Cytoplasmic Tail Phosphorylation of the α-Factor Receptor Is Required for Its Ubiquitination and Internalization

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Abstract. G protein–coupled (GPC) receptors are phosphorylated in response to ligand binding, a modification that promotes receptor desensitization or downregulation. The α-factor pheromone receptor (Ste2p) of *Saccharomyces cerevisiae* is a GPC receptor that is hyperphosphorylated and ubiquitinated upon binding α-factor. Ubiquitination triggers Ste2p internalization into the endocytic pathway. Here we demonstrate that phosphorylation of Ste2p promotes downregulation by positively regulating ubiquitination and internalization. Serines and a lysine are essential elements of the Ste2p SINNDAKSS internalization signal that can mediate both constitutive and ligand-stimulated endocytosis. The SINNDAKSS serines are required for receptor phosphorylation which, in turn, facilitates ubiquitination of the neighboring lysine. Constitutive phosphorylation is required to promote constitutive internalization, and is also a prerequisite for ligand-induced phosphorylation at or near the SINNDAKSS sequence. Mutants defective in yeast casein kinase I homologues are unable to internalize α-factor, and do not phosphorylate or ubiquitinate the receptor, indicating that these kinases play a direct or indirect role in phosphorylating the receptor. Finally, we provide evidence that the primary function of phosphorylation controlled by the SINNDAKSS sequence is to trigger receptor internalization, demonstrating that phosphorylation-dependent endocytosis is an important mechanism for the downregulation of GPC receptor activity.

Cell surface receptors coupled to heterotrimeric G proteins receive and transmit extracellular signals into the interior of the cell through the recognition and binding of specific ligands. Once cells receive and act upon a signal transmitted through receptor–ligand binding, they must return to a basal, unstimulated state for the appropriate regulation of growth and differentiation. Cells become desensitized to a signal and downregulate their response to it by a variety of mechanisms. Several components involved in initiating signal transduction are targets of downregulation, including the heterotrimeric G protein and the G protein–coupled (GPC)[1] receptor itself. These components are generally downregulated by either modification and/or degradation.

Both G protein subunits and receptors become phosphorylated in response to ligand binding and this modification plays an important role in signal desensitization (Cole and Reed, 1991; Lefkowitz, 1993). Phosphorylation of GPC receptors promotes desensitization by both uncoupling the receptor from its heterotrimeric G protein (for review see Dohlman et al., 1991) and by facilitating receptor internalization (Ferguson et al., 1995; Naik et al., 1997; Pals-Rylaarsdam and Hosey, 1997). However, although most G protein–coupled receptors undergo ligand-stimulated phosphorylation, the role of phosphorylation in receptor desensitization varies. In addition, the signals that stimulate GPC receptor internalization, and the fate of the protein once it enters the cell, differ from receptor to receptor.

The downregulation of the G protein–coupled α-factor receptor of *Saccharomyces cerevisiae* is triggered by a novel internalization signal that requires modification of the receptor tail with the polypeptide ubiquitin (Hicke and Riezman, 1996). The α-factor receptor (Ste2p), which is expressed on the surface of **a** cells, stimulates the mating response pathway upon binding the 13-amino acid pheromone secreted by α cells (for review see Bardwell et al., 1994). This receptor is constitutively internalized and degraded in the lysosome-like vacuole in the absence of ligand, and its internalization rate is stimulated ~10-fold in the presence of pheromone. Ligand-stimulated internalization also results in transport of the receptor to the vacuole; there is no evidence that Ste2p recycles from endosomes to the plasma membrane (Jenness et al., 1986; Singer and Riezman, 1990; Schandel and Jenness, 1994). Ste2p is modified in two ways in response to α-factor binding: (*a*) its cytoplasmic tail becomes hyperphosphorylated

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1. *Abbreviations used in this paper*: CIP, calf intestinal alkaline phosphatase; GPC, G protein–coupled.

on serine and threonine residues, and (*b*) it is ubiquitinated on lysine residues (Reneke et al., 1988; Hicke and Riezman, 1996).

Ubiquitination of the α-factor receptor signals its ligand-stimulated entry into the endocytic pathway. The role of internalization signal is a new function for ubiquitin, a highly conserved 76-amino acid polypeptide that has been well characterized as a recognition tag for the degradation of intracellular proteins by the multisubunit proteolytic particle known as the 26S proteasome (for review see Ciechanover, 1994; Hochstrasser, 1996). Ubiquitin serves as an internalization signal for multiple plasma membrane proteins in yeast (Galan et al., 1996; Roth and Davis, 1996; Kölling and Losko, 1997), and ubiquitin-dependent internalization has also been shown to occur in mammalian cells to promote the downregulation of a tyrosine kinase signaling receptor (Strous et al., 1996).

In previous work we observed that the mutation of serine residues within a well-defined α-factor receptor internalization signal, the SINNDAKSS sequence, abrogated the ability of this sequence to be ubiquitinated and to promote receptor internalization (Hicke and Riezman, 1996). In this paper we report that receptor phosphorylation controlled by the SINNDAKSS serines promotes receptor internalization. Phosphorylation of the receptor cytoplasmic tail positively regulates ubiquitination at neighboring lysines and these modifications are required for both constitutive and stimulated receptor internalization.

Materials and Methods

Strains, Plasmids, Media, and Reagents

Mutations were introduced into the wild-type or 345Stop truncated form of Ste2p as described (Rohrer et al., 1993) except Pfu DNA polymerase (Stratagene, La Jolla, CA) was used. The sequence of the PCR-amplified part of the resulting plasmids was determined to ensure that the expected mutations had been introduced and not any others. Plasmids containing the *STE2* variants were introduced into the *ura3* locus of strain RH3162 by single-step gene transplacement. The mutant Ste2 proteins were each able to complement the *ste2Δ* mating defect of the parent strain. Two individual transformants of each mutant were assayed for their ability to internalize α-factor and in each case both transformants demonstrated similar internalization kinetics.

The *yck1Δ yck2-2* strain was generously provided by L. Robinson (Louisiana State University Medical Center, New Orleans, LA) and was crossed twice to our wild-type background to generate strains RH3589 (*yck1Δ yck2-2*) and RH3992 (*YCK1 YCK2*). The genotypes of all strains used for the experiments described in this paper are listed in Table I.

YPUAD rich medium and SD minimal medium have been described (Zanolari et al., 1992; Hicke and Riezman, 1996). SD-no phos buffer was SD medium with KCl substituted for potassium phosphate and buffered with 7 mM succinate, pH 5.8. SD-low phos buffer was SD-no phos buffer with the addition of 50 mM KH_2PO_4.

Protein phosphatase 1 was obtained from Boehringer Mannheim GmbH (Mannheim, Germany) and calf intestinal alkaline phosphatase (CIP) was from New England Biolabs Inc. (Beverly, MA). EXPRE^{35}S^{35}S protein labeling mix was from New England Nuclear Life Science Products (Boston, MA), $H_3{}^{32}PO_4$ and Tran^{35}SLabel were from ICN Pharmaceuticals Inc. (Irvine, CA), and $H_2{}^{35}SO_4$ was from Amersham Pharmacia Biotech. Inc. (Piscataway, NJ). The purification of ^{35}S-labeled α-factor (Singer and Riezman, 1990; Dulic et al., 1991) and of affinity-purified Ste2p antiserum (Hicke and Riezman, 1996) have been described previously.

α-Factor Internalization Assays

All assays were performed as previously described (Dulic et al., 1991) on strains that were propagated overnight in YPUAD. Briefly, cells were grown at 24° or 30°C to a density of 0.5–2 × 10^7 cells/ml. Cells were harvested, washed in YPUAD, and then resuspended to 5 × 10^8 cells/ml in ice-cold YPUAD. ^{35}S-labeled α-factor was added and allowed to bind to cells for 45 min on ice. Unbound pheromone was removed by centrifugation at 4°C, and cells were resuspended to 5 × 10^8 cells/ml in YPUAD prewarmed to 30°C. Aliquots of cells were withdrawn after different times, washed in pH 1.0 buffer to remove surface-bound α-factor, or in pH 6.0 buffer, filtered, and then the amount of cell-associated radioactivity was determined by scintillation counting. To assay internalization in the *end4* and *yck1Δ yck2-2* mutant strains, cells were harvested and washed as above, then resuspended in 37°C YPUAD and incubated for 15 min at 37°C. ^{35}S-labeled α-factor was added and the assay was performed as described above. A time course of internalization was generated for each strain by expressing the amount of internalized α-factor as a ratio of cpm detected in pH 1.0–washed cells to that detected in pH 6.0–washed cells at each time point.

Table I. Yeast Strains

Strain*	Genotype
RH448	*ura3 leu2 his4 lys2 bar1-1*
RH1298	*ste2::LEU2 ura3 leu2 his4 bar1-1*
RH1597	*end4-1 ura3 leu2 his4 bar1-1*
RH1965	*end4::LEU2 ura3 leu2 his4 lys2 bar1-1*
RH3162	*ste2::LEU2 ura3 leu2 his3 trp1 bar1-1*
RH3180	*ura3::ste2-345Stop[URA3],* same as RH3162
RH3181	*ura3::ste2-K337R,345Stop[URA3],* same as RH3162
RH3333	*ura3::ste2-S331A,S338A,S339A,345Stop[URA3],* same as RH3162
RH3510	*ura3::ste2-S331E,S338E,S339E,345Stop[URA3],* same as RH3162
RH3511	*ura3::ste2-S331D,S338D,S339D,345Stop[URA3],* same as RH3162
RH3589	*yck1Δ yck2-2 ura3 leu2 his3 ade2 bar1-1*
RH3687	*ura3::ste2-S331A,S338A,S339A[URA3],* same as RH3162
RH3992	*ura3 leu2 his3 lys2 trp1 bar1-1*
LHY636	*ura3::ste2-S331E,S338E,S339E,345Stop[URA3],* same as RH3162
LHY638	*ura3::ste2-345Stop[URA3],* same as RH3162
LHY639	*ura3::ste2-S331A,S338A,S339A,345Stop[URA3],* same as RH3162

*All strains listed are *MATa*.

Receptor Clearance Assays

The measurement of receptors cleared from the cell surface in the absence of α-factor was performed as described (Jenness and Spatrick, 1986; Rohrer et al., 1993) with the following modifications: cells were propagated as described for internalization assays, collected by centrifugation, and then resuspended in YPUAD to 5 × 10^6 cells/ml. After incubation for 5 min at 30°C, cycloheximide was added to 20 μg/ml to inhibit new receptor synthesis and then incubation was continued at 30°C. To measure ligand-stimulated receptor clearance, α-factor was added to a final concentration of 10^{-6} M. At different time points, aliquots of 5 × 10^7 cells were removed and then filtered onto nitrocellulose disks. The filters were incubated in YPUAD with 10 mM NaN_3 and 10 mM NaF to remove α-factor that remained bound to cell surface receptors. The filters were removed and the cell suspension was centrifuged, resuspended in YPUAD/10 mM NaN_3 with 10 mM NaF, and then incubated with ^{35}S-labeled α-factor alone or ^{35}S-labeled α-factor plus 4 × 10^{-5} M unlabeled α-factor. Receptor clearance assays were performed two or three times for each experiment. Although the rate of clearance for each strain varied slightly from experiment to experiment, the differences in clearance rates between strains were reproducible.

α-Factor Recovery Assays

α-Factor recovery assays were performed in a manner similar to that described previously (Rohrer et al., 1993). Briefly, cells were propagated overnight in YPUAD at 24°C to ~10^7 cells/ml, diluted to 2 × 10^6 cells/ml in YPUAD, and then incubated with 2.5 × 10^{-8} M synthetic α-factor at 30°C for 2 h to arrest cells in the G1 phase of the cell cycle. Cells arrest at this phase of the cell cycle without buds. Cells were washed with medium

harvested from a *MAT**a*** yeast culture that contained the secreted Bar1 protease. Bar1p degrades α-factor and this medium was used to remove all pheromone bound to the arrested cells. Washed cells were resuspended in the same volume of YPUAD used for α-factor incubation and then incubated at 30°C. To measure recovery from the pheromone-induced G1 arrest, the number of budded cells in each culture was determined by counting cells in a hemacytometer at various times after resuspension.

Immunoblots and Immuneprecipitations

Immunoblots on *end4Δ*, *ste2Δ*, and wild-type cell extracts to detect the α-factor receptor were carried out as described previously (Hicke and Riezman, 1996). The immunoblot performed on extracts of *end4-1* and *yck1Δ yck2-2* cells was done as described except cells were preincubated for 15 min at 37°C before harvesting the no α-factor sample. Incubation was continued at 37°C after the addition of α-factor.

For the phosphatase treatment of radiolabeled α-factor receptors, immuneprecipitations of receptors from cell extracts prepared before and after exposure to α-factor were performed as described (Hicke and Riezman, 1996), except only a single round of precipitation was done, and the washed immuneprecipitates were not immediately eluted from protein A–Sepharose beads. For treatment with CIP, the precipitates were washed twice in CIP buffer (50 mM Tris-HCl, pH 8.6, 1 mM $MgCl_2$, 1 mM PMSF, 1 μg/ml each of pepstatin, antipain, and leupeptin), resuspended in 50 μl of the same buffer, and then incubated with 30 U CIP for 1 h at 37°C. For treatment with protein phosphatase 1, the beads were washed with protein phosphatase 1 buffer (50 mM Tris-HCl, pH 7.0, 0.1 mM EDTA, 5 mM DTT, 0.2 mM $MnCl_2$, 200 μg/ml BSA, 1 mM PMSF, 1 μg/ml each of pepstatin, antipain, and leupeptin), resuspended in 30 μl of the same buffer, and then incubated with 1 mU protein phosphatase 1 for 30 min at 30°C. After the phosphatase treatment, the precipitated proteins were eluted from the beads in 9 M urea, 40 mM Tris-HCl, pH 6.8, 0.1 mM EDTA, 5% SDS by heating for 10 min at 37°C and then resolved by SDS-PAGE on 10 or 12.5% acrylamide gels. The proteins were visualized by exposure of the dried gel to X-OMAT film (Eastman Kodak Co., Rochester, NY).

Parallel radiolabeling of strains with $H_3{}^{32}PO_4$ and Tran^{35}SLabel was performed as described previously (Zanolari et al., 1992) with the following exceptions: After incubation in SD-low phos buffer, cells were harvested, washed in SD-no phos, and then split into two aliquots. One aliquot was resuspended in 2 ml of SD-no phos and subsequently labeled with 1 mCi $H_3{}^{32}PO_4$; the other aliquot was resuspended in 2 ml SD-low phos and then radiolabeled with 100 μCi Tran^{35}SLabel. Radiolabeling was performed for 1 h and then each labeled sample was split into two equal volumes. One volume was immediately transferred to a tube containing a one-tenth vol of 10×Stop and then frozen in liquid N_2. The other volume was treated with 10^{-6} M synthetic α-factor for 10 min at 30°C and then collected in the same manner. After lysis of cells in each sample by mechanical agitation with glass beads in 100 μl of breaking buffer (9 M urea, 40 mM Tris-HCl, pH 6.8, 0.1 mM EDTA, 140 mM 2-mercaptoethanol), an additional 100 μl of breaking buffer and SDS to 1% were added, and then the extracts were heated to 37°C for 10 min. Immuneprecipitations of receptors from these extracts were performed as described. Radiolabeled receptors were detected using a Storm 860 PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

Results

Serines and Lysine Are Essential Components of the SINNDAKSS Internalization Signal Required for Both Constitutive and Ligand-stimulated Endocytosis

A truncated version of Ste2p (345Stop) that has lost approximately two-thirds of its cytoplasmic tail, yet is internalized rapidly in response to pheromone, carries a single well-defined internalization signal (SINNDAKSS) that is both necessary and sufficient for receptor endocytosis (Rohrer et al., 1993). The sequence of the truncated receptor tail is shown in Fig. 1. Ubiquitination of the SINNDAKSS lysine (K337) is required to trigger 345Stop receptor internalization (Hicke and Riezman, 1996). Mutation of single serine residues within the SINNDAKSS sequence leads to small effects on the rate of receptor internalization (Rohrer et al., 1993) and simultaneous mutation of all three SINNDAKSS serines to alanine severely retards ligand-stimulated receptor internalization, indicating that these residues serve a redundant function within the internalization sequence. These serines are required for internalization because they promote ubiquitination at K337 in the SINNDAKSS sequence (Hicke and Riezman, 1996). Like the wild-type receptor, the 345Stop receptor undergoes slow constitutive endocytosis in the absence of pheromone. To determine whether the serines and lysine within the SINNDAKSS sequence also mediate constitutive internalization of the receptor, we assayed the ability of mutant receptors carrying the triple serine to alanine mutation 3S→A, 345Stop (S331A, S338A, S339A, 345Stop), or a mutation of K337 to arginine (K337R, 345Stop), to be cleared from the cell surface in the absence of α-factor. The receptor clearance assay we used measured the number of α-factor binding sites that remained on the cell surface after cells were treated with cycloheximide to inhibit new receptor synthesis. The 345Stop truncated receptor was constitutively internalized with a half-time of ~1 h. In contrast, both the K337R and 3S→A variants of this receptor were not internalized constitutively (Fig. 2 *A*). These data suggested that modification of the 345Stop receptor tail with ubiquitin was required for constitutive internalization. Previously we demonstrated that ubiquitinated forms of Ste2p accumulated in response to α-factor stimulation in *end4Δ* mutant cells that cannot internalize proteins from the plasma membrane. To test whether Ste2p is ubiquitinated in the absence of α-factor, we performed immunoblots on extracts prepared from wild-type and *end4Δ* cells expressing full-length receptor that had never been exposed to pheromone. Fig. 2 *B* shows that higher molecular weight forms of Ste2p were detected in *end4Δ* cells. These forms were previously shown to be ubiquitinated Ste2p by demonstrating that they were diminished in cells that lack ubiquitin-conjugating machinery and that they specifically precipitated with antiubiquitin antiserum (Hicke and Riezman, 1996). Ubiquitinated Ste2p forms were absent in *ste2Δ* cells that did not express the receptor and were observed at a very low level in wild-type cells. The detection of ubiquitinated full-length Ste2p in unstimulated cells suggests that ubiquitination can signal constitutive internalization of the full-length α-factor receptor as well as that of the 345Stop truncated receptor.

345Stop	AANNASKTNTITSDFTTSTDRFYRGTLSSPQTD **SINNDAKSS** LRSRL
K337R,345Stop	AANNASKTNTITSDFTTSTDRFYRGTLSSPQTD **SINNDARSS** LRSRL
3S→A,345Stop	AANNASKTNTITSDFTTSTDRFYRGTLSSPQTD **AINNDAKAA** LRSRL
3S→D,345Stop	AANNASKTNTITSDFTTSTDRFYRGTLSSPQTD **DINNDAKDD** LRSRL
3S→E,345Stop	AANNASKTNTITSDFTTSTDRFYRGTLSSPQTD **EINNDAKEE** LRSRL

Figure 1. Sequences of mutant Ste2p cytoplasmic tails.

Serines within the SINNDAKSS Internalization Signal Are Required for Constitutive Receptor Phosphorylation, Which Is a Prerequisite for Ligand-stimulated Hyperphosphorylation and Ubiquitination

Serine phosphorylation has been shown to positively regu-

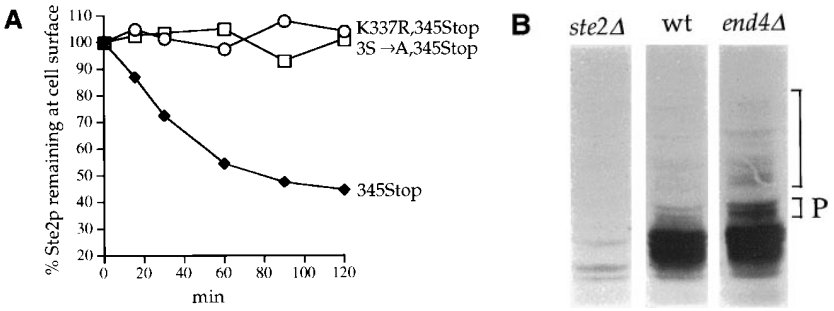


Figure 2. Ubiquitination mediates constitutive receptor internalization. (*A*) The SINNDAKSS serines and lysine are required for internalization of the 345Stop truncated receptor in the absence of α-factor. Strains expressing different forms of the 345Stop receptor (RH3180, RH3181, and RH3333) were treated with cycloheximide to inhibit new receptor synthesis and then harvested in the presence of energy poisons at various times. The relative number of receptors remaining at the plasma membrane at each time was determined by incubating cells with radiolabeled α-factor to measure the extent of cell surface pheromone binding. This value is expressed as the amount of α-factor bound at each time point relative to that bound at $t = 0$ min. This experiment is representative of three independent experiments performed on these strains. 345Stop, (*closed diamonds*); K337R, 345Stop, (*open circles*); 3S→A, 345Stop (*open squares*). (*B*) The α-factor receptor is ubiquitinated in the absence of pheromone. Cell extracts were prepared from wild-type (RH448), *end4Δ* (RH1965), and *ste2Δ* (RH1298) strains that had never been exposed to α-factor. Immunoblots were prepared from these extracts and then probed with anti-Ste2p antiserum. Ubiquitinated and phosphorylated forms of the receptor are indicated by brackets (Hicke and Riezman, 1996; Terrell et al., 1998).

late the ubiquitination of cytosolic proteins, such as the cyclins and the transcription factor inhibitor IκB, which undergo stimulated or regulated modification with ubiquitin (Chen et al., 1995; Deshaies et al., 1995; Yaglom et al., 1995). The α-factor receptor is known to be constitutively phosphorylated and to become hyperphosphorylated in response to α-factor binding (Reneke et al., 1988). These two observations led to the idea that the SINNDAKSS serines may be required for receptor internalization because they are sites of phosphorylation that regulate ubiquitination at the SINNDAKSS lysine (Hicke and Riezman, 1996). To test whether mutations in these serines affect phosphorylation of the 345Stop truncated receptor, we immunoprecipitated different variants of the 345Stop receptor before and after exposure to α-factor, and then treated the immunoprecipitates with protein phosphatase (Fig. 3). The mobility of the 345Stop truncated receptor that had not been exposed to α-factor was increased upon incubation with protein phosphatase 1 (Fig. 3, lanes *1* and *3*), an enzyme that dephosphorylates serine and threonine residues. This indicated that the truncated receptor was constitutively phosphorylated. The dephosphorylated 345Stop receptor migrated as a doublet, as does the full-length receptor, perhaps due to heterogeneous glycosylation of the protein (Blumer et al., 1988; Konopka et al., 1988; David et al., 1997). Upon binding pheromone, several new species of the 345Stop receptor with decreased mobility appeared (Fig. 3, lane *2*). These new species were due to serine/threonine hyperphosphorylation because the pheromone-stimulated receptor was reduced to the same relative mobility as unstimulated receptor by incubation with protein phosphatase (Fig. 3, lane *4*). Like the 345Stop receptor, the K337R, 345Stop receptor was phosphorylated constitutively and in response to α-factor (Fig. 3, lanes *5–8*). Since the K337R, 345Stop receptor is unable to be ubiquitinated, this observation indicates that ubiquitination is not required for receptor phosphorylation. In contrast, the 3S→A, 345Stop receptor migrated with a lower molecular weight than the 345Stop receptor in both the absence and presence of α-factor (Fig. 3, lanes *9* and *10*). In addition, the mobility of the 3S→A, 345Stop receptor did not shift upon incubation with phosphatase in either case (Fig. 3, lanes *11* and *12*). These results indicated that the 3S→A, 345Stop receptor was not phosphorylated constitutively or in response to pheromone binding.

To test the role of the SINNDAKSS serines in the full-length Ste2p cytoplasmic tail, which contains a total of fifteen serines and nineteen threonines, we mutated these serines in wild-type Ste2p and then tested the ability of the 3S→A receptor (S331A, S338A, S339A) to be internalized in the absence and presence of α-factor. Fig. 4, *A* and *B* shows assays that measured the ability of wild-type receptors and 3S→A receptors to be cleared from the cell surface in the absence and presence of pheromone. The 3S→A mutations reduced the rate of constitutive internalization of the full-length receptor approximately threefold (Fig. 4 *A*). These mutations reduced the rate of ligand-stimulated receptor removal from the cell surface to a small extent (Fig. 4 *B*). A direct assay of α-factor ligand internalization confirmed the slower rate of stimulated endocytosis by the 3S→A receptor (Fig. 4 *C*). Receptor clearance assays measure the number of receptors present

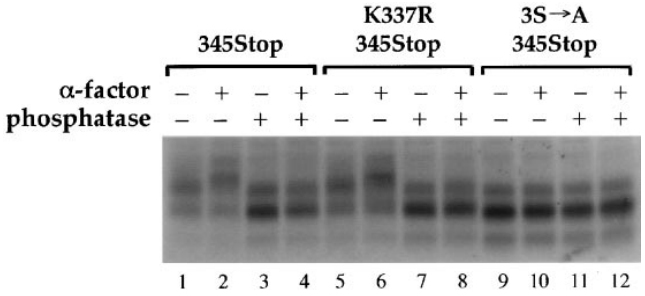


Figure 3. The SINNDAKSS serines are required for receptor phosphorylation. Incubation of mutant 345Stop receptors with protein phosphatase. Cells expressing different variants of the 345Stop α-factor receptor were radiolabeled with EXPRE^{35}S^{35}S protein–labeling mix and then lysed before (−) or 10 min after (+) exposure to α-factor. The receptors were then isolated by immunoprecipitation, incubated with phosphatase (+) or subjected to a mock incubation (−), resolved by SDS-PAGE, and then detected by autoradiography. The experiment shown here was performed with protein phosphatase 1 that is specific for serine/threonine phosphorylation; however, identical results were obtained with alkaline phosphatase.

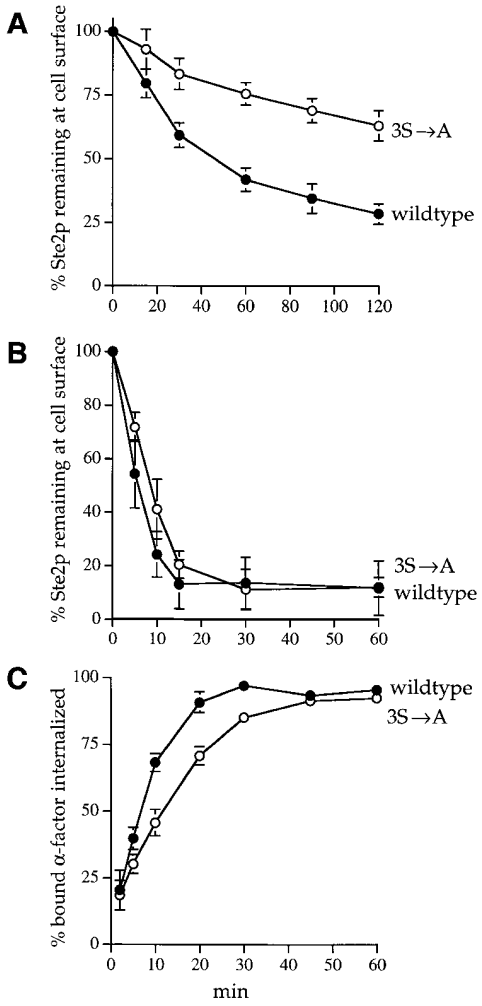


Figure 4. Mutation of the SINNDAKSS serines diminishes both constitutive and stimulated receptor internalization rates in the context of the full-length Ste2p tail. (*A*) The clearance of wild-type (RH448, *closed circles*) and 3S→A (RH3687, *open circles*) receptors from the cell surface in the absence of α-factor.(*B*) The clearance of wild-type (*closed circles*) and 3S→A (*open circles*) receptors from the cell surface in the presence of α-factor. (*C*) α-Factor ligand internalization assays performed on cells expressing either wild-type (*closed circles*) or 3S→A receptors (*open circles*). All curves represent the average of three independent experiments.

at the cell surface that have not been internalized, in addition to those that may have been returned to the cell surface by recycling. The wild-type α-factor receptor is not recycled after internalization (Jenness and Spatrick, 1986; Schandel and Jenness, 1994); however, it was possible that a mutation in the receptor may divert it into a putative recycling pathway. The almost complete clearance of the 3S→A receptor from the cell surface in the presence of α-factor (Fig. 4 *B*) suggested that these mutations did not induce the receptor to recycle but impaired its ability to be internalized. In addition, the 3S→A receptor that was constitutively cleared from the cell surface was degraded (data not shown) and not accumulated intact in the cell as would be expected for a receptor on a recycling pathway. Therefore, the SINNDAKSS serines are important regulators of internalization within the wild-type tail, primarily in the absence of ligand. There may be additional serines distal to amino acid 345 that also serve this function.

Negatively Charged Amino Acids Partially Substitute for Serines in the SINNDAKSS Internalization Signal

The inability of the 3S→A, 345Stop mutant to be phosphorylated suggested that the three SINNDAKSS serines are sites of phosphorylation that positively regulate ubiquitination and internalization. To determine whether negatively charged amino acids that might mimic phosphorylated serines substitute for the SINNDAKSS serines in regulating internalization, we mutated these serines to aspartate or glutamate (refer to Fig. 1). Fig. 5 *A* shows α-factor internalization assays on cells expressing the 3S→D, 345Stop, 3S→E, 345Stop, or 3S→A, 345Stop mutants. The mutations to aspartate or glutamate partially restored the ability of the 345Stop receptor to be internalized in response to pheromone binding. We then assayed the ability of the 3S→E, 345Stop mutant to be internalized in the absence of pheromone. Fig. 5 *B* indicates that this mutation did not restore constitutive internalization. The same result was observed for the 3S→D, 345Stop receptor (data not shown). To determine why the substitution of glutamate for serine in the SINNDAKSS sequence restored stimulated endocytosis, but not constitutive endocytosis, we analyzed the phosphorylation state of the 3S→E, 345Stop receptor by treating radiolabeled immunoprecipitated receptors with phosphatase. Fig. 6 *A* shows that, unlike the 345Stop receptor, the unstimulated 3S→E, 345Stop receptor migrated with a similar, though not identical, mobility before and after incubation with alkaline phosphatase, indicating that this receptor was not constitutively phosphorylated to the same extent as the 345Stop receptor (Fig. 6 *A*, compare lanes *1* and *5*). After treatment with α-factor, higher molecular weight forms of the 3S→E, 345Stop receptor appeared (Fig. 6 *A*, lane *2*). The size of these higher molecular weight forms of 3S→E, 345Stop receptor decreased upon treatment with phosphatase (Fig. 6 *A*, lane *8*), indicating that these forms were phosphorylated.

A more quantitative measurement of the relative phosphorylation state of the 3S→E, 345Stop, 3S→A, 345Stop, and 345Stop receptors was obtained by radiolabeling cells expressing the different receptors with $H_3{}^{32}PO_4$ followed by immunoprecipitation of the phosphorylated receptors. To compare the levels of receptors expressed in the different strains, identical aliquots of each culture were also radiolabeled with ^{35}[S]methionine. Fig. 6 *B* shows the ^{32}P-labeled and ^{35}S-labeled receptors isolated before and after treatment with pheromone. The 345Stop receptor was labeled strongly with radioactive phosphate in the presence and absence of α-factor (Fig. 6 *B*, lanes *3* and *4*). The 3S→A, 345Stop receptor was not labeled significantly with phosphate (Fig. 6 *B*, lanes *5* and *6*), confirming that this receptor underwent neither constitutive or stimulated phosphorylation. The 3S→E, 345Stop receptor was not phosphorylated as heavily as the 345Stop receptor, although phosphorylation increased upon binding of α-factor (Fig. 6 *B*, lanes *1* and *2*). The level of 3S→E, 345Stop receptor expression, however, as measured by the amount of ^{35}S-labeled

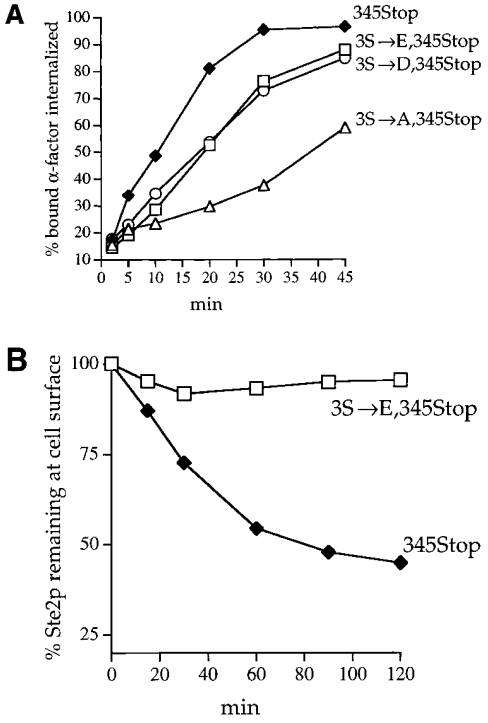
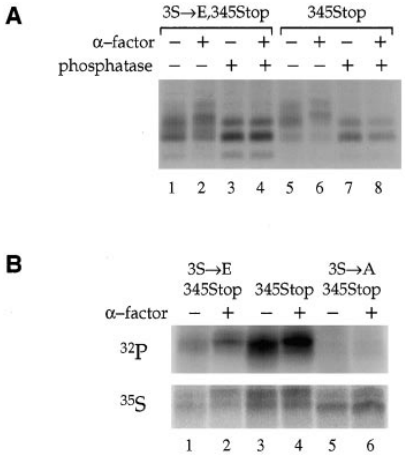


Figure 5. The substitution of negatively charged amino acids for the SINNDAKSS serines rescues ligand-stimulated but not constitutive receptor internalization. (*A*) α-Factor internalization assays performed on strains expressing 345Stop (*closed diamonds*), 3S→A, 345Stop (*open triangles*), 3S→E, 345Stop (*open squares*), or 3S→D, 345Stop (*open circles*) receptors. These curves represent the averages of at least two independent experiments. (*B*) Receptor clearance assays performed in the absence of pheromone on strains expressing 345Stop (*closed diamonds*) or 3S→E, 345Stop (*open squares*) receptors. These curves are representative of three independent experiments.



C

Receptor	αF	Level of phosphorylation (arbitrary units normalized to amount of expressed receptor)	Phosphorylation relative to 345Stop receptor
345Stop	—	1,680,000	1
345Stop	+	1,860,000	1.1
3S→A, 345Stop	—	347,000	0.2
3S→A, 345Stop	+	161,000	0.1
3S→E, 345Stop	—	986,000	0.6
3S→E, 345Stop	+	1,380,000	0.8

Figure 6. The substitution of negatively charged amino acids for the SINNDAKSS serines rescues ligand-stimulated receptor phosphorylation. (*A*) Cells expressing 345Stop (RH3180) or 3S→E, 345Stop (RH3510) receptors were radiolabeled with EXPRE^{35}S^{35}S protein–labeling mix and then lysed before (−) or 10 min after (+) incubation with α-factor. Radiolabeled receptors were isolated from cell extracts by immunoprecipitation. The immunoprecipitates were then incubated with alkaline phosphatase (+) or subjected to a mock incubation (−), resolved by SDS-PAGE, and then receptors were detected by autoradiography. (*B*) Cultures of cells expressing 345Stop (LHY638), 3S→A, 345Stop (LHY639), or 3S→E, 345Stop (LHY636) receptors were propagated and then split into two identical aliquots. One aliquot was metabolically radiolabeled with $H_3{}^{32}PO_4$; the other aliquot was radiolabeled with Tran^{35}SLabel protein–labeling mix. Radiolabeled cells were lysed before (−) or 10 min after (+) incubation with α-factor and then receptors were immunoprecipitated and resolved by SDS-PAGE. The mobility difference between phosphorylated and nonphosphorylated forms of the receptor is not as pronounced as observed in Fig. 6 *A* because a different percentage polyacrylamide gel was used to resolve these receptors. (*C*) The amount of radioactive receptor detected in each immunoprecipitation was determined by analysis with ImageQuant software (Molecular Dynamics). The level of phosphorylated receptor precipitated was normalized to the amount of ^{35}S-labeled receptor precipitated from each strain to correct for differences in the expression level of different receptors. The relative level of phosphorylation in each sample is expressed as a ratio of the phosphorylation of the 345Stop receptor in the absence of α-factor.

receptor precipitated from each strain, appeared to be lower that than of the other receptors. To quantify the relative levels of phosphorylation, the amount of ^{32}P-labeled receptor detected was normalized to the level of ^{35}S-labeled receptor precipitated from the same strain (Fig. 6 *C*). These data indicate that the 3S→E, 345Stop receptor was constitutively phosphorylated approximately twofold less than the 345Stop receptor, and underwent ligand-stimulated phosphorylation to a level that was ~75% of that of the stimulated 345Stop receptor. These observations suggest that constitutive phosphorylation, or negatively charged amino acids, in the SINNDAKSS sequence are required for phosphorylation at non-SINNDAKSS serines. The stimulated phosphorylation that occurs at non-SINNDAKSS serines in the 3S→E mutant can explain the ability of this receptor to undergo ligand-stimulated internalization.

α-Factor Receptor Modification and Internalization Requires Yeast Casein Kinase I Activity

The kinase(s) that phosphorylates the α-factor receptor has eluded identification. However, Panek and co-workers have recently demonstrated that constitutive internalization of the **a**-factor receptor (Ste3p), which is also phosphorylated and ubiquitinated (Roth and Davis, 1996), is blocked in a mutant that lacks activity of the yeast casein kinase I homologues (Yck1p and Yck2p) (Panek et al., 1997). To determine whether mutations in the *YCK* genes also affect Ste2p internalization, we assayed the ability of a mutant that lacks the *YCK1* gene and carries a temperature-sensitive allele of the *YCK2* gene, *yck2-2*, to internalize α-factor. Fig. 7 *A* shows pheromone internalization as-

says performed on wild-type and *yck1Δ yck2-2* cells after incubation at 24° or 37°C. Wild-type cells internalized α-factor rapidly and to a similar extent at both temperatures. *yck1Δ yck2-2* cells internalized α-factor slowly at 24°C and not at all at 37°C, similar to the behavior of *end4* cells that are defective in endocytosis.

We then tested whether the Yck kinases are required to phosphorylate and ubiquitinate Ste2p. Fig. 7 *B* shows an immunoblot of extracts prepared from *yck1Δ yck2-2* and *end4* cells before and after the addition of α-factor at 37°C. Phosphorylated and ubiquitinated forms of the stimulated receptor accumulated in *end4* mutants that modify the receptor but are unable to internalize and degrade the protein (Hicke and Riezman, 1996) (Fig. 7 *B*, lane *4*). In contrast, modified forms of the stimulated receptor were not observed in the *yck1Δ yck2-2* mutant even though the receptor was not internalized. The mobility of the receptor expressed in this mutant was affected very little by the binding of α-factor (Fig. 7 *B*, lanes *1* and *2*), indicating that the receptor was phosphorylated to only a small extent. To confirm that receptor phosphorylation was deficient in the *yck1Δ yck2-2* mutant, we immunoprecipitated receptors from *end4* and *yck1Δ yck2-2* mutants incubated at the nonpermissive temperature and then treated the precipitated receptors with protein phosphatase I. Fig. 7 *C* shows that the mobility of receptors expressed in *end4* cells before exposure to α-factor increased upon phosphatase treatment, indicating that the receptor was constitutively phosphorylated (Fig. 7 *C*, lanes *5* and *7*). In response to α-factor binding the mobility of the receptor decreased (Fig. 7 *C*, lane *6*). This mobility shift was due to phosphorylation because treatment of stimulated receptor with phosphatase increased its mobility (Fig. 7 *C*, lane *8*). The receptor precipitated from *yck1Δ yck2-2* cells was not constitutively phosphorylated (Fig. 7 *C*, lanes *1* and *3*) because there was very little mobility shift upon treatment of unstimulated receptor with phosphatase. The mobility of the receptor shifted slightly upon pheromone binding to α-factor at the nonpermissive temperature (Fig. 7 *C*, lane *2*), and this shift was reversed by incubation with phosphatase (lane *4*). Thus, there was some increase in receptor phosphorylation in response to ligand binding, but to a much smaller extent than in *end4* cells. These data demonstrate that receptor phosphorylation is severely compromised in *yck1Δ yck2-2* cells. The Yck proteins are required for phosphorylation and ubiquitination of the wild-type α-factor receptor and its internalization, demonstrating that in the absence of phosphorylation, the receptor is not internalized even when it carries an intact SINNDAKSS sequence.

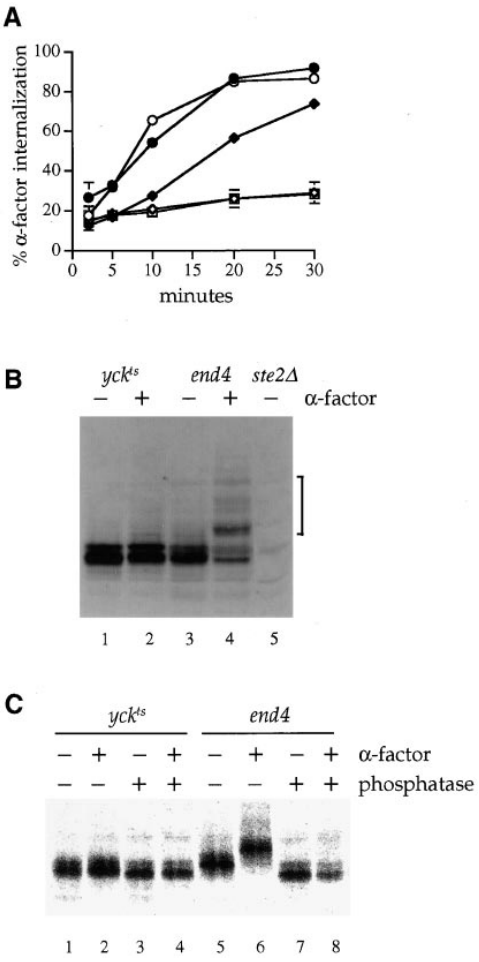


Figure 7. Mutants carrying defective Yck kinases are unable to modify or internalize α-factor receptor. (*A*) α-Factor internalization assays performed on wild-type (RH3992), *end4* (RH1965), and *yck1Δ yck2-2* (RH3589) strains preincubated for 15 min at 24° or 37°C. Wild-type, 24°C, *closed circles*; wild-type, 37°C, *open circles*; *yck1Δ yck2-2ρ*, 24°C, *closed diamonds*; *yck1Δ yck2-2*, 37°C, *open diamonds*; *end4*, 37°C, *open squares.* (*B*) Immunoblot of extracts prepared from *yck1Δ yck2-2* (RH3589) and *end4* (RH1597) cells before (−) or 10 min after (+) exposure to α-factor. Each strain was preincubated for 15 min at 37°C. Cells were removed for the no α-factor sample and then incubation was continued at 37°C after the addition of α-factor. Extracts were resolved by SDS-PAGE, transferred to nitrocellulose, and then probed with α-Ste2p antiserum. Modified forms of the receptor are designated by the bracket. (*C*) Immunoprecipitates of Ste2p from *end4* and *yck1Δ yck2-2* strains incubated at 37°C. Cells were labeled with EXPRE^{35}S^{35}S protein–labeling mix for 30 min at 30°C, chase mix was added, and then the cells were shifted to 37°C for 15 min before withdrawing samples before (−) and 10 min after (+) exposure to α-factor. Cell extracts were prepared and precipitated with Ste2p antiserum. The precipitates were incubated with protein phosphatase 1 (+) or mock-treated with buffer alone (−) and then resolved by SDS-PAGE. The precipitated proteins were visualized by autoradiography.

The Primary Function of SINNDAKSS-dependent Phosphorylation in Signal Downregulation Is to Promote Receptor Internalization

The phosphorylation of GPC receptors has been proposed to function in the activation and downregulation of signal response. Because α-factor receptor lacking its cytoplasmic tail is capable of transducing signal, the phosphorylation of receptor tail serines is not required for signal transmission (Konopka et al., 1988; Reneke et al., 1988). Instead, we have presented evidence demonstrating that the phosphorylation of serines within the Ste2p cytoplasmic tail is required for receptor internalization. It has been previously shown that internalization of Ste2p is a primary mechanism by which cells recover from α-factor stimula-

tion. Cells expressing the K337R, 345Stop receptor, which is unable to be endocytosed, recover from α-factor–induced growth arrest much more slowly than cells expressing the 345Stop receptor. However, these cells do eventually recover (Fig. 8; Rohrer et al., 1993). It is possible that phosphorylation of the SINNDAKSS serines functions in signal downregulation not only by stimulating receptor internalization but by other phosphorylation-dependent mechanisms (for example see Chen and Konopka, 1996). To test whether the SINNDAKSS serines promote signal downregulation by mechanisms other than receptor internalization, we analyzed the ability of cells expressing the 3S→A, 345Stop receptor, which is internalization defective, to recover from the cell cycle arrest induced by exposing *MAT**a*** cells to α-factor pheromone. We incubated cultures of cells expressing different receptors with α-factor to activate the growth arrest that occurs in cells stimulated with pheromone. α-Factor was then washed away from the cells and then the number of budded cells in the culture was counted at different times after wash-out as an assay of recovery from growth arrest. Fig. 8 shows that cells expressing the 345Stop receptor were fully recovered (>95% of budded cells) 1 h after α-factor had been removed. Cells expressing the K337R, 345Stop receptor recovered very slowly. They were ~70% budded after 4 h. Expression of the 3S→A, 345Stop receptor also allowed cells to recover slowly (Fig. 8). Expression of a 3S→A, K337R, 345Stop receptor, which is unable to be phosphorylated or ubiquitinated and is not internalized at all, resulted in recovery with the same kinetics as that observed for the K337R, 345Stop receptor (data not shown). These data indicated that the SINNDAKSS serines were not required for the recovery that occurred independent of receptor internalization observed with the K337R mutant. The recovery of cells expressing the 3S→A receptor was only slightly faster than the recovery of cells expressing the K337R mutant. Internalization of the 3S→A receptor was also slightly faster than that of K337R (Hicke and Riezman, 1996). The 3S→E mutations, which partially restored ligand-stimulated receptor endocytosis, also largely restored recovery from α-factor arrest (Fig. 8). The effect of mutations in the SINNDAKSS serines on α-factor recovery mirror their effects on α-factor internalization, supporting the contention that the primary, if not the only, function of the SINNDAKSS serines is to downregulate the α-factor signal by internalization of Ste2p through internalization into the endocytic pathway.

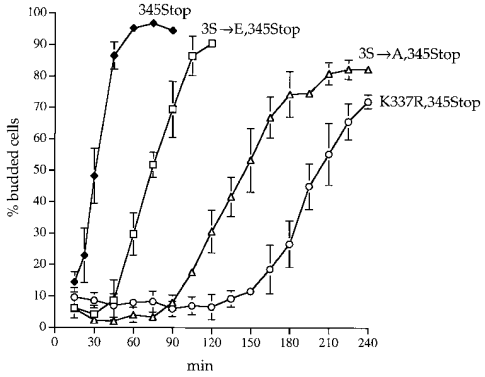


Figure 8. Cells expressing receptors that are internalized inefficiently are severely defective in their ability to recover from cell cycle arrest induced by α-factor binding. Cells expressing 345Stop (*closed diamonds*), K337R, 345Stop (*open circles*), 3S→A, 345Stop (*open triangles*), or 3S→E, 345Stop (*open squares*) receptors were incubated with α-factor until the entire population lacked buds, indicating a full arrest of growth in the G1 phase of the cell cycle. α-Factor was then washed away from the cells and the number of budded cells in each culture was counted after various periods of time as a measure of the ability of cells to recover from growth arrest and resume growing. Each recovery curve is an average of at least three independent experiments. The standard deviation at each time point is indicated by error bars.

Discussion

In this paper we present evidence that phosphorylation of the G protein–coupled α-factor receptor positively regulates receptor internalization by promoting the ubiquitination of lysine residues within the receptor cytoplasmic tail. We showed previously that mutation of the three serines within the SINNDAKSS internalization signal in a truncated receptor abolishes the ability of the receptor to be ubiquitinated and internalized (Hicke and Riezman, 1996). Now we demonstrate that these three serine residues are required for constitutive phosphorylation and that this, in turn, is required for the truncated receptor to undergo ligand-induced hyperphosphorylation. The SINNDAKSS serines are required for both constitutive and ligand-stimulated internalization of the truncated receptor.

Phosphorylation of serine residues, rather than just their presence, is required for receptor ubiquitination and endocytosis because the *yck1Δ yck2-2* mutant, which is severely compromised in its ability to phosphorylate the α-factor receptor, cannot ubiquitinate or internalize wild-type receptor even though it carries an intact SINNDAKSS sequence. This conclusion is also supported by the analysis of a receptor in which the SINNDAKSS serines have been replaced with negatively charged glutamates. Glutamate replacements of the serines restores ligand-stimulated phosphorylation to a level of ~75% of that of the 345Stop receptor. The replacements also partially rescue ligand-stimulated internalization of the receptor, presumably by facilitating ligand-induced phosphorylation on residues outside the SINNDAKSS sequence. Although we have not directly demonstrated that the SINNDAKSS serines are sites of phosphorylation, it is likely that one or more of these residues are constitutively phosphorylated because negatively charged amino acids substitute for their function in promoting ligand-stimulated phosphorylation. The 3S→E, 345Stop mutant was constitutively phosphorylated on non-SINNDAKSS serines at a low level, but it was not constitutively internalized. The constitutive phosphorylation level of this mutant may be below the threshold level required to promote internalization, or the 3S→E mutations may shift constitutive phosphorylation to residues that are not normally modified and may not function efficiently to promote receptor internalization.

The SINNDAKSS serines regulate internalization not only in the 345Stop truncated receptor but also in the wild-type tail, most likely through a similar mechanism. Muta-

tion of the three SINNDAKSS serines to alanines in the full-length receptor leads to a defect in constitutive internalization even though the receptor tail carries numerous serine (15) and threonine (19) residues, and can be ubiquitinated at many of its eight tail lysines.

We propose that the following series of events controls internalization of the α-factor receptor from the plasma membrane into the cell. The receptor is constitutively phosphorylated on serine residues. Constitutive phosphorylation and ubiquitination mediate the slow constitutive uptake of the receptor. Upon stimulation, the receptor becomes hyperphosphorylated and modified further with ubiquitin. Phosphorylation precedes and regulates ubiquitination at the SINNDAKSS lysine because ubiquitination at this lysine does not occur in a mutant lacking the SINNDAKSS serines. In contrast, phosphorylation is normal in a mutant lacking the ubiquitination site. Our results demonstrate that constitutive and stimulated internalization are mediated not by different signals but by modulating the level of the same signal, phosphorylation-dependent ubiquitination. It is not known how receptor phosphorylation effects an increase in receptor ubiquitination. Phosphorylated serines may recruit ubiquitination machinery to the receptor by providing favorable sites of interaction with ubiquitin-conjugating enzymes or a ubiquitin protein ligase.

Ligand-stimulated phosphorylation may occur at sites that become available for modification due to the conformation change in the receptor induced by pheromone binding (Bukusoglu and Jenness, 1996). Negative charge in the SINNDAKSS sequence, provided either by constitutively phosphorylated serines or by glutamate, may be required for this conformational change. Alternatively, negative charge in the SINNDAKSS sequence may facilitate recognition of the receptor by its kinase. Kinases that phosphorylate Ste2p have not been identified. *S. cerevisiae* does not carry homologues of the GPC receptor kinases (GRKs) that phosphorylate many mammalian GPC receptors, and the Npr1 kinase that appears to be involved in the ubiquitin-dependent downregulation of the general amino acid permease (Vandenbol et al., 1990; Hein et al., 1995) is not required for α-factor receptor endocytosis (our unpublished data). However, we have shown that mutants lacking casein kinase I activity do not constitutively phosphorylate the receptor. Since constitutive phosphorylation is required for both constitutive and ligand-induced internalization, this can explain the inability of the *yck1Δ yck2-2* mutant to internalize α-factor. The Yck kinases may directly phosphorylate the receptor or they may phosphorylate another protein that regulates receptor phosphorylation. The Yck proteins may also phosphorylate other components of the endocytic machinery (Panek et al., 1997).

Although several functions have been proposed for GPC receptor phosphorylation, we show here that phosphorylation at or near the membrane proximal SINNDAKSS sequence of the Ste2p tail functions to promote receptor internalization. The SINNDAKSS serines probably do not promote downregulation of Ste2p by mechanisms other than receptor internalization because the 3S→A, 345Stop mutant recovers from pheromone stimulation with the same slow kinetics as the internalization-defective K337R, 345Stop mutant. Our results do not rule out that phosphorylation of sites more distal, in the last quarter of the receptor tail, may function in an alternate mechanism of receptor downregulation (Chen and Konopka, 1996).

Phosphorylation has been shown to regulate the ubiquitination of cytosolic proteins that are targeted for degradation by the proteasome. Serine phosphorylation positively regulates the ubiquitination of cyclins that undergo regulated degradation at specific stages of the cell cycle (Deshaies et al., 1995; Yaglom et al., 1995). In addition, the relationship between serine phosphorylation and the ubiquitination and degradation of the transcription factor inhibitor, IκB, is well documented. IκB is a cytosolic protein that undergoes regulated degradation by the ubiquitin–proteasome pathway in response to a number of extracellular signals (Chen et al., 1995; Roff et al., 1996). These signals induce the phosphorylation of specific serine residues, which is required for the protein to be ubiquitinated at neighboring lysines (Brown et al., 1995). Like the cytosolic IκB, Ste2p also undergoes phosphorylation-dependent ubiquitination, indicating that this may be a general mechanism for triggering the ubiquitination of both cytosolic and membrane proteins that undergo regulated ubiquitin-dependent destruction.

Many, if not all, eukaryotic GPC receptors are phosphorylated on serine residues in response to binding of their cognate ligand. Phosphorylation of the β_2 adrenergic receptor by kinases of the GRK family promotes its interaction with arrestin, which is required for internalization of the protein (Ferguson et al., 1995, 1996; Goodman et al., 1996; Ménard et al., 1996). Yeast do not have an obvious arrestin homologue and we show here that phosphorylation of the α-factor receptor promotes internalization by positively regulating receptor ubiquitination. The phosphorylation of some mammalian receptors appears to play no role in their internalization (Holtmann et al., 1996; Oppermann et al., 1996). Thus, ligand-induced phosphorylation of GPC receptors is likely to have different functions from receptor to receptor, and the type and location of phosphate modification may specify the mechanism(s) by which a receptor is downregulated. Although the ubiquitin-dependent internalization of mammalian GPC receptors has not been described, a number of receptor tyrosine kinases undergo ligand-stimulated ubiquitination that may mediate stimulated internalization (Mori et al., 1992; Miyazawa et al., 1994; Yee et al., 1994; Galcheva-Gargova et al., 1995; Strous et al., 1996). A subset of mammalian GPC receptors may also be regulated by phosphorylation-dependent ubiquitination.

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Note Added in Proof. While this manuscript was under review, Marchal et

al. (Marchal, C., R. Haguenauer-Tsapis, and D. Urban-Grimal. 1998. *Mol. Cell. Biol.* 18:314–321) described experiments suggesting that serine residues within a PEST-like sequence of the yeast uracil permease are required for the phosphorylation and ubiquitin-dependent internalization of the permease.

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