

# A Modulatory Role for Clathrin Light Chain Phosphorylation in Golgi Membrane Protein Localization during Vegetative Growth and during the Mating Response of *Saccharomyces cerevisiae*

Diana S. Chu,<sup>\*†‡</sup> Babak Pishvaei,<sup>\*</sup> and Gregory S. Payne<sup>\*‡§</sup>

<sup>\*</sup>Department of Biological Chemistry and <sup>†</sup>The Molecular Biology Institute, University of California Los Angeles, Los Angeles, California 90095-3717

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The role of clathrin light chain phosphorylation in regulating clathrin function has been examined in *Saccharomyces cerevisiae*. The phosphorylation state of yeast clathrin light chain (Clc1p) in vivo was monitored by [<sup>32</sup>P]phosphate labeling and immunoprecipitation. Clc1p was phosphorylated in growing cells and also hyperphosphorylated upon activation of the mating response signal transduction pathway. Mating pheromone-stimulated hyperphosphorylation of Clc1p was dependent on the mating response signal transduction pathway MAP kinase Fus3p. Both basal and stimulated phosphorylation occurred exclusively on serines. Mutagenesis of Clc1p was used to map major phosphorylation sites to serines 52 and 112, but conversion of all 14 serines in Clc1p to alanines [S(all)A] was necessary to eliminate phosphorylation. Cells expressing the S(all)A mutant Clc1p displayed no defects in Clc1p binding to clathrin heavy chain, clathrin trimer stability, sorting of a soluble vacuolar protein, or receptor-mediated endocytosis of mating pheromone. However, the *trans*-Golgi network membrane protein Kex2p was not optimally localized in mutant cells. Furthermore, pheromone treatment exacerbated the Kex2p localization defect and caused a corresponding defect in Kex2p-mediated maturation of the  $\alpha$ -factor precursor. The results reveal a novel requirement for clathrin during the mating response and suggest that phosphorylation of the light chain subunit modulates the activity of clathrin at the *trans*-Golgi network.

## INTRODUCTION

In eukaryotic cells, the assembly of clathrin coats on specific membrane organelles leads to the formation of vesicles that engage in selective intracellular protein transport (Schmid, 1997). At the plasma membrane clathrin participates in receptor-mediated endocytosis, whereas at the Golgi complex clathrin is involved in the sorting of newly synthesized lysosomal proteins to the lysosome and localization of *trans*-Golgi network (TGN) resident membrane proteins (Pearse and Robinson, 1990; Wilsbach and Payne, 1993a; Schmid,

1997). Clathrin coats comprise individual clathrin molecules, termed triskelions, that associate to form a polyhedral lattice (Kirchhausen and Harrison, 1981; Ungewickell and Branton, 1981). The clathrin triskelion itself is a protracted three-legged structure composed of three heavy chain subunits (HCs) of 180 kDa and three light chains (LCs) of 30 kDa (Schmid, 1997). The extended HC molecules constitute the main structural component of the coat lattice. In mammalian cells two forms of HC have been identified. The first is ubiquitously expressed and has been extensively characterized (Schmid, 1997). Recently, a second form has been discovered in humans that shows restricted tissue distribution limited primarily to skeletal muscle (Brodsky, 1997). In contrast, the yeast *Saccharomyces cerevisiae* contains only a single clathrin heavy chain

<sup>†</sup> Present address: Department of Molecular Biology and Cell Biology, University of California, Berkeley, CA 94720-3204.

<sup>§</sup> Corresponding author.

gene (*CHC1*) encoding an HC that shares 50% amino acid identity with the mammalian ubiquitous HC (Payne and Schekman, 1985; Lemmon *et al.*, 1991). There are two forms of LC in mammalian cells, LCa and LCb, which have an identity of 60% (Brodsky *et al.*, 1991). Yeast has one gene (*CLC1*) encoding an LC, which, although only 18% identical to mammalian LCs, shares many properties with LCa and LCb such as size, acidic composition, acid and heat stability, and binding to calcium and calmodulin (Nathke *et al.*, 1988; Silveira *et al.*, 1990; our unpublished results).

The LC subunit has been proposed to regulate clathrin function based on several potential regulatory features, including phosphorylation, calcium binding, and interactions with calmodulin, the Hsp70-uncoating ATPase, and the HC trimerization domain (Brodsky *et al.*, 1991; Pishvae *et al.*, 1997). In yeast, deletion of *CLC1* (*clc1Δ*) causes defects in receptor-mediated endocytosis and localization of resident TGN membrane proteins (Chu *et al.*, 1996; Huang *et al.*, 1997). These defects are similar to those observed in *chc1* mutant strains, consistent with the idea that the HC and LC subunits act in concert within the cell. The protein trafficking defects in *clc1Δ* cells can be attributed to the decreased stability of clathrin trimers, altered Chc1p membrane association, and loss of clathrin-coated vesiculation that occur in the absence of Clc1p (Chu *et al.*, 1996; Huang *et al.*, 1997). These results point to the critical role that Clc1p plays in clathrin trimer formation or stability and in trimer association with membranes. Interestingly, Clc1p also appears to have functions independent of Chc1p, because overexpression of Clc1p can suppress the growth defect caused by deletion of *CHC1* in the appropriate strain background (Huang *et al.*, 1997).

One possible mechanism for regulating clathrin function is through phosphorylation of LC. LC has been shown to be phosphorylated *in vivo* in rat liver, rat reticulocytes, and Chinese hamster ovary cells (Cantournet *et al.*, 1987; Bar-Zvi *et al.*, 1988; Corvera and Capocasale, 1990). *In vitro*, LCb can be phosphorylated by casein kinase II, a kinase that copurifies with coated vesicles (Schook and Puszkun, 1985; Usami *et al.*, 1985; Bar-Zvi and Branton, 1986; Cantournet *et al.*, 1987; Merrese *et al.*, 1990). The sites of *in vitro* phosphorylation have been mapped to serines at positions 11 and 13 located within casein kinase II consensus recognition sequences (Hill *et al.*, 1988). LCa is also phosphorylated, but to a lesser extent than LCb. LCa phosphorylation occurs on as yet unidentified serine residues (Wilde and Brodsky, 1996) and, in a regulated manner, on tyrosine residues (Mooibroek *et al.*, 1992). Little data are available that address the function of LC phosphorylation. In rat reticulocytes, phosphorylated LC was detected in both assembled and soluble clathrin. However, the level of LC phosphorylation in the assembled fraction was slightly higher than in the

soluble fraction, perhaps implicating phosphorylation in regulating the assembly state of clathrin (Bar-Zvi *et al.*, 1988). *In vitro*, phosphorylated LCb has been reported to activate a phosphatase that acts on a 50-kDa coated vesicle protein, presumably a member of the clathrin adaptor complex, although the effect of this activation was not established (Hanson *et al.*, 1990). LCa can be phosphorylated in response to epidermal growth factor (EGF) stimulation by the EGF receptor-associated tyrosine kinase (Mooibroek *et al.*, 1992), but the consequences of this modification were not determined. Thus, the function of LC phosphorylation remains obscure.

We have applied biochemical and genetic approaches to investigate the effects of phosphorylation on LC function in yeast. Here we show that yeast Clc1p is constitutively phosphorylated *in vivo* at multiple serines scattered throughout the protein. Unexpectedly, activation of the mating response pathway results in Clc1p hyperphosphorylation. Mutagenesis of all 14 serine residues to eliminate phosphorylation resulted in no detectable differences in clathrin trimer stability, cell growth, receptor-mediated endocytosis, or vacuolar protein targeting. However, resident TGN protein localization was affected by these mutations, particularly in the presence of pheromone. These results suggest that Clc1p phosphorylation plays a modulatory role that becomes more important when pheromone activation of the mating response signal transduction pathway elicits the complex program of cellular changes that allow mating.

## MATERIALS AND METHODS

### Plasmids

Plasmid constructions were carried out using standard molecular biology techniques (Sambrook *et al.*, 1989). Site-directed mutagenesis was done as described by Kunkel *et al.* (1987). All mutations were introduced onto the plasmid pBKSCLC. Sequential mutagenesis and subcloning were used to convert multiple serine to alanine residues to create the S(52,112)A and S(all)A *clc1* mutants. pRSCLC05 wild-type and mutant versions were created by inserting a 1.5-kbp *SmaI-SacI* fragment containing wild-type or mutant *CLC1* from pBKSCLC into pRS305 digested with *XhoI-SacI*.

### Yeast Strains and Media

Yeast strains used in this study are listed in Table 1. GPY680, 681, and 682 were generated by transformation of YPH499, YDM200, and YDM600 (Ma *et al.*, 1995) with the plasmid pJGsst1 (Reneke *et al.*, 1988) digested with *SalI* and *EcoRI* to disrupt the *SST1* gene. GPY915 and 916 were derived similarly from YDH6 and YDH8. GPY1034-19D was obtained as a meiotic progeny of the diploid formed by mating GPY74-15C and GPY986-2A. GPY1946, 1947, and 1949 were generated by transformation of GPY986-2A with pRSCLC05 linearized by digestion with *PstI* for wild-type *CLC1* and the S(52,112)A *clc1* mutant and with *BstXI* for the S(all)A *clc1* mutant to integrate wild-type or mutant versions of the *CLC1* gene at the *LEU2* locus. To obtain these strains without pgalCLCURA3 (Chu *et al.*, 1996), cells were selected by growth on media containing

**Table 1.** Yeast strains used in this study

Strain	Genotype	Source
GPY74-15C	<i>MATa ura3-52 leu2-3,112 trp1-289 his4-519 sst1-3</i>	Rad <i>et al.</i> , 1995
GPY449	<i>MATα ura3-52 leu2-3,112 trp1 can1 pep4::LEU2</i>	Tan <i>et al.</i> , 1993
LSY89.2-4C	<i>MATα ura3-52 leu2-3,112 trp1 clc1Δ::URA3</i>	L. Silveira
SM1581	<i>MATa ura3 leu5 his4 trp1 can1 pSM219</i>	S. Michaelis
FC279	<i>MATa ura3-52 leu2-3,112 trp1-289 his2 ace1 sst1::LEU2</i>	Chang and Herskowitz, 1990
FC280	<i>MATa ura3-52 leu2-3,112 trp1-289 his2 ace1 sst1::LEU2 far1::URA3</i>	Chang and Herskowitz, 1990
RD680	<i>MATa ura3-52 leu2-3,112 reg1-501 pep4-3 prd-1-1122 GAL+ gal-STE4::LEU2</i>	I. Herskowitz
YPH499	<i>MATa ura3-52 leu2-Δ1 trp1-Δ63 his3-Δ200 ade2-101 lys-801</i>	Sikorski and Hieter, 1989
YDM200	<i>MATa ura3-52 leu2-Δ1 trp1-Δ63 his3-Δ200 ade2-101 lys2-801 fus3::LEU2</i>	D. Ma and J. Thorner
YDM600	<i>MATa ura3-52 leu2-Δ1 trp1-Δ63 his3-Δ200 ade2-101 lys2-801 kss1::hisg</i>	D. Ma and J. Thorner
GPY680	<i>MATa ura3-52 leu2-Δ1 trp1-Δ63 his3-Δ200 ade2-101 lys2-801 sst1::LYS2</i>	This study
GPY681	<i>MATa ura3-52 leu2-Δ1 trp1-Δ63 his3-Δ200 ade2-101 lys2-801 fus3::LEU2 sst1::LYS2</i>	This study
GPY682	<i>MATa ura3-52 leu2-Δ1 trp1-Δ63 his3-Δ200 ade2-101och lys2-801 kss1::hisg sst1::LYS2</i>	This study
YDH6	<i>MATa ura3-52 leu2-Δ1trp1-Δ1 ade2-101 lys2-801 cka1-Δ1::HIS3 cka2-Δ1::TRP1 pCEN6/LEU2CKA2</i>	Hanna <i>et al.</i> , 1995
YDH8	<i>MATa ura3-52 leu2-Δ1trp1-Δ1 ade2-101 lys2-801 cka1-Δ1::HIS3 cka2-Δ1::TRP1 pCEN6/LEU2cka2-8</i>	Hanna <i>et al.</i> , 1995
GPY915	<i>MATa ura3-52 leu2-Δ1 trp1-Δ1 ade2-101 lys2-801 cka1-Δ1::HIS3 cka2-Δ1::TRP1 pCEN6/LEU2CKA2 sst1::URA3</i>	This study
GPY916	<i>MATa ura3-52 leu2-Δ 1trp1-Δ1 ade2-101 lys2-801 cka1-Δ1::HIS3 cka2-Δ1::TRP1 pCEN6/LEU2cka2-8 sst1::URA3</i>	This study
GPY986-2A	<i>MATα ura3-52 leu2-3,112 trp1-Δ200 ade2-101 suc2-Δ9 clc1::HIS3 pgalCLCURA3</i>	Chu <i>et al.</i> , 1996
GPY1034-19D	<i>MATa ura3-52 leu2-3,112 trp1 his4-519 and/or his3-Δ200 sst1-3 ade2-101 clc1Δ::HIS3 pgalCLCURA3</i>	This study
GPY1080	<i>MATa ura3-52 leu2-3,112 trp1 his4-519 and/or his3-Δ200 sst1-3 ade2-101 clc1Δ::HIS3 pRSCLC14 (TRP1)</i>	This study
GPY1081	<i>MATa ura3-52 leu2-3,112 trp1 his4-519 and/or his3-Δ200 sst1-3 ade2-101 clc1Δ::HIS3 pgalCLCURA3 pRS314</i>	This study
GPY1118	<i>MATa leu2-3,112 ura3-52 his4-519 trp1 can1 pep4::LEU2 YE<sub>p</sub>CHCCLCURA3</i>	Chu <i>et al.</i> , 1996
GPY1946	<i>MATα ura3-52 leu2-3,112::pRSCLC05 trp1-Δ200 ade2-101 suc2-Δ9 clc1::HIS3</i>	This study
GPY1947	<i>MATα ura3-52 leu2-3,112PpRSCLC05(S52,112A) trp1-Δ200 ade2-101 suc2-Δ9 clc1::HIS3</i>	This study
GPY1949	<i>MATα ura3-52 leu2-3,112::pRSCLC05(Salla) trp1-Δ200 ade2-101 suc2-Δ9 clc1::HIS3</i>	This study
GPY1954	<i>MATa ura3-52 leu2-3,11::pRSCLC14(S52T) trp1 his4-519 and/or his3-Δ200 sst1-3 ade2-101 clc1Δ::HIS3</i>	This study
GPY1955	<i>MATa ura3-52 leu2-3,112::pRSCLC14(S112T) trp1 his4-519 and/or his3Δ200 sst1-3 ade2-101 clc1Δ::HIS3</i>	This study
GPY1970	<i>MATa ura3-52 leu2-3,112::pRSCLC05 trp1 his4-519 and/or his3-Δ200 sst1-3 ade2-101 clc1Δ::HIS3</i>	This study
GPY1971	<i>MATa ura3-52 leu2-3,112::pRSCLC05(S52,112A) trp1 his4-519 and/or his3-Δ200 sst1-3 ade2-101 clc1Δ::HIS3</i>	This study
GPY1973	<i>MATa ura3-52 leu2-3,112::pRSCLC05(Salla) trp1 his4-519 and/or his3-Δ200 sst1-3 ade2-101 clc1Δ::HIS3</i>	This study

5-fluoro-orotic acid. GPY1950, 1954, 1970, 1971, and 1973 were generated in the same way from GPY1034-19D.

Standard media preparation (Difco, Detroit, MI) and yeast growth were conducted as described by Sherman *et al.* (1974). DNA transformations were performed by the lithium acetate procedure (Ito *et al.*, 1982; Gietz and Schiestl, 1995).

SD is 0.67% yeast nitrogen base without amino acids (Difco) and 2% dextrose. SDYE is SD supplemented with 0.2% yeast extract. SDCAA medium is SD containing 5 mg/ml vitamin assay casamino acid mix (Difco) with 15 μg/ml adenine and 20 μg/ml methionine, histidine, uracil, and tryptophan. SDCAA-ura is SDCAA without uracil. SDCAA-trp is SDCAA without tryptophan. YP medium is 1% bacto-yeast extract and 2% bacto-peptone. YPD is YP with 2% dextrose. YP-phosphate was prepared according to the method of Rubin (1975). Cell densities in liquid culture were measured in a

1-cm plastic cuvette using a Beckman (Fullerton, CA) DU-62 spectrophotometer. One  $A_{500}$  unit is equivalent to  $2.3 \times 10^7$  cells/ml.

### Size Exclusion Chromatography, Coimmunoprecipitations, and Immunoblotting

Preparation of cellular lysates, size exclusion chromatography, and coimmunoprecipitation with Clc1p antibodies were carried out as previously described (Chu *et al.*, 1996; Pishvae *et al.*, 1997). Immunoblotting was carried out according to the method of Burnette (1981) with secondary antibodies coupled to alkaline phosphatase (ALP; Bio-Rad, Richmond, CA). Antibodies were visualized using color development for ALP. Immunoblot signals were quantitated using a Molecular Dynamics (Sunnyvale, CA) densitometer.

### Radiolabeling and Immunoprecipitations

In general for *in vivo* phosphate labeling, *sst1Δ* cells were grown in YP-phosphate plus 2% dextrose to midlogarithmic phase at 30°C. Cells were then incubated with or without 2.5 μM α-factor for 20 min at 30°C. Fifty microcuries of [<sup>32</sup>P]inorganic phosphate (P<sub>i</sub>) were added per 1 × 10<sup>7</sup> cells for 20 min at 30°C. Cells were lysed in 2% SDS and 0.2-ml glass beads in the presence of 10 μM sodium orthovanadate. For Gal-Ste4p experiments using the strain RD680, cells were shifted to 2% galactose-containing media for 1 h in lieu of α-factor addition before labeling. To test the role of casein kinase II in Clc1p phosphorylation, cells were grown on agar medium at 24°C and then resuspended in YP plus 1 M sorbitol. Unbudded cells were isolated by centrifugation for 3 min at 1000 × *g* and then washed in YPD. Cells were then shifted to 24°C for 90 min or 37°C for 60 min before treatment with or without 2.5 μM α-factor for 30 min. Labeling time was 30 min.

Immunoprecipitations were performed as described by Seeger and Payne (1992b) using affinity-purified polyclonal antisera against Clc1p, or Kss1p antibodies (a gift from J. Thorner, University of California, Berkeley, CA). Quantitation of <sup>32</sup>P incorporated into Clc1p was accomplished using a Molecular Dynamics PhosphorImager. In all cases, the level of phosphorylation was normalized to protein recovery as measured by immunoblotting. Phosphoamino acid analysis was conducted as described by Boyle *et al.* (1991) and visualized using either autoradiography or a Molecular Dynamics PhosphorImager.

For metabolic labeling of α-factor, cells were grown to midlogarithmic phase in SDYE at 30°C. Cells were then resuspended in SDYE or preconditioned α-factor-containing medium for 1 h. Preconditioned medium was prepared by growing SM1581 in SDCAA-ura overnight at 30°C. SM1581 overexpresses α-factor from a multicopy plasmid carrying the *MFA1* gene (pSM219; a gift from Susan Michaelis, Johns Hopkins University, Baltimore, MD). The medium was removed from cells, and 2% dextrose and 2% YE were added. Cells were labeled at 30°C. Labeling and immunoprecipitation of α-factor was performed as described by Seeger and Payne (1992b), except that the labeling period was 30 instead of 10 min.

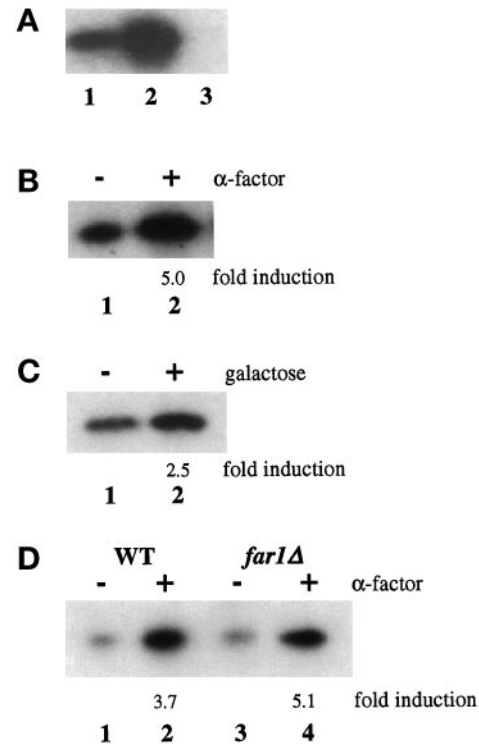
Metabolic labeling of Kex2p was performed according to the method of Wilsbach and Payne (1993b), except that labeling time was 15 instead of 20 min. Kex2p and the various forms of α-factor were quantified using a Molecular Dynamics PhosphorImager.

Endocytosis assays were performed as described by Chu *et al.* (1996).

## RESULTS

### Clc1p Is Phosphorylated *In Vivo* and Hyperphosphorylated in the Presence of Mating Pheromone

To determine whether Clc1p is phosphorylated *in vivo* in yeast, incorporation of <sup>32</sup>P-labeled P<sub>i</sub> into Clc1p was analyzed. Label was added to cells grown to midlogarithmic phase in medium depleted of phosphate. Clc1p was immunoprecipitated from these cells, subjected to SDS-PAGE and immunoblotting, and then analyzed by phosphoimaging. Incorporation of [<sup>32</sup>P]phosphate into immunoprecipitated material indicated that Clc1p is phosphorylated *in vivo* (Figure 1A, lane 1). Two control experiments established the specificity of the immunoprecipitation. First, no signal was detected in *clc1Δ* cells, which harbor a disruption of the *CLC1* gene (Figure 1A, lane 3). Second, overexpression of clathrin trimers by introduction of *CLC1* and *CHC1* genes together on a multicopy plasmid



**Figure 1.** Phosphorylation and hyperphosphorylation of Clc1p *in vivo*. (A) Phosphorylation of Clc1p *in vivo*. Wild-type cells (GPY449, lane 1), wild-type cells overexpressing Clc1p and Chc1p (GPY1118, lane 2), and *clc1Δ* cells (LSY89.1, lane 3) were grown to midlogarithmic phase and labeled with [<sup>32</sup>P]P<sub>i</sub> at 30°C for 1 h. Clc1p was immunoprecipitated, separated by SDS-PAGE, and analyzed by autoradiography. (B) Clc1p is hyperphosphorylated in the presence of mating pheromone. *MATa sst1Δ* cells (GPY74-15) were grown at 30°C for 30 min without (lane 1) or with (lane 2) 2.5 μM α-factor. Cells were labeled with [<sup>32</sup>P]P<sub>i</sub> for 30 min, and Clc1p was immunoprecipitated, separated by SDS-PAGE, and transferred to nitrocellulose. After determination of radiolabel incorporation through phosphoimaging, protein content was determined by probing with polyclonal Clc1p antibodies and densitometry. Label incorporated was then normalized to protein content. Values obtained for α-factor-treated cells are divided by values obtained from untreated cells to determine “fold induction” values. (C) Clc1p hyperphosphorylation requires activation of the mating response pathway. Cells (RD680) harboring a galactose-inducible copy of Ste4p were incubated with 2% glucose (lane 1) or 2% galactose (lane 2) for 1 h before incubation with [<sup>32</sup>P]P<sub>i</sub> for 30 min at 30°C. Clc1p was analyzed as described in B. (D) Clc1p hyperphosphorylation is not dependent on cell cycle arrest. Wild-type (WT; FC279, lanes 1 and 2) or *far1Δ* (FC279, lanes 3 and 4) cells were labeled, and Clc1p was analyzed as described in B.

resulted in an increased signal (Figure 1A, lane 2). These data demonstrate that Clc1p is phosphorylated in growing cells.

We also observed that treatment of cells with the mating pheromone α-factor caused an increase in the amount of phosphate incorporated into Clc1p. For this experiment, cells were used that contain a disrupted version of the *SST1* gene. *SST1* encodes a secreted



protease that degrades  $\alpha$ -factor, so the *ssf1* $\Delta$  disruption confers increased sensitivity to the pheromone (Chan and Otte, 1982; MacKay *et al.*, 1988). When *ssf1* $\Delta$  mating type a (*MATa*) cells were treated with  $\alpha$ -factor for 30 min before a 30-min labeling period in the continued presence of pheromone, Clc1p phosphorylation increased up to fivefold over levels in untreated cells (Figure 1B). This hyperphosphorylation was evident as quickly as 5 min after pheromone addition and was sustained until pheromone was removed (our unpublished data). The magnitude of Clc1p hyperphosphorylation varied from experiment to experiment. We found that the genetic background of different strains and the specific biological activity of the commercial  $\alpha$ -factor preparations contributed to the variability. Nevertheless, in every case,  $\alpha$ -factor-treated cells incorporated more [ $^{32}$ P]phosphate into Clc1p than untreated cells. Furthermore, similar increases in phosphorylation were also observed in *MATa* cells treated with  $\alpha$ -factor-containing medium (our unpublished data).

#### **The Mating Response Signal Transduction Pathway Is Responsible for Clc1p Hyperphosphorylation**

Mating pheromones trigger a well-defined signal transduction pathway to elicit the mating response by binding to plasma membrane receptors that are members of the G protein-coupled receptor family (Sprague and Thorner, 1992). Pheromone binding to the receptors catalyzes nucleotide exchange on the heterotrimeric G protein  $\alpha$  subunit (Gpa1p), causing release of the G $\beta\gamma$  subunit complex (consisting of Ste4p and Ste18p, respectively). Free  $\beta\gamma$  complexes then stimulate a kinase cascade that results in activation of a MAP kinase module. This mating response signal transduction pathway also can be activated in the absence of pheromone by overexpression of the G $\beta$  subunit (Cole *et al.*, 1990; Nomoto *et al.*, 1990; Whiteway *et al.*, 1990). It is thought that high levels of G $\beta$  compete with endogenous  $\beta\gamma$  complexes for binding to  $\alpha$  subunits, thereby increasing levels of free  $\beta\gamma$  to stimulate the pathway. To avoid variation caused by different preparations of  $\alpha$ -factor and to obtain independent evidence that Clc1p is a target for the mating response kinase pathway, we examined Clc1p phosphorylation in a strain capable of overexpressing the G $\beta$  subunit from a galactose-inducible copy of *STE4*. Cells carrying an integrated copy of *STE4* under control of the *GALI* promoter were incubated for 60 min in galactose to induce expression or in glucose to maintain repression. After a 30-min labeling period with  $^{32}$ P, immunoprecipitation of Clc1p revealed hyperphosphorylation in galactose-treated cells (Figure 1C, lane 2) compared with the glucose-treated control (Figure 1C, lane 1). Together, the results shown in Figure 1 indicate that Clc1p is constitutively phos-

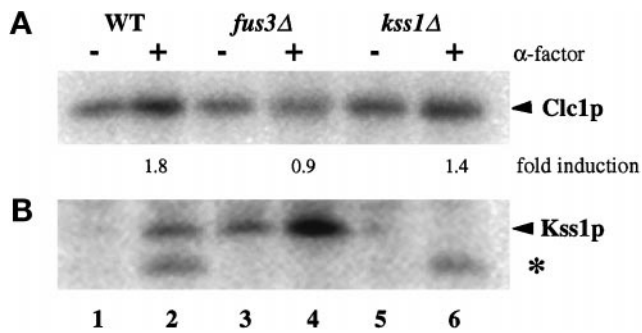
phorylated in growing cells and is hyperphosphorylated upon activation of the mating response pathway.

#### **Cell Cycle Arrest Is Not Necessary for Clc1p Hyperphosphorylation**

Pheromone triggering of the mating response pathway leads not only to morphological changes and transcriptional activation but also to arrest of cells in the G1 phase of the cell cycle (Sprague and Thorner, 1992). It was possible that the hyperphosphorylation of Clc1p could be a direct consequence of arrest in G1 and, therefore, a secondary effect of mating pathway activation. To test this possibility, Clc1p hyperphosphorylation was examined in a strain unable to undergo pheromone-induced cell cycle arrest because of disruption of the *FAR1* gene. *FAR1* encodes a cyclin-dependent kinase inhibitor that is necessary for G1 arrest in response to mating pheromones (Chang and Herskowitz, 1990; Peter *et al.*, 1993). When treated with pheromone, cells carrying a *FAR1* disruption (*far1* $\Delta$ ) exhibit morphological changes and transcriptional activation but continue to proceed through the cell cycle (Chang and Herskowitz, 1990). Wild-type and congenic *far1* $\Delta$  cells were incubated with or without  $\alpha$ -factor and labeled with  $^{32}$ P. As shown in Figure 1D, the absence of Far1p (Figure 1D, lanes 3 and 4) did not inhibit the increased phosphorylation of Clc1p stimulated by  $\alpha$ -factor treatment. Therefore, cell cycle arrest is not required for pheromone-stimulated hyperphosphorylation of Clc1p, suggesting that hyperphosphorylation is directly dependent on the mating response pathway.

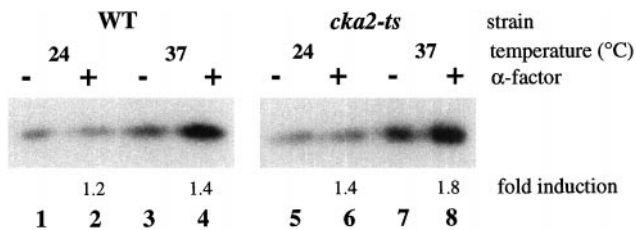
#### **Clc1p Hyperphosphorylation Is Dependent on the Fus3p MAP Kinase**

The mating response pathway propagates the pheromone-induced signal through two partially redundant MAP kinases, Fus3p and Kss1p (Sprague and Thorner, 1992). Cells lacking either kinase alone will respond to pheromone, but cells lacking both are unresponsive (Elion *et al.*, 1991a,b). Cells carrying disruptions of either *FUS3* and *KSS1* were examined for Clc1p hyperphosphorylation. In the experiment shown in Figure 2,  $\alpha$ -factor stimulated Clc1p phosphorylation by 1.8-fold in the congenic wild-type strain (Figure 2A, lanes 1 and 2). Because the MAP kinases are activated by phosphorylation, immunoprecipitates of Kss1p were analyzed in parallel. Indicative of mating response pathway activation, Kss1p displayed a strong increase in phosphorylation in the cells treated with  $\alpha$ -factor (Figure 2B, lanes 1 and 2). A faster-migrating band of undetermined identity that correlates with the presence and size of Fus3p was also detected by our methods in the samples from the  $\alpha$ -factor-treated cells precipitated with antibodies against Kss1p (Figure 2B, asterisk). This cross-reacting band also served as a



**Figure 2.** Clc1p hyperphosphorylation depends on Fus3p. Congenic wild-type (GPY680, WT, lanes 1 and 2), *fus3Δ* (GPY681, lanes 3 and 4), or *kss1Δ* (GPY682, lanes 5 and 6) cells were grown to midlogarithmic phase, incubated with or without 2.5  $\mu$ M  $\alpha$ -factor for 30 min, and labeled with [ $^{32}$ P]P<sub>i</sub> for 30 min. (A) Immunoprecipitation of Clc1p. (B) Immunoprecipitation of Kss1p. Asterisk, cross-reacting Fus3-dependent phosphoprotein.

useful control for pathway activation. Disruption of *FUS3* completely eliminated pheromone-dependent Clc1p hyperphosphorylation (Figure 2A, lanes 3 and 4), although activation of the pathway was apparent from the increased level of Kss1p phosphorylation (Figure 3B, lanes 3 and 4). Disruption of *KSS1* had only a moderate effect on the level of Clc1p hyperphosphorylation (Figure 2A, lanes 5 and 6). Qualitatively similar results were obtained in multiple experiments, although quantitative variations occurred in the level of pheromone-stimulated Clc1p hyperphosphorylation in wild-type and *kss1Δ* strains. Importantly, in each experiment Clc1p hyperphosphorylation was not observed in *fus3Δ* cells. These results demonstrate that Clc1p hyperphosphorylation is dependent on the Fus3p kinase and suggest that either Fus3p or a downstream kinase is responsible for modifying Clc1p in response to pheromone.



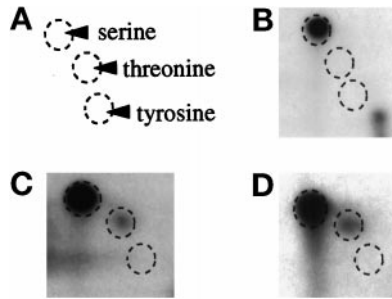
**Figure 3.** Casein kinase II is not responsible for Clc1p phosphorylation. Clc1p phosphorylation in a *cka1Δ cka2Δ* strain carrying either a wild-type (GPY987, WT) or temperature-sensitive (GPY988, *cka2-ts*) copy of *CKA2*. Wild-type cells were grown at 24°C and then incubated at the permissive temperature (24°C) for 90 min or the nonpermissive temperature (37°C) for 60 min. Cells were then treated with or without 2.5  $\mu$ M  $\alpha$ -factor for 30 min and labeled with [ $^{32}$ P]P<sub>i</sub> for 30 min.

### Casein Kinase II Is Not Responsible for Constitutive Phosphorylation of Clc1p

Casein kinase II associates with clathrin-coated vesicles in mammalian cells and can phosphorylate LCB in vitro (Schook and Puszkin, 1985; Bar-Zvi and Branton, 1986; Merrese *et al.*, 1990). The availability of yeast strains with mutant forms of casein kinase II allowed us to investigate the role of this enzyme in the in vivo phosphorylation of Clc1p. Yeast casein kinase II is a complex of two catalytic subunits ( $\alpha$  and  $\alpha'$ ) and two regulatory subunits ( $\beta$  and  $\beta'$ ) encoded by the genes *CKA1*, *CKA2*, *CKB1*, and *CKB2*, respectively (Glover *et al.*, 1994). Simultaneous disruption of both of the catalytic genes (*cka1Δ cka2Δ*) is lethal, whereas single disruptions have no deleterious effects (Chen-Wu *et al.*, 1988; Padmanabha *et al.*, 1990; Bidwai *et al.*, 1995). Strains with conditional alleles of different subunits have been used to demonstrate that casein kinase II functions in cell cycle progression during both G1 and G2-M transitions, cell polarity, and ion homeostasis (Bidwai *et al.*, 1995; Hanna *et al.*, 1995; Rethinaswamy *et al.*, 1998). To assess the role of casein kinase II in Clc1p phosphorylation, *cka1Δ cka2Δ* strains sustained by either wild-type (*CKA2*) or temperature-sensitive alleles of *CKA2* (*cka2-ts*) were incubated at permissive (24°C) or nonpermissive (37°C) temperature for 60 min before a 30-min  $^{32}$ P-labeling period. Immunoprecipitation of Clc1p revealed no significant reduction in the amount of label incorporated into Clc1p in *cka2-ts* cells compared with cells with wild-type *CKA2* at either temperature (Figure 3, odd-numbered lanes). Analysis of pheromone-stimulated Clc1p hyperphosphorylation in these strains was complicated by the relatively low level of hyperphosphorylation exhibited by the wild-type strain. However, no consistent effect of the temperature-sensitive mutation on pheromone-stimulated hyperphosphorylation was observed (Figure 3, even-numbered lanes). Although it is possible that casein kinase II may be a minor contributor to Clc1p phosphorylation, it does not appear to be the kinase responsible for the bulk of the modification.

### Serines 52 and 112 Are Major Phosphorylation Sites of Clc1p

The sites of phosphorylation in Clc1p were characterized by subjecting in vivo-labeled Clc1p to phosphoamino acid analysis. Labeled Clc1p was immunoprecipitated and subjected to acid hydrolysis. Two-dimensional thin-layer cellulose electrophoresis of the hydrolysate showed that phosphorylation occurred solely on serine residues (Figure 4, A and B). There are 14 serine residues present in Clc1p (Figure 5). Because serine kinases will often also recognize threonine, a site-directed strategy to convert each serine to threonine was adopted (Kennelly, 1994). Phosphoamino acid analysis of each of the 14 individual S to T *clc1*



**Figure 4.** Clc1p is phosphorylated on serines, primarily at residues 52 and 112. Cells expressing wild-type Clc1p (GPY1080, B), Clc1p S52T (GPY1954, C) or Clc1p S112T (GPY1955, D) were labeled as described in Figure 1A. Immunoprecipitated Clc1p was subjected to acid hydrolysis and two-dimensional thin-layer cellulose chromatography electrophoresis to separate labeled amino acids. (A) Positions of phosphoamino acid standards.

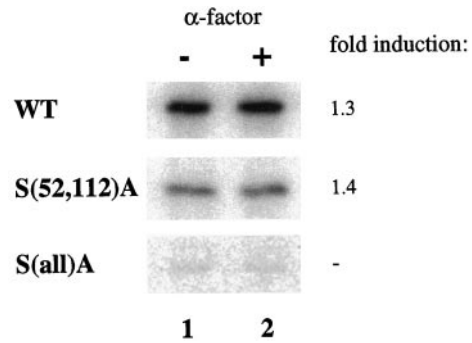
mutants detected phosphorylation of threonines only at positions 52 and 112 (Figure 4, C and D; our unpublished results). In these two mutants, the level of threonine phosphorylation relative to serine phosphorylation did not change in cells treated with  $\alpha$ -factor (our unpublished results). This result suggests that the pheromone-stimulated Clc1p kinase(s) recognizes both serine and threonine. The absence of phosphorylation of threonine replacements at sites other than 52

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TAGATACATA TATATATA ATG TCA GAG AAA TTC CCT CCT TTG GAA GAT CAA
M S E K F P P P L E D Q
2
AAC ATT GAT TTC ACA CCC AAC GAC AAA AAA GAT GAC GAC ACC GAT TTT TTA
N I D F T P N D K K D D D T D F L
AAA AGA GAA GCA GAG ATA CTT GGA GAC GAG GTT CAA GAC GAA CAG GAT GAC
K R E A E I L G D E F K T E Q D D
ATA TTG GAA ACG GAG GCT TCC CCC GCC AAA GAT GAT GAC GAA ATT AGG GAT
I L E T E A S P A K D D D E I R D
52
TTT GAA GAG CAA TTT CCA GAT ATC AAC TCC GCA AAT GGC GCA GTC TCG AGC
F E E Q F P D I N S A N G A V S S
72
GAT CAA AAT GGT AGT GCT ACT GTA TCT AGT GGT AAT GAC AAT GGC GAG GCA
D Q N G S A T V S S G N D N G E A
84
GAT GAT GAT TTT TCC ACA TTC GAA GGT GCC AAT CAG AGT ACA GAA TCC GTT
D D D F S T F E G A N Q S T E S
101
AAA GAG GAT CGT TCT GAA GTT GTA GAC CAA TGG AAA CAA CGT CGT GCT GTA
K E D R S E V V D Q W K Q R R A V
118
GAG ATT CAC GAA AAG GAT TTA AAG GAC GAG GAG TTA AAA AAG GAA TTG CAG
E I H E K D L K D E E L K K E L Q
GAT GAA GCT ATC AAA CAT ATT GAC GAT TTT TAC GAT TCT TAC AAT AAA AAG
D E A I K H I D D F Y D S Y N K K
160
AAG GAA CAA CAA TTG GAA GAT GCT GCT AAG GAA GCT GAG GCT TTC TTA AAG
K E Q Q L E D A A K E A E A F L K
AAA AGA GAT GAA TTT TTT GGT CAA GAC AAT ACG ACC TGG GAT CGT GCA CTT
K R D E F F G Q D N T T W D R A L
CAA TTA ATT AAC CAA GAT GCC GAT ATC AIT GGG GGT AGA GAC AGG TCT
Q L I N Q D D A D I I G G R D R S
215
AAG CTT AAA GAA ATT CTT TTG AGA TTG AAA GGT AAC GCG AAG GCT CCC GGT
K L K E I L L R L K G N A K A P G
GCT TAAATGATGATGAATAATAAGACCATTAATGAACCTAAGGAAGAGAAAGGGCTTAT
A

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**Figure 5.** Amino Acid sequence of Clc1p. Serine residues are shown in outline.



**Figure 6.** S to A mutations in Clc1p reduce phosphorylation. Cells expressing wild-type Clc1p (GPY1970), Clc1p S(52,112)A (GPY1971), or Clc1p S(all)A (GPY1973) integrated at the *LEU2* locus were analyzed as described in Figure 1B.

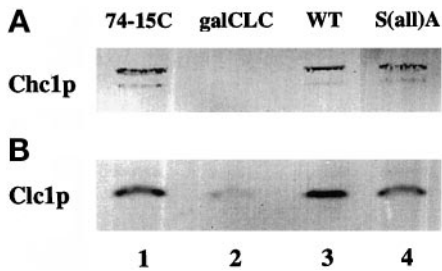
and 112 may be accounted for by kinase(s) that show an especially strong predilection for serines over threonines in a specific context (Kemp *et al.*, 1977; Tuazon and Traugh, 1991).

To further characterize phosphoacceptor sites, the 14 serine residues were also mutated to alanines. Single substitutions did not result in dramatic decreases in the amount of  $^{32}$ P incorporated into Clc1p, even when serine 52 or 112 was changed (our unpublished results). When both serines 52 and 112 were converted to alanine in combination, the level of Clc1p phosphorylation dropped to a level approximately one-third that of wild-type Clc1p, but the degree of hyperphosphorylation was not significantly affected [Figure 6, S(52A,112)A]. Combinations of the S(52,112)A mutations with various S to A changes at other sites revealed that Clc1p is phosphorylated to a small extent on many serine residues throughout the protein. Because of the large number of serine phosphoacceptor sites, and the slight changes in phosphorylation levels resulting from each mutation, we were unable to ascertain exact sites of phosphorylation. Mutagenesis of all serine residues to alanines was necessary to essentially eliminate phosphorylation of Clc1p [Figure 6, S(all)A]. Our results indicate that Clc1p is phosphorylated on multiple serines throughout the protein, with serines 52 and 112 constituting major sites of phosphorylation.

#### *S(all)A Clc1p Associates with HC in Stable Clathrin Trimers*

Because the S(52,112)A mutant retained a substantial level of phosphorylation, we focused on cells expressing the S(all)A mutant to investigate the structural and functional roles of phosphorylation. For this approach, a congenic set of mutant and wild-type strains was constructed. Each strain contained a disrupted version of the endogenous *CLC1* gene and either a single copy





**Figure 7.** Steady-state levels of Chc1p in Clc1p mutants are not significantly altered. Protein extracts were prepared from GPY74-15C (which carries endogenous *CLC1*, lane 1) or GPY1034-19D (*clc1Δ* cells containing pgalCLCURA3, lane 2), GPY1970 (wild-type [WT] *CLC1* integrated at the *LEU2* locus, lane 3), and GPY1973 [S(all)A *clc1* integrated at the *LEU2* locus, lane 4]. Growth of GPY1034-19D cells in 2% glucose repressed Clc1p expression. Extracts were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with either monoclonal antibodies to Chc1p or polyclonal antibodies to Clc1p. The lower band corresponds to a Chc1p degradation product.

of the S(all)A mutant or wild-type *CLC1* integrated at the *LEU2* locus (see MATERIALS AND METHODS). Because steady-state levels of clathrin heavy chain and the stability of clathrin trimers are dependent on Clc1p (Chu *et al.*, 1996; Huang *et al.*, 1997), we analyzed the levels of Clc1p and Chc1p by immunoblotting. Densitometric quantitation of multiple immunoblots demonstrated that steady-state levels of expression from the *LEU2*-integrated wild-type Clc1p are approximately twofold higher than from the endogenous *CLC1* (an example is shown in Figure 7B, lane 1 vs. 3). The levels of Clc1p protein expressed from the *LEU2*-integrated S(all)A Clc1p were decreased by approximately twofold compared with that from the integrated wild-type *CLC1* (Figure 7B, lane 3 vs. 4), resulting in levels comparable with those in cells with only the endogenous *CLC1* (Figure 7B, lane 1 vs. 4). The cause of the differences in Clc1p levels is not clear; the decreased quantity of mutant Clc1p may be due to instability of the mutant protein or, alternatively, due to decreased synthesis caused by mutagenesis, perhaps from the presence of nonideal codons. It is unlikely that the differences detected by immunoblotting reflect differential antibody recognition of mutant and wild-type Clc1p antibodies, because bacterially expressed S(all)A Clc1p was detected by  $\alpha$ -Clc1p antibodies as effectively as bacterially expressed wild-type Clc1p (our unpublished results).

Importantly, the level of Chc1p in mutant cells remained equal to those of wild-type strains, indicating that the slight fluctuation in Clc1p protein levels between strains has no deleterious effects on Chc1p stability (Figure 7A, lanes 1, 3, and 4). By comparison, the steady-state level of Chc1p was undetectable when Clc1p levels were dramatically reduced by glucose repression of cells expressing *CLC1* under control of

the *GAL1* promoter (Figure 7, A and B, lane 2). In addition, when Clc1p was visualized by immunofluorescence, the only difference detected between wild-type and S(all)A strains was a slightly decreased signal for the S(all)A mutant (our unpublished results). Punctate distribution of Clc1p throughout the cytoplasm was consistent between strains in the absence or presence of pheromone.

A coimmunoprecipitation protocol was used to examine the effects of the S(all)A mutations on Clc1p binding to Chc1p. In native conditions, antibodies specific for Clc1p will coprecipitate Chc1p (Pishvaei *et al.*, 1997). However, in this assay, recovery of Chc1p is substoichiometric, because antibody binding to Clc1p appears to destabilize the Chc1p–Clc1p interactions. This destabilizing effect makes coprecipitation of Chc1p highly sensitive to mutations that perturb Clc1p binding to Chc1p; single amino acid changes in either Chc1p or Clc1p can abolish Chc1p coprecipitation (Pishvaei *et al.*, 1997; our unpublished observations). Extracts of wild-type and S(all)A cells were incubated with Clc1p antibodies, and the resulting precipitates were analyzed by immunoblotting with Clc1p and Chc1p antibodies. As controls, the starting extracts were analyzed directly for levels of Chc1p and Clc1p (Figure 8A, lanes 2 and 4). No difference was evident in the levels of Chc1p coprecipitated with Clc1p from the two extracts (Figure 8A, lanes 1 and 3).

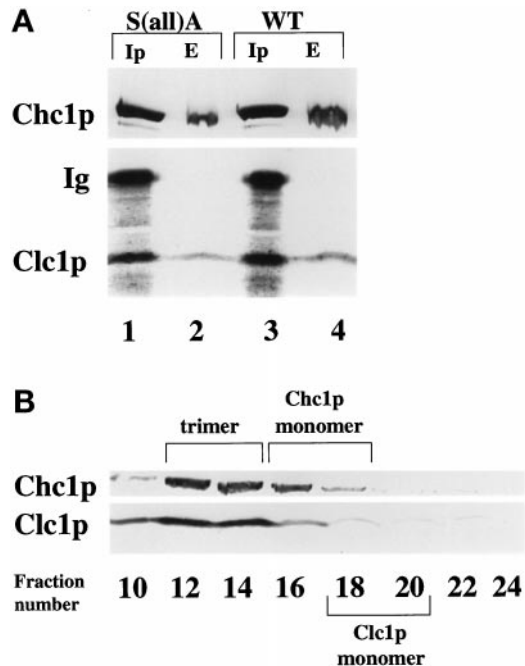
Because Clc1p is necessary for the stability of clathrin trimers (Chu *et al.*, 1996; Huang *et al.*, 1997), we monitored the status of trimerization by size exclusion chromatography on Sepharose CL4B (Figure 8B). Both Chc1p and Clc1p in the S(all)A Clc1p strain coeluted in fractions in which wild-type clathrin trimers elute (Chu *et al.*, 1996; Pishvaei *et al.*, 1997). Thus mutagenesis of the 14 serines in Clc1p to alanines, and the concomitant loss of phosphorylation, does not overtly affect the association of Clc1p with Chc1p to form stable clathrin trimers.

#### *A TGN Membrane Protein Localization Defect Caused by S(all)A Clc1p*

Clathrin-mediated transport processes were assessed in the S(all)A mutant cells to ascertain whether the absence of Clc1p phosphorylation affects clathrin function. Previous analyses of *clc1Δ* strains determined that Clc1p is required for efficient receptor-mediated endocytosis (Chu *et al.*, 1996; Huang *et al.*, 1997). To determine whether Clc1p phosphorylation affects this process, we monitored  $^{35}\text{S}$ -labeled  $\alpha$ -factor internalization in S(all)A mutant and wild-type strains. No difference was apparent in the rate or extent of  $\alpha$ -factor uptake over a 20-min time course in the two strains (Figure 9).

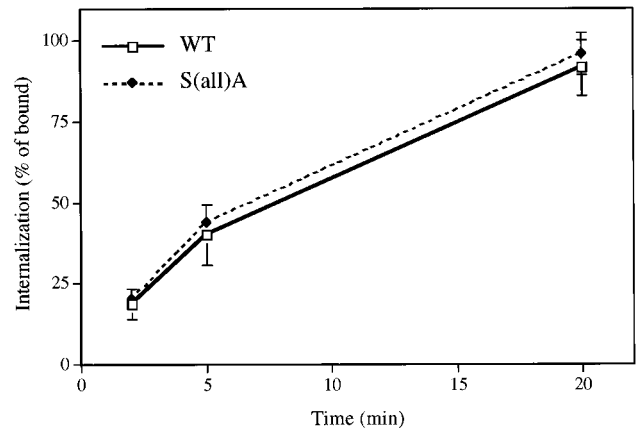
Clathrin-mediated functions at the Golgi complex include sorting of vacuolar proteins to the vacuole and





**Figure 8.** S(all)A Clc1p associates with Chc1p in stable trimers. (A) Antibodies specific for Clc1p were used to coimmunoprecipitate Chc1p (Ip lanes) from extracts of S(all)A mutant cells (GPY1973, lane 1) or wild-type (WT) cells (GPY1970, lane 3). Total extracts (E lanes) representing ~20% of the extract used for coimmunoprecipitation were analyzed directly in lanes 2 (GPY1973) and 4 (GPY1970). The band marked Ig corresponds to Ig heavy chain from the immunoprecipitates that reacts with the secondary antibody used in the immunoblot. (B) Sepharose CL4B column chromatography of extract from GPY1973 cells expressing S(all)A Clc1p. Extracts were prepared according to MATERIALS AND METHODS and fractionated by Sepharose CL4B column chromatography. Selected fractions were precipitated with 10% trichloroacetic acid and analyzed by immunoblotting with monoclonal antibodies to Chc1p or polyclonal antibodies to Clc1p. Column fraction numbers are indicated at the top of the panels. Wild-type clathrin displayed the same fractionation pattern.

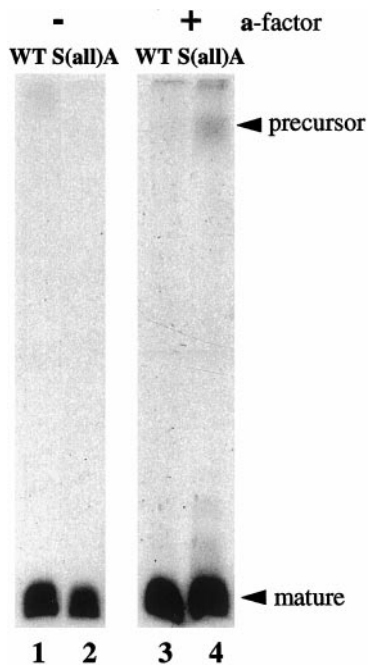
localization of resident TGN membrane proteins (Payne and Schekman, 1989; Seeger and Payne, 1992a,b). We found that the S(all)A Clc1p had no adverse effect on the sorting or delivery of carboxypeptidase Y, a vacuolar protein, to the vacuole (our unpublished results). Clathrin is also required for the efficient localization of TGN proteins such as the Kex2p endoprotease (Payne and Schekman, 1989; Seeger and Payne, 1992b). Kex2p initiates proteolytic maturation of the  $\alpha$ -factor precursor as the precursor passes through the TGN (Fuller *et al.*, 1988). In cells with compromised clathrin function, Kex2p is mislocalized to the cell surface and is less stable than in wild-type cells (Payne and Schekman, 1989; Seeger and Payne, 1992b; Redding *et al.*, 1996b). The consequence of this mislocalization is a decrease in the efficiency of  $\alpha$ -factor precursor maturation leading to secretion of the intact precursor. Thus, defects in



**Figure 9.** Internalization of  $\alpha$ -factor is normal in S(all)A Clc1p cells. Uptake of radiolabeled  $\alpha$ -factor was measured as described in MATERIALS AND METHODS in congenic *clc1* $\Delta$  cells containing an integrated copy of wild-type (WT) *CLC1* (GPY1970) or S(all)A *clc1* (GPY1973). Each experiment was performed in duplicate, and the values at each time point were averaged. Data are the mean  $\pm$  SD for three experiments.

Kex2p localization can be conveniently monitored by evaluating the form of  $\alpha$ -factor that is secreted into the culture medium. After metabolic labeling with [ $^{35}$ S]methionine, only mature  $\alpha$ -factor was immunoprecipitated from the medium of wild-type and S(all)A mutant cells (Figure 10, lanes 1 and 2). Because pheromone treatment stimulates Clc1p hyperphosphorylation, we considered the possibility that phosphorylated Clc1p might be more important in cells undergoing a mating response. To test this idea, maturation of  $\alpha$ -factor was investigated in cells treated with pheromone. Because  $\alpha$ -factor is synthesized only in *MAT* $\alpha$  cells, we treated S(all)A or wild-type *MAT* $\alpha$  strains with preconditioned medium from an  $\alpha$ -factor-overproducing strain. A 1-h pretreatment with  $\alpha$ -factor-conditioned media resulted in a small but reproducible  $\alpha$ -factor maturation defect in the S(all)A mutant (Figure 10, lanes 3 and 4).

Based on the results from the  $\alpha$ -factor maturation experiments, the status of Kex2p was investigated directly by measuring Kex2p stability with a pulse-chase immunoprecipitation protocol. Wild-type and S(all)A cells were pulse labeled with [ $^{35}$ S]methionine/cysteine for 15 min and then subjected to a chase with unlabeled amino acids for 45 or 90 min. Kex2p stability was decreased in the S(all)A mutant cells; at the 90-min chase point the level of Kex2p in mutant cells declined to approximately half the starting level, whereas the level in wild-type cells only dropped to 70% (Figure 11A). When cells were treated with pheromone before the pulse-chase analysis, the stability of Kex2p in the mutant cells was slightly, but reproducibly, decreased even further (Figure 11B). Although small, the difference between Kex2p stability in mu-

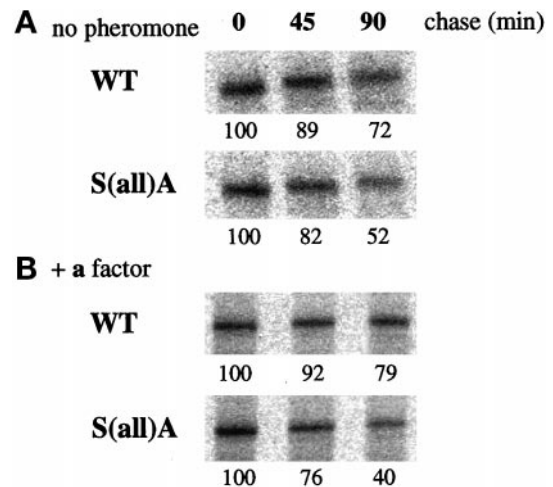


**Figure 10.** Maturation of  $\alpha$ -factor precursor is incomplete in S(all)A Clc1p cells treated with pheromone. GPY1946 (WT *CLC1*, lanes 1 and 3) and GPY1949 [S(all)A *clc1*; lanes 2 and 4] were grown at 30°C, incubated without (lanes 1 and 2) or with (lanes 3 and 4) a-factor for 1 h, and metabolically labeled with [<sup>35</sup>S]cysteine/methionine for 30 min.  $\alpha$ -Factor was immunoprecipitated from the culture supernatant and subjected to SDS-PAGE and phosphoimaging.

tant and wild-type cells and between mutant cells with or without pheromone treatment was observed in three separate experiments. These results suggest that mutation of Clc1p serines to alanines results in a subtle defect in TGN protein localization that is exacerbated in cells undergoing the mating response.

## DISCUSSION

Yeast clathrin light chain, like its mammalian counterparts, is a phosphoprotein. This finding has allowed a genetic approach to probe the functional significance of clathrin light chain phosphorylation *in vivo*. Our results indicate that Clc1p is constitutively phosphorylated in growing cells and undergoes hyperphosphorylation upon activation of the mating response MAP kinase signal transduction pathway. Elimination of Clc1p phosphorylation by mutagenesis of serine residues revealed only a modest defect in the clathrin-mediated localization of a TGN membrane protein. However, in cells expressing the phosphorylation-deficient Clc1p mutant, activation of the mating response pathway exacerbates the TGN membrane protein localization defect. These results suggest a role for Clc1p phosphorylation in modulating clathrin function at the TGN.



**Figure 11.** Kex2p stability is decreased in the S(all)A Clc1p mutant. Congenic strains containing an integrated copy of wild-type (WT) *CLC1* (GPY1946) or S(all)A *clc1* (GPY1949) were grown at 30°C, incubated without (A) or with (B) a-factor, and metabolically labeled with [<sup>35</sup>S]cysteine/methionine for 15 min. Excess unlabeled amino acids were added, and an aliquot of cells was harvested from each sample at 0-, 45-, or 90-min intervals (chase). Cells were lysed, and Kex2p was immunoprecipitated and subjected to SDS-PAGE and phosphoimaging. Numbers below each lane correspond to the relative percentage of Kex2p remaining, normalized to the 0-min chase value.

Both basal and pheromone-stimulated phosphorylation of Clc1p occurs exclusively on serine residues, with major sites mapped to serines at positions 52 and 112. Mutagenesis of S52 and S112 to alanines revealed that a substantial amount of both basal and stimulated phosphorylation, approximately one-third of wild-type levels, occurs at multiple other phosphoacceptor sites. Phenotypic analysis of S(52,112)A cells did not reveal defects in clathrin-dependent transport pathways, even in cells treated with pheromones (our unpublished observations). Thus, all 14 serine residues in Clc1p were converted to alanine to eliminate phosphorylation. Cells expressing this mutant exhibited a defect in localization of the TGN membrane protein Kex2p, suggesting a role for Clc1p phosphorylation in this process. However, the extent of this mutagenesis raises concern that any mutant phenotype could be due to a general perturbation of Clc1p structure by the 14 alanine residues rather than due to a specific effect on phosphate modification. This concern is allayed in part by the naturally resilient structure of wild-type Clc1p; both yeast and mammalian Clcs remain soluble and functional after incubation at 100°C (Silveira *et al.*, 1990; Brodsky *et al.*, 1991). Furthermore, the effect of the S(all)A mutations was relatively specific. Except for defective localization of Kex2p, the mutant Clc1p did not alter growth, receptor-mediated internalization, or vacuolar protein sorting. Additionally, the mutations did not affect Clc1p binding to heavy chain,

clathrin trimer stability, light chain heat stability, or binding to calmodulin (Figures 8 and 9; our unpublished results). These properties argue that the mutations did not have a nonspecific, global effect on Clc1p structure and support our interpretation that the mutant phenotype can be attributed to an absence of Clc1p phosphorylation.

Mislocalization of Kex2p in cells expressing the S(all)A Clc1p provides an indication that Clc1p phosphorylation plays a role in clathrin-mediated localization of membrane proteins in the TGN. The prevailing model for TGN membrane protein localization in yeast suggests that the steady-state distribution of proteins to the TGN involves dynamic cycling between the TGN and a prevacuolar endosomal compartment (Wilsbach and Payne, 1993a; Nothwehr and Stevens, 1994). Clathrin has been proposed to act in this pathway at the TGN by collecting TGN membrane proteins into clathrin-coated vesicles targeted to the endosomes (Wilsbach and Payne, 1993a). In this scenario, phosphorylation of Clc1p could contribute to Kex2p localization in several ways. Phosphorylated Clc1p could slow the assembly of clathrin coats at the TGN, thereby increasing the residence time of Kex2p in the TGN. Phospho-Clc1p could also slow egress of Kex2p from the TGN by shifting the assembled state of clathrin from polyhedral cages into hexagonal planar coats. Planar coats could serve as a stable matrix to anchor Kex2p in the TGN. Alternatively, phospho-Clc1p could promote uncoating of TGN-derived, clathrin-coated vesicles to increase the rate of delivery to endosomes. Fractionation experiments to assess the relative distribution of phospho-Clc1p in soluble and assembled clathrin pools did not reveal significant enrichment in either fraction (our unpublished observations). Additional experiments will be necessary to distinguish between the various models for the role of Clc1p phosphorylation.

Pheromone-stimulated hyperphosphorylation of Clc1p revealed a possible link between modification of Clc1p and clathrin function during the mating response. Consistent with this possibility, the Kex2p localization defect in S(all)A mutant cells was enhanced by pheromone treatment. Although the decline in Kex2p stability caused by pheromone appears small, studies of localization-defective forms of Kex2p indicate that slight changes in Kex2p stability can have profound consequences on the mating process (Redding *et al.*, 1996a). The enhanced localization defect in S(all)A cells treated with pheromones implies that the complex cellular changes that occur during mating place an increased demand on clathrin function at the TGN. The basis for this heightened requirement for clathrin is currently unclear. Possibilities include increased membrane flux through the TGN or recruitment of clathrin to other transport steps such as endocytosis.

One change known to occur upon activation of the mating response pathway is an increase in transcription of the genes encoding precursor  $\alpha$ -factor (Sprague and Thorner, 1992). The resulting rise in levels of  $\alpha$ -factor precursor traversing the TGN might challenge the processing capacity of Kex2p. Such a situation could underlie the  $\alpha$ -factor maturation defect that accompanies the small decline in Kex2p stability in pheromone-treated S(all)A *clc1* cells. This line of reasoning suggests one way in which functional requirements for clathrin could change during mating: to counter the increase in substrate, the level of Kex2p resident in the TGN could be increased through modulation of the clathrin-mediated localization mechanism.

Our results provide some insight into the kinase(s) responsible for basal or pheromone-stimulated Clc1p phosphorylation. From analysis of cells expressing a temperature-sensitive form of casein kinase II, we conclude that this kinase, which has been implicated in phosphorylation of mammalian Lcb, does not contribute significantly to yeast Clc1p phosphorylation or hyperphosphorylation. Preliminary results also eliminate protein kinase C as a Clc1p kinase (our unpublished data). We have observed that disruption of the mating response pathway MAP kinase Fus3p essentially abolished pheromone-stimulated hyperphosphorylation. In contrast, disruption of the partly redundant MAP kinase Kss1p had lesser effects. These results are consistent with recent findings indicating that Fus3p is the MAP kinase dedicated to the mating response, whereas Kss1p principally is involved in the signal transduction pathway mediating invasive growth (Cook *et al.*, 1997; Madhani *et al.*, 1997; Tedford *et al.*, 1997). The strong dependence of Clc1p hyperphosphorylation on Fus3p identifies Clc1p as a target of the mating branch of the MAP kinase cascade and a potentially direct substrate for Fus3p. However, preliminary results from *in vitro* kinase assays did not show Clc1p phosphorylation by Fus3p (Bardwell, Chu, Payne, and Thorner, unpublished results). Thus it is possible that an additional kinase (or kinases) acts as an intermediate between Fus3p and Clc1p. Given the rapidity of pheromone-stimulated Clc1p hyperphosphorylation, such a kinase is likely to be directly activated by the MAP kinase cascade rather than to require transcriptional induction through activation of the mating pathway transcription factor Ste12p. The same argument can be applied against the possibility that pheromone-dependent cross-activation of the cell integrity MAP kinase Mpk1p is responsible for Clc1p hyperphosphorylation, because cross-activation requires new transcription and protein synthesis (Buehrer and Errede, 1997). There is also recent evidence that the mating response pathway and the osmoregulatory MAP kinase pathway share a common MAP kinase kinase kinase, but cross-activation of the



osmoregulatory pathway by pheromone does not occur, making it unlikely that osmoregulatory pathway kinases are involved in Clc1p modification (Posas and Saito, 1997). Thus, the kinase mediating pheromone-elicited Clc1p hyperphosphorylation awaits identification. Nonetheless, hyperphosphorylation of Clc1p represents a novel connection between the mating pathway MAP kinase pathway and membrane trafficking machinery.

Like yeast Clc1p, mammalian LCa and LCb are phosphorylated on serine residues (Usami *et al.*, 1985; Hill *et al.*, 1988; Wilde and Brodsky, 1996). Differential phosphorylation of the mammalian LCs has been proposed to provide a regulatory distinction between the two subunits (Brodsky *et al.*, 1991). LCb is phosphorylated *in vitro* by a coated vesicle-associated casein kinase II-like activity on serines 11 and 13, whereas the phospho-acceptor sites on LCa have not been determined (Schook and Puszkun, 1985; Usami *et al.*, 1985; Bar-Zvi and Branton, 1986; Cantournet *et al.*, 1987; Hill *et al.*, 1988). The absence of specific N-terminal phosphorylation sites in yeast Clc1p and the casein kinase II independence of Clc1p phosphorylation resemble the properties of LCa, which lacks serines 11 and 13 and is not a substrate for casein kinase II *in vitro* (Bar-Zvi and Branton, 1986). Another possible analogy between Clc1p and LCa, phosphorylation induced by extracellular stimuli, is provided by the observation that LCa, but not LCb, is subject to ligand-activated phosphorylation by the EGF receptor *in vitro* (Mooibroek *et al.*, 1992). These similarities suggest that yeast Clc1p may reflect regulatory features of LCa more closely than LCb.

In mammalian cells, phosphorylation of other clathrin coat subunits has been observed (Keen and Black, 1986; Bar-Zvi *et al.*, 1988; Corvera and Capocasale, 1990; Wilde and Brodsky, 1996). In particular, phosphorylation of the adaptor (AP) complexes has been studied. Adaptor complexes play a central role in coat assembly by mediating clathrin binding to membranes through a direct interaction between the AP  $\beta$  subunits and clathrin (Schmid, 1997). Phosphorylation of the AP  $\beta$  subunits inhibits clathrin binding, thus potentially promoting coat disassembly (Wilde and Brodsky, 1996). Together with our results, these findings argue that the multiple phosphorylation targets in clathrin coats provide an important regulatory framework for modulating clathrin-mediated protein traffic.

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