains can therefore be attributed to the single variable, the presence or absence of clathrin heavy chain. Invertase export in sets of congenic strains was followed by assessing secretory stage-specific carbohydrate

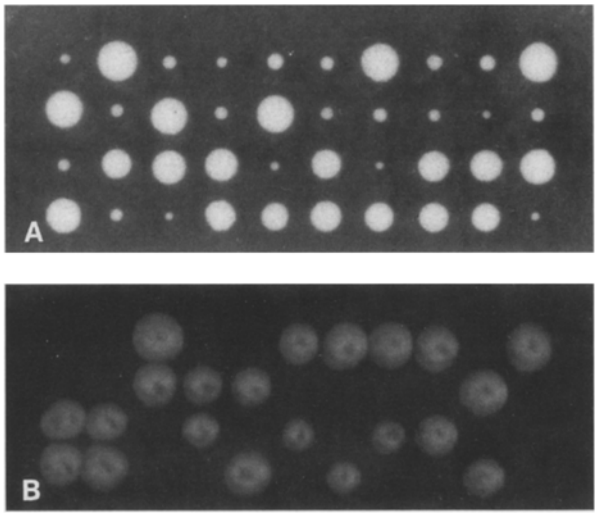

Fig. 3. (*A*) Tetrads from a diploid heterozygous for a disruption of the *CHC1* gene and homozygous for SCD^V. The *chc1* SCD^V cells are viable but form small colonies. (Reproduced from Payne et al., 1987, with permission of American Society for Microbiology.) (*B*) Tetrads from a diploid heterozygous for a *CHC1* disruption and homozygous for SCD^L. The *chc1* SCD^L cells are inviable and do not form colonies

modifications and by measuring the appearance of the glycoprotein at the cell surface. Pulse-chase experiments indicated that invertase transport from the ER to the Golgi complex occurred at identical rates in each strain. Mutant cells displayed a two-fold slower rate of invertase transfer from the Golgi apparatus to the periplasm when compared to wild-type partners. Similar results were obtained when export of the mating pheromone α-factor was analyzed (Payne & Schekman, 1989). The viability of clathrin-deficient cells and their ability to secrete invertase and α-factor indicates that clathrin is not required for the formation of transport vesicles which move cell surface and secreted proteins through the secretory pathway in yeast.

Can the conclusions concerning clathrin's role in the yeast secretory pathway be extended to mammalian cells? Morphological in situ analyses and characterization of requirements for in vitro transport through the Golgi complex indicate that viral membrane glycoprotein transport through the mammalian secretory pathways does not involve clathrin-coated membranes (Wehland et al., 1982; Griffiths et al., 1985; Orci, Glick & Rothman, 1986; Malhotra et al., 1989). On the other hand, it has been shown that purified clathrin-coated vesicles can contain newly-made secretory proteins (Kinnon & Owen, 1983; Benson et al., 1985; Fishman & Fine, 1987). However, since the clathrin-coated vesicles carry only a very small amount of the total cell-associated secretory proteins, the possibility cannot be discounted that secretory proteins present in the Golgi complex at the time of cell disruption are nonspecifically trapped within Golgi-derived clathrin-coated structures which are released by homogenization. Taken together, the experiments in yeast and mammalian cells argue that, although some percentage of newly-synthesized secretory proteins may travel in clathrin-coated vesicles, bulk transfer of proteins between secretory pathway organelles is not mediated by clathrin-coated vesicles (Rothman, 1986; Brodsky, 1988).

Additional Genes Can Affect the Viability of Clathrin-Deficient Yeast

Lemmon and Jones (1987) also disrupted *CHC1* in diploid yeast and conducted tetrad analysis of the resulting heterozygous diploid cells. Whereas Payne and Schekman (1985) found that most *chc1* segregants were viable, Lemmon and Jones (1987) observed that only about half of the *chc1* segregants from their diploid were viable. This ratio of viable to inviable *chc1* segregants suggested that a second gene (not *chc1*) was capable of influencing the viability of *chc1* cells, and it was present in the original diploid as two alleles, one which allowed *chc1* cell growth and one which did not. Further genetic analysis established that a second gene could influence the viability of *chc1* cells; this gene was designated *SCD1* (*s*uppressor of *c*lathrin *d*eficiency). Because the dominant/recessive relationship between the viable and lethal versions of the gene has not been determined they will be referred to here as $SCD1^V$ (viable allele) and $SCD1^L$ (lethal allele). No effect of either allele of *SCD1* on the growth of *CHC1* cells has been reported. An example of the growth properties of segregants from a diploid homozygous for $SCD1^L$ and heterozygous for a gene disruption of *CHC1* is shown in Fig. 3*B*. The two *CHC1* $SCD1^L$ segregants grow normally while the two *chc1* SCD^L segregants fail to produce colonies.

Some confusion arose from the use of the term suppressor for *SCD1* (Schekman & Payne, 1988; Lemmon & Jones, 1988). $SCD1^V$ does not arise *de novo* as a "suppressor" mutation in SCD^L when *CHC1* is disrupted in either haploid or diploid cells (Payne et al., 1987; Lemmon & Jones, 1987). Rather, *SCD1* preexists as either a lethal or viable allele in laboratory strains of yeast. A limited survey of over 20 strains from different laboratories has failed to uncover a strain with $SCD1^L$ (Payne et al., 1987; G. Payne, *unpublished results*) suggesting that the lethal allele is rare. The most simple inter-

pretation of the results with *SCD1* is that a mutation of $SCD1^V$ has produced $SCD1^L$, which is lethal in the presence of *chc1* but has no obvious effect on cells carrying *CHC1*. The significance of *SCD1* is unclear. It does not exhibit a high degree of sequence homology to *CHC1* since low stringency hybridizations with a *CHC1* probe failed to detect related sequences. Even so, *SCD1* could encode a protein which can functionally substitute for clathrin heavy chain, allowing enough intracellular transport to sustain growth. In the absence of the *SCD1* gene product (that is, in strains carrying $SCD1^L$) an essential pathway would be blocked in *chc1* cells, resulting in cell death. Phenotypic analysis of $SCD1^L$ strains carrying conditional alleles of *chc1* (for instance, a temperature-sensitive *chc1* allele) should allow a test of this possibility. Also, molecular cloning of *SCD1* may provide insight into its function. Another, less informative, explanation of *SCD1* must be considered. Strains carrying *chc1* are debilitated and consequently more sensitive to traumatic growth conditions. High temperature, low pH or low levels of a toxic drug (canavanine) that are not lethal to *CHC1* cells can completely inhibit the growth of *chc1* strains (Payne et al., 1987). These observations raise the possibility that perturbation of a process unrelated to clathrin function could impose sufficient stress to kill a cell already weakened by the *chc1* mutation. In this fashion $SCD1^L$ could result in *chc1* cell lethality even though it plays no direct role in intracellular protein transport or any other aspect of clathrin function. A likely example of this sort of nonspecific "euthanasia" has recently been uncovered in a second strain (W303) that is unable to grow in the absence of clathrin heavy chain (L. Silveira, *personal communication*). Like *chc1* mutations, deletion of a protein phosphatase-encoding gene, *PPH1* (previously *SIT4*) in strain W303 is lethal while the same disruption in other strains yields viable, slow-growing cells (A. Sutton, *personal communication*). There is no apparent functional relationship between *CHC1* and *PPH1*; genetic studies of *PPH1* suggest that it may interact with RNA polymerase to affect transcriptional activity (Arndt, Styles & Fink, 1989) and also is required for progression through the G_1 phase of the cell-cycle (A. Sutton, *personal communication*). Since deletion of either *CHC1* or *PPH1* in W303 is lethal, the strain may harbor a cryptic alteration that confers enhanced sensitivity to mutations that retard cell growth. In view of this finding, care must be exercised when interpreting the relationship between two genes that display this type of synthetic lethality. Further characterization of *SCD1* and the lesion in W303 is needed to resolve the nature of their relationship to clathrin heavy chain.

Vacuolar Protein Localization Appears Normal in *chc1* Yeast

Vacuoles are the major hydrolytic organelles in yeast and can be considered analogs of mammalian lysosomes (*reviewed in* Rothman & Stevens, 1988). Vacuolar proteases are synthesized as inactive precursors that traverse the secretory pathway into the Golgi complex. In the Golgi complex the precursors are diverted from the secretory pathway and transported to vacuoles where they undergo proteolytic maturation (*reviewed in* Schekman, 1985). This transport pathway is analogous to that followed by lysosomal hydrolase precursors in mammalian cells (*reviewed in* von Figura & Hasilik, 1986; Kornfeld & Mellman, 1989).

In mammalian cells, more details of the pathway are known (*reviewed in* von Figura & Hasilik, 1986; Kornfeld & Mellman, 1989). Mannose-6-phosphate residues on the carbohydrate side chains of lysosomal precursors are recognized by specific mannose-6-phosphate receptors (M6PR) in the Golgi complex and the complexes are segregated from the secretory pathway and packaged into transport vesicles in the *trans* Golgi network (TGN). The transport vesicles then deliver their contents to an acidic endosomal compartment where the hydrolases dissociate from the receptors. It is thought that the receptors are recycled back to the Golgi complex from this compartment while the hydrolases are shuttled to lysosomes. By analogy with receptor-mediated endocytosis, clathrin-coated Golgi membranes may participate in selective packaging of lysosomal hydrolase precursors into transport vesicles that form from the TGN. Interaction between the cytoplasmic tails of the mannose-6-phosphate receptors and the clathrin APs could mediate this sorting event. In addition, immunocytochemical and histochemical studies have revealed lysosomal proteins and mannose-6-phosphate receptors in coated regions of the Golgi complex and in coated vesicles, although the coats have not been unequivocally shown to consist of clathrin (Friend & Farquhar, 1967; Brown, Constantinescu & Farquhar, 1984; Brown & Farquhar, 1984; Geuze et al., 1985; Brown, Goodhouse & Farquhar, 1986; Griffiths et al., 1988). Also, hydrolase precursors and the M6PR have been demonstrated in preparations of purified clathrin-coated vesicles (Campbell & Rome, 1983; Shulze-Lohoff, Hasilik & von Figura, 1985; Lemansky et al., 1987).

Sorting of vacuolar hydrolases in yeast differs in some respects from targeting of lysosomal precursors. Unlike lysosomal hydrolases, vacuolar hydrolase precursors do not require mannose-6-phosphate residues for accurate targeting (*reviewed in* Rothman & Stevens, 1988). For two soluble vacuo-

lar proteins, sequences in the precursor domain of the protein are necessary for sorting, implying that specific amino acids or a particular conformation acts as the sorting determinant (Johnson, Bankaitis & Emr, 1987; Valls et al., 1987; Klionsky, Banta & Emr, 1988). The putative receptor for the vacuolar sorting determinant has not been identified. Also, it is not presently known whether vacuolar hydrolase precursors proceed through intermediate compartments, such as endosomes, between the Golgi complex and vacuoles.

Given the compelling circumstantial evidence that clathrin participates in delivery of lysosomal enzymes, and given the similar routes taken by proteins bound for lysosomes or vacuoles, it was not unreasonable to suspect that targeting of newly synthesized vacuolar proteins would be abnormal in *chc1* cells. Indeed, vacuoles in *chc1* cells have altered morphologies, appearing fragmented and multi-vesicular when compared to vacuoles in wild-type cells (Payne et al., 1987, 1988). Analysis of vacuolar protein targeting in *chc1* strains relied on studies of yeast cells carrying *vps* (*v*acuolar *p*rotein *s*orting) mutations. In *vps* cells, the vacuolar protease precursors are not sorted from the secretory pathway but, instead, continue along the pathway to the cell surface (Rothman & Stevens, 1986; Bankaitis, Johnson & Emr, 1986; Robinson et al., 1988; Rothman, Howard & Stevens, 1989). The precursors are proteolytically activated at the cell surface but with much slower kinetics than in wild-type cells (Rothman & Stevens, 1986). Thus, two conveniently assayed phenotypes are available to diagnose missorting of vacuolar hydrolase precursors: increased levels of secreted hydrolase precursors and a decreased rate of precursor maturation. *chc1* Cells exhibited neither phenotype (Payne et al., 1988). Newly synthesized vacuolar protease precursors were converted to mature forms with similar kinetics in congenic sets of *chc1* and *CHC1* strains. Also in contrast to *vps* cells, *chc1* cells did not secrete abnormal amounts of protease precursors. Finally, immunolocalization experiments showed that the bulk of a particular vacuolar protease, carboxypeptidase Y, was located within morphologically identifiable vacuolar structures in *chc1* cells (Payne et al., 1988). Preliminary results also suggest that maturation of the precursor to a vacuolar membrane protein, alkaline phosphatase, also occurs at wild-type rates in *chc1* cells (D. Klionsky, *personal communication*). These results argue that clathrin is not required for sorting and delivery of vacuolar proteins.

Again, the results from yeast do not conform to expectations based on studies of mammalian cells. In considering possible resolutions of this discrepancy, one of three distinct interpretations may apply: (i) the conclusions from experiments with mammalian cells require revision; (ii) the conclusions from experiments with yeast require revision; or (iii) the pathways in yeast and mammals are different.

The conclusions from mammalian cell experiments are based on substantial indirect evidence that sorting and delivery of newly-made lysosomal precursors to lysosomes is mediated by clathrin coats. However, the complexity of traffic routes followed by the M6PR makes unequivocal conclusions difficult. M6PR are found in the Golgi complex, endosomes and the cell surface and they cycle between all three compartments (*reviewed in* Kornfeld & Mellman, 1989). Also, although most newly made lysosomal precursors are efficiently sorted to lysosomes, a small fraction (5%) escape sorting and travel to the plasma membrane. Thus, the origin and destination of clathrin-coated vesicles that contain lysosomal hydrolases cannot be unambiguously determined. Given the results in yeast, functional assays for lysosomal hydrolase sorting are necessary to evaluate clathrin's role in this pathway.

In yeast cells, clathrin may normally be involved in vacuolar protein delivery, but when clathrin is eliminated, an alternative transport pathway may be activated. Alternatively, in the absence of clathrin, lysosomal hydrolase precursors might accumulate in the Golgi complex or TGN, leading to formation of a hybrid organelle that can function in the secretory pathway and also is able to mature and retain vacuolar hydrolase precursors. These possibilities can be addressed by characterization of the immediate consequences of a clathrin heavy chain deficiency in cells carrying a conditional *chc1* allele. Also, further analysis of the vacuolar organelles in *chc1* cells may help in distinguishing between the various hypotheses.

Finally, clathrin could function in different pathways in mammalian cells and yeast. A clathrin-independent mechanism may have evolved in yeast to insure segregation of vacuolar proteins from the secretory pathway.

chc1 Cells Carry Out Endocytosis

Studies of receptor-mediated endocytosis in mammalian cells have provided a compelling demonstration of clathrin's association with membrane vesiculation. Receptor-mediated endocytosis may occur during yeast mating (Chvatchko, Howald & Reizman, 1986; Jenness & Spatrick, 1986). Haploid yeast cells exhibit one of two mating types, mating type α (*MAT*α) or mating type **a** (*MAT***a**) (*reviewed in* Cross et al., 1988) Cells of opposite mating type can conjugate and form diploids. Each haploid cell

type secretes a peptide pheromone, α-factor or **a**-factor, respectively, that interacts with specific cell-surface receptors on cells of the opposite mating type to elicit physiological responses necessary for mating. *MAT***a** cells take up α-factor (Chvatchko et al., 1986; Jenness & Spatrick, 1986). Internalization bears the hallmarks of receptor-mediated endocytosis: uptake depends on time, temperature, energy and specific cell-surface receptors (Chvatchko et al., 1986; Jenness & Spatrick, 1986). Also, peptide uptake is accompanied by a concomitant loss of cell-surface receptor activity, presumably due to receptor-ligand internalization (Jenness & Spatrick 1986). However, proof of a receptor-mediated endocytic mechanism in yeast awaits the demonstration of a vesicular intermediate in the uptake of α-factor. Processing of receptor-bound **a**-factor by *MAT*α cells has not been characterized.

Uptake of radiolabeled α-factor by *chc1 MAT***a** cells was used as a means to measure receptor-mediated endocytosis (Payne et al., 1988). α-Factor was taken up by *chc1* cells at 36–50% of the levels internalized by congenic *CHC1 MAT***a** cells. The reduction in pheromone uptake by mutant cells was not due to fewer cell surface α-factor receptors since mutant and wild-type cells displayed equivalent numbers of receptors. If, as seems likely, α-factor internalization occurs by receptor-mediated endocytosis, then uptake of α-factor by *chc1* cells argues that clathrin cannot be the sole mediator of plasma membrane vesiculation during endocytosis. The reduced uptake of α-factor by *chc1* cells can be interpreted in several ways. First, clathrin polymerization may not provide the force which drives vesiculation of the plasma membrane but, instead, could expedite endocytosis through other capacities such as clustering the receptors in domains of the plasma membrane undergoing vesiculation. Second, clathrin may normally drive vesiculation but, in its absence, another protein can provide the same function. Third, both clathrin-dependent and -independent pathways may exist and α-factor is internalized by the independent pathway. Until more is known about the endocytic pathway in yeast, distinguishing between these possibilities is difficult.

When α-factor binds to its receptor on *MAT***a** cells, it triggers cell cycle arrest, surface expression of agglutinins and alterations of cell surface and nuclear morphologies (*reviewed in* Cross et al., 1988). *MAT***a** cells can also adapt to the continuous presence of α-factor and resume normal mitotic growth (*reviewed in* Cross et al., 1988). The cell cycle arrest response of *chc1* cells is indistinguishable from the response of wild-type cells, suggesting that the receptor is able to transmit the ligand-induced signal and undergo complete adaptive response in the absence of clathrin.

The amino-acid sequence of the α-factor receptor, deduced from the sequence of the gene, contains seven potential membrane spanning domains and a carboxy-terminal cytoplasmic tail (Nakayama, Miyajima & Arai, 1985). This structure is similar to that of a class of receptors in mammalian cells which includes β-adrenergic receptors (*reviewed in* Sibley et al., 1987). Although β-adrenergic receptors are internalized, it is unclear whether the uptake mechanism involves clathrin-coated intermediates. Thus, by analogy to the β-adrenergic receptors, the α-factor receptor may not be a likely candidate for clathrin-mediated endocytosis. In contrast to the β-adrenergic receptors, the nutrient and growth factor receptors that are known to be internalized via clathrin-coated pits and vesicles consist of polypeptides that span the membrane once. Cell-surface receptors with single membrane-spanning domains have not been identified in yeast.

Mislocalization of a Golgi Protein in *chc1* cells

In wild-type cells, production of both α-factor and **a**-factor requires proteolytic excision of the active peptide from a larger precursor, but maturation and export of the two pheromones occur by different routes (*reviewed in* Fuller, Sterne & Thorner, 1988). The α-factor pheromone follows the conventional secretory pathway whereas **a**-factor bypasses secretory organelles and is apparently directly translocated across the plasma membrane. The biosynthetic intermediates in the maturation of α-factor, and the proteases responsible for maturation, have been well characterized. Two genes encode α-factor precursor polypeptides that differ primarily in the number of peptide repeats located at the carboxyl termini, the more abundant precursor carries four copies and the minor form carries two copies. After acquiring carbohydrate modifications in the ER and Golgi, precursor α-factor is subjected to a series of proteolytic scissions that release the mature peptide (Fig. 4). Maturation begins in the Golgi complex and is thought to proceed as the precursor is packaged into secretory vesicles. The first cleavages are carried out by the *KEX2* endoprotease (Kex2p), which cuts after pairs of basic amino acids to generate peptides with 6 amino-terminal amino acids and 2 carboxl-terminal amino acids flanking the mature sequence. The extra carboxyl-terminal aa's are severed by the *KEX1* carboxypeptidase. The amino-terminal extension is removed by the *STE13*-encoded dipeptidyl aminopeptidase. The

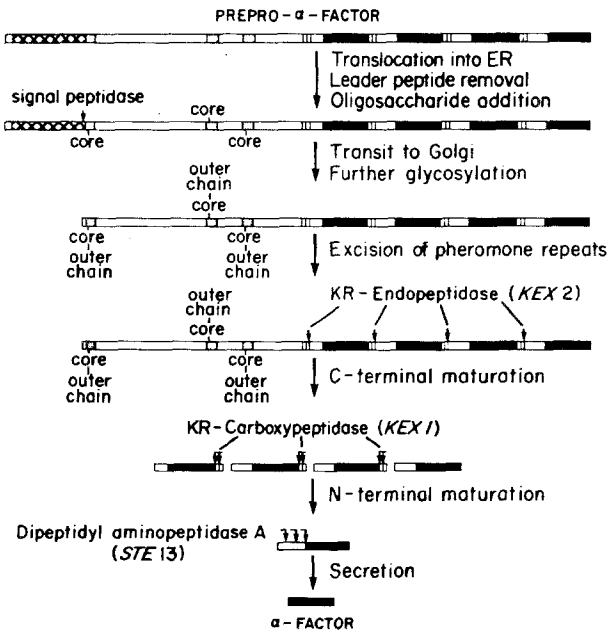

Fig. 4. Biosynthesis of α-factor mating pheromone. The pheromone is synthesized as part of a larger precursor which is glycosylated then subjected to proteolytic cleavage to release the mature pheromone. (Reproduced from Fuller et al. (1988) with permission from the *Annual Review of Physiology*, Vol. 50, copyright 1988 by Annual Reviews, Inc.

action of all three proteases is required for production of biologically active α-factor.

Examination of mating pheromone biosynthesis in *chc1* cells uncovered a striking defect (Payne & Schekman, 1989). In a three-hour period, *chc1* *MATα* cells produce about 16-fold less biologically active α-factor than congenic wild-type *MATα* cells. In contrast, the disruption of *CHC1* in *MAT***a** cells does not reduce production of **a**-factor by more than twofold in the same time period. This difference is mirrored in the mating efficiencies of *MATα* and *MAT***a** cells bearing the *chc1* mutation. The α-factor production deficit is due to a defect in maturation; *chc1* cells secrete the same highly glycosylated form of α-factor precursor that is secreted by cells carrying a disruption of the *KEX2* gene. This result suggested that a lack of clathrin heavy chain prevents cleavage of the α-factor precursor by the *KEX2* endoprotease.

In wild-type cells, Kex2p assumes a transmembrane configuration with a cytoplasmic carboxyl-terminal tail, a single membrane-spanning domain and a large lumenal domain that contains the active site (Fuller et al., 1988). Kex2p activity is latent in wild-type cells unless they are permeabilized with detergent, suggesting that the enzyme resides in an intracellular secretory compartment (Fuller et al., 1988). Since Kex2p first acts on α-factor in the Golgi complex it is thought to reside in Golgi membranes. Two lines of evidence suggest that Kex2p is aberrantly localized in *chc1* cells, an anomally that could explain the α-factor maturation defect (Payne & Schekman, 1989). First, using a membrane-impermeant Kex2p substrate, 60–90% of the total cell-associated Kex2p activity in *chc1* cells was detected when intact cells were assayed. The absence of latency implies that the enzyme is located at the cell surface. Approximately 70% of the Kex2p in *chc1* cells could also be iodinated under conditions that preferentially label cell surface proteins. No Kex2p was labeled in CHC1 cells. These experiments reveal that the bulk of Kex2p in *chc1* cells resides at the plasma membrane and could explain the mating-type specific effect of the *chc1* mutation since **a**-factor maturation does not require Kex2p activity. Clathrin therefore appears to be required for the intracellular retention of Kex2p, probably in the Golgi complex. In this capacity clathrin may serve to orchestrate maturation of the α-factor precursor.

Three models for the role of clathrin in Kex2p localization shown in Fig. 5. Model 1 (Fig. 5*A*, *1*) suggests that clathrin collects Kex2p at sites where α-factor precursor is being packaged into secretory vesicles. Clathrin-coated vesicles are postulated to bud from the nascent secretory vesicle, removing Kex2p from the secretory route and recycling it to Golgi membranes. This model is similar to a model proposed for the formation of secretory granules from the TGN in mammalian endocrine cells (Burgess & Kelly, 1987). The observation of clathrin patches on nascent but not mature secretory granules suggests that the formation of clathrin-coated vesicles from the patches could retrieve membrane, and perhaps proteins, from the granule for return to the TGN. In the second model (Fig. 5*A*, *2*), stable hexagonal arrays of clathrin on Golgi membranes act to tether Kex2p in the Golgi complex. This model does not account for clathrin's ability to assemble into cages in vitro or its documented association with vesiculating membranes in mammalian cells. However, planar sheets of clathrin have been observed in mammalian cells (Heuser, 1980), and the function of such arrays could be different from that of clathrin cages. The last model (Fig. 5*B*) has clathrin retrieving Kex2p from the plasma membrane by receptor-mediated endocytosis. In this view, Kex2p normally is transported to the plasma membrane in secretory vesicles but is efficiently recycled to the Golgi complex. Localization of clathrin and Kex2p in wild-type yeast cells and in

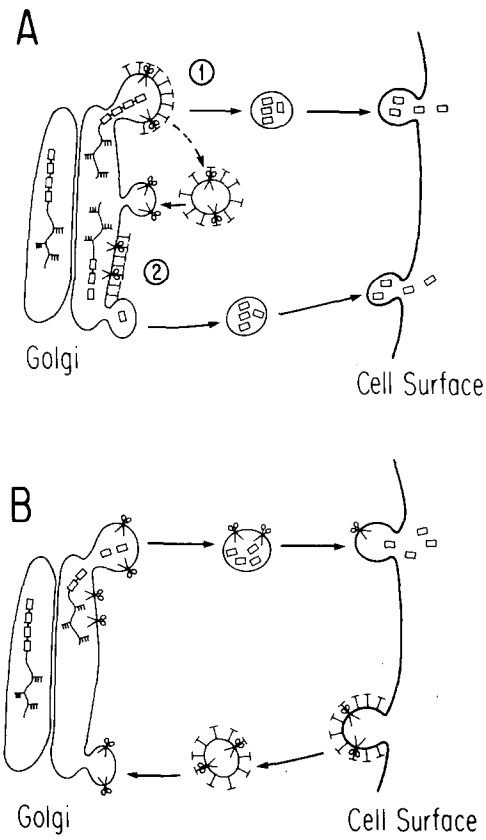

Fig. 5. Possible mechanisms for clathrin-mediated retention of Kex2p in the Golgi complex. (*A*) Kex2p is continuously retained intracellularly. (*B*) Kex2p reaches the plasma membrane but is efficiently endocytosed. Glycosylated pro-α-factor is shown within the Golgi cisternae, with combs representing asparagine-linked carbohydrate chains and boxes signifying mature peptide units. Clathrin is shown as *T*s coating the cytoplasmic surfaces of the Golgi complex and a Golgi complex-derived vesicle in (*A*), and an endocytic pit and vesicle in (*B*). Kex2p is drawn as scissors which interact with clathrin coats. (Reproduced from Payne & Schekman (1989), copyright by the AAAS

membranes isolated from those cells, and analysis of Kex2p in cells carrying reversible, temperature-sensitive *chc1* mutations may aid in discerning the validity of these various models.

Structural Aspects of Yeast Clathrin Heavy Chain Can be Studied in Vivo

Viable cells lacking clathrin heavy chain provide a means to analyze the structural determinants of clathrin heavy chain function. The *CHC1* gene can be mutagenized in vitro and then introduced into *chc1* cells to determine the consequences of the mutation. The functional integrity of different mutant alleles can be assessed by determining the ability of each mutant to reverse the growth defect or α-factor precursor processing defect in cells carrying a deletion of the *CHC1* gene. Each mutation can be monitored for its effect on triskelion formation, assembly of triskelions into coats and disassembly of clathrin coats. From an analysis of this sort, domains of clathrin heavy chain that are important in each of these processes can be identified. For instance, in vitro experiments support the idea that the amino-terminal region of clathrin heavy chain, a globular domain that forms the foot of each triskelion leg, is dispensable for coat formation but necessary for coat disassembly (*reviewed in* Rothman & Schmid, 1986). Removal of the terminal domain (and light chains) from mammalian triskelions by trypsin treatment does not perturb the ability of triskelions to rebind to uncoated membranes (Hanspal, Luna & Branton, 1984). Clathrin cages treated with trypsin to remove the terminal domain (and light chains) are stable, although their structure may be slightly more irregular (Kirchhausen & Harrison, 1984; Vigers, Crowther & Pearse, 1986). Cages lacking terminal domains are not, however, substrates for the 70-kD heat shock family proteins (HSP70) that, in vitro, depolymerize native clathrin cages in an ATP-dependent reaction (Rothman & Schmid, 1986). The function of the terminal domain can now be investigated in vivo by deleting the *CHC1* sequences that encode this domain, introducing the mutant allele into *chc1* yeast and monitoring the assembly state of the mutant protein in the cells.

Clathrin heavy chains are joined at their carboxyl-termini to form the vertex of a triskelion (Kirchhausen et al., 1987). From analysis of the rat clathrin heavy chain gene, Kirchhausen et al. (1987) proposed that triskelion formation involves an unusual stretch of amino acids at the carboxyl-terminal end of rat clathrin heavy chain that contains high numbers of prolines and glycines. Analysis of sequences required for triskelion formation can be performed by mutagenesis of the yeast *CHC1* gene.

Yeast Clathrin Light Chain

Most mammalian cells express two distinct light chains (*reviewed in* Brodsky, 1988). The two chains appear to be randomly distributed in purified triskelions, and no significant functional distinctions have been made between the two polypeptides. The contribution of light chains to triskelion structure and triskelion assembly into cages is unresolved. Light chains have been removed from triskelions by either limited proteolysis with elastase or treatment with chaotropic salts (Kirchhausen & Harrison, 1981; Schmid et al., 1982; Winkler and Stanley,

1983). The results of proteolysis on triskelion structure have varied; in one case no effect on triskelion conformation was observed, in another, the rigidity of the angle in triskelion legs was reduced (Kirchhausen & Harrison, 1981; Schmid et al., 1982). In both cases, light chain-free triskelions were incapable of assembling into regular cages. Chemical extraction of the light chains yields triskelions which are structurally normal and fully capable of reassembly into cages, but this treatment may not quantitatively remove light chains from triskelions (Winkler & Stanley, 1983; Schmid et al., 1984). Light chains are not apparently required for the structural integrity of preformed cages (Kirchhausen & Harrison, 1981; Vigers et al., 1986). Clathrin light chains may play a role in the disassembly of clathrin coats. ATP-dependent depolymerization of clathrin coats in vitro by HSP70s requires clathrin light chains (Schmid et al., 1984). The significance of this observation to uncoating in vivo has not been established (*see* next section).

Unlike most mammalian cells, yeast triskelions contain only one species of light chain (Payne & Schekman, 1985; L. Silveira, *personal communication*). The yeast gene encoding light chain (*CLC1*) has been cloned and used to disrupt the single chromosomal gene in a manner analogous to that used for *CHC1* (L. Silveira, *personal communication*). Elimination of *CLC1* in most strains results in viable, slow-growing cells similar in their growth characteristics to cells lacking *CHC1*. Cells carrying both *clc1* and *chc1* mutations grow at the same rate as cells with either mutation alone. Curiously, *clc1* is not lethal in cells with SCD^L, but is lethal in the W303 strain. Interpretation of these findings awaits genetic tests to determine whether W303 harbors a version of the SCD^L allele. Viable *clc1* cells will allow analysis of triskelion formation, coat assembly and disassembly in vivo in the complete absence of light chains. This approach should clarify some of the discrepancies which have arisen during the in vitro analyses of mammalian light chain function.

Uncoating ATPase

Electron microscopic studies of mammalian cells suggest that clathrin-coated endocytic vesicles quickly shed their coats. This may be necessary for vesicle fusion with target membranes, since, in vitro, the clathrin coat prevents lipid bilayer fusion (Alstiel & Branton, 1983). Using depolymerization of empty clathrin cages as an assay, an ATP-dependent cytosolic uncoating activity was identified and purified to homogeneity (*reviewed in* Rothman & Schmid, 1986). The purified protein was shown to be a member of the HSP70 class of mammalian stress proteins (Chappell et al., 1986). Cytosolic fractions obtained from extracts of yeast cells also display ATP-dependent uncoating activity against mammalian clathrin cages (Chappell et al., 1986), and antibodies raised against bovine brain uncoating ATPase cross-react with members of the HSP70 class of yeast stress proteins, suggesting that they may be responsible for the cytosolic uncoating activity (Chappell et al., 1986). Unfortunately, the complexity of the HSP70 gene family in yeast has made this proposal difficult to prove. Phenotypic analysis of yeast mutants lacking various HSP70 members indicates that the gene family can be divided into at least three subfamilies, *SSA, SSB,* and *SSC* (Werner-Washberne, Stone & Craig, 1988). The *SSA* subfamily consists of four members, *SSA1, SSA2, SSA3,* and *SSA4*. *SSB1* and *SSB2* comprise the second subfamily and *SSC1* is the only member of its group. The predominant proteins in yeast cytosol which are recognized by antibody specific for the bovine brain uncoating ATPase are encoded by *SSA1* and *SSA2* (Chappell et al., 1986). Cells carrying disruptions of both these genes are viable and express wild-type levels of uncoating ATPase against mammalian cages, but the *SSA4* gene is induced in these cells, is also immunologically related to the bovine brain uncoating protein and could supply the uncoating activity (Chappell et al., 1986). Cells lacking *SSA1, SSA2,* and *SSA4* are not viable, but growth is restored by expression of *SSA1* under control of a repressible promoter (Werner-Washburne et al., 1988). Repression of *SSA1* expression in such a strain should allow determination of uncoating ATPase activity in cells devoid of *SSA* gene products. An additional confounding observation is that the antibodies raised against the bovine brain protein also react with products of the *SSB* genes (Werner-Washburne et al., 1988). A possible interpretation of these data is that uncoating ATPase activity is provided by the products of the *SSA* genes and disruption of three of the members reduces activity below a threshold necessary for cell growth. On the other hand, Deshaies et al. (1988) have shown that the *SSA* gene products are involved in vivo in the post-translational import of precursor polypeptides into both mitochondria and the lumen of the ER. Both the uncoating function of HSP70s and their involvement in translocation can be accounted for by the proposal of Pelham (1986), elaborated by Deshaies et al. (1988) and Rothman (1989), that HSP70s couple ATP hydrolysis to disruption of tertiary structure, quaternary structure in multi-subunit complexes or denatured aggregates. It remains to be determined

whether HSP70s participate in clathrin coat disassembly in vivo.

Summary

The use of yeast mutants to study the function and dynamics of clathrin-coated membranes has offered new insights into clathrin's role in the secretory pathway and has raised additional questions. Most strains of yeast can incur a disruption of clathrin heavy or light chain genes and remain viable. However, in rare cases, alleles of genes other than clathrin affect the viability of clathrin-deficient cells. The relationship of the products of these genes to clathrin awaits clarification. Phenotypic characterization of clathrin-deficient yeast mutants suggests that clathrin is not essential for the generation of secretory pathway transport vesicles at the ER or the Golgi complex but is required for the intracellular retention of a Golgi membrane protein, Kex2p. With this genetic evidence for clathrin's function in vivo, biochemical and genetic experiments can be designed to address the mechanism by which clathrin effects retention of Kex2p.

Clathrin-deficient yeast carry out protein secretion, receptor-mediated endocytosis of mating pheromone, and efficient targeting of newly synthesized vacuolar proteins. These observations challenge aspects of clathrin's proposed involvement in protein transport through the secretory pathway and to lysosomes in mammalian cells. However, the differences are beginning to recede in the face of additional experiments; the formation of clathrin coated vesicles is no longer commonly thought to be obligately coupled to transport through the secretory pathway in mammalian cells (Rothman, 1986; Brodsky, 1988), and the role of clathrin in retaining a Golgi membrane protein in yeast may have its precedents in receptor-mediated endocytosis by mammalian cells or in secretory granule formation in endocrine cells. A unified theory of clathrin function is emerging (Brodsky, 1988) which suggests that the clathrin coat assemblage (clathrin heavy and light chains and the associated proteins) acts as a facilitator of intracellular protein transport by sorting and concentrating cargo molecules. The results from studies of clathrin-deficient yeast support this theory. Future experiments will determine whether clathrin provides its functions at different transport stages in different organisms or whether all eukaryotic cells employ clathrin at the same stages of intracellular protein transport.

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