Mutational Analysis of the Role of N-Glycosylation in α-Factor Receptor Function†

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ABSTRACT: The α-factor mating pheromone receptor (encoded by *STE2*) activates a G protein signaling pathway that stimulates the conjugation of *Saccharomyces cerevisiae* yeast cells. The α-factor receptor is known to undergo several forms of post-translational modification, including phosphorylation, mono-ubiquitination, and N-linked glycosylation. Since phosphorylation and mono-ubiquitination have been shown previously to play key roles in regulating the signaling activity and membrane trafficking of the α-factor receptors, the role of N-linked glycosylation was investigated in this study. The Asn residues in the five consensus sites for N-linked glycosylation present in the extracellular regions of the receptor protein were mutated to prevent carbohydrate attachment at these sites. Mutation of two sites near the receptor N-terminus (N25Q and N32Q) diminished the degree of receptor glycosylation, and the corresponding double mutant was not detectably N-glycosylated. The nonglycosylated receptors displayed normal function and subcellular localization, indicating that glycosylation is not important for wild-type receptor activity. However, mutation of the glycosylation sites resulted in improved plasma membrane localization for the Ste2-3 mutant receptors that are normally retained intracellularly at elevated temperatures. These results suggest that N-glycosylation may be involved in the sorting process for misfolded Ste2 proteins, and may similarly affect certain mutant receptors whose altered trafficking is implicated in human diseases.

The α-factor pheromone receptor (Ste2p) activates a G protein signal pathway that stimulates the highly regulated process of conjugation during which haploid yeast cells of opposite mating type fuse together to form a diploid cell (*1*−*3*). The components of the pheromone signal pathway, particularly the pheromone receptors, are under multiple forms of regulation that act in concert to enable cells to select an appropriate mating partner and fuse efficiently (*4*, *5*). Several different mechanisms have been identified that control the signaling activity and membrane trafficking of the α-factor receptors. One mechanism is mediated by Afr1p, a negative regulator of signaling that is anchored at the neck of mating projections by interaction with the Cdc12p septin (*6*−*8*). In addition, ligand-bound receptors are negatively regulated by phosphorylation on C-terminal residues which promotes receptor desensitization (*9*, *10*). Phosphorylation also appears to promote mono-ubiquitination of the receptor C-terminus (*11*) which then stimulates receptor endocytosis and subsequent trafficking of the receptors to the vacuole for destruction (*12*).

The α-factor receptors are also modified by N-linked glycosylation (*13*, *14*), but the role of this modification in receptor function has not been studied previously. Glycosylation is thought to be capable of influencing a number of different aspects of membrane protein structure and function, including effects on protein folding, stabilizing the structures of mature proteins, and protection from extracellular proteases (*15*, *16*). In the case of some adhesion molecules and hormone receptors, it also plays a direct role in ligand recognition. N-Glycosylation has also been found to serve important roles intracellularly where it is needed for the proper trafficking of many membrane proteins to the cell surface, and for the recognition of misfolded proteins in the ER (*17*). Like Ste2p, many of the G protein-coupled receptors (GPCRs)[1] found in other organisms are also glycosylated on their extracellular domains. Mutational analysis has shown that N-glycosylation is required for proper membrane trafficking and/or receptor function of some GPCRs (*18*−*20*), but for others, N-glycosylation does not seem to be important (*21*).

Since α-factor receptors are highly regulated during mating, we set out to determine whether N-glycosylation plays a role in their function. Site-directed mutagenesis was employed to identify which of the five potential sites for N-linked glycosylation (Asn-X-Ser/Thr) were utilized and to analyze the effects of these mutations on the signaling and trafficking of an otherwise wild-type α-factor receptor. To study the role of glycosylation in the trafficking of misfolded receptors, the consensus sites for N-linked glycosylation were mutated in *ste2-3*, which encodes a temperature-sensitive mutant receptor that is active, but displays a trafficking defect at restrictive temperatures (*13*). At elevated temperatures, Ste2-3p is apparently recognized by a post-ER sorting pathway that causes these mutant receptors

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[1] Abbreviations: GPCR, G protein-coupled receptor; PAGE, polyacrylamide gel electrophoresis; MW, molecular weight.

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Table 1: Yeast Strains Used in This Study

strain	genotype
YLG123	*MATa ade2-1^o his4-580^a lys2^o trp1^a tyr1^o leu2 ura3 bar1-1 mfa2::FUS1-lacZ ste2::LEU2*
YJL7	*MATa bar1 far1 his3::FUS1-lacZ mfaZ::FUS1-lacZ ade2-1 leu2-3 trp1-1 ura3-1 can1-100 ste2*Δ
PMY5	isogenic to YLG123 but *stp22::TRP1*
PMY6	isogenic to YLG123 but *cne1::kan^R*
lys1α	*MAT*α *lys1*

Table 2: Plasmids Constructed in This Study

plasmid	*STE2* allele	parent plasmid
pPK1	*STE2-N25Q*	pDB02
pPK2	*STE2-N32Q*	pDB02
pPK9	*STE2-N105Q*	pDB02
pPK4	*STE2-N25/32Q* (*STE2-2NQ*)	pPK1
pPK8	*STE2-N25/105Q*	pPK1
pPK7	*STE2-N32/105Q*	pPK2
pPK6	*STE2-N25/32/105Q* (*STE2-3NQ*)	pPK4

to be retained intracellularly (*22*). Rather than being targeted to the plasma membrane, Ste2-3p is redirected by a sorting pathway in *Saccharomyces cerevisiae* that has been shown to cause certain misfolded membrane proteins to transit via the Golgi apparatus to the vacuole for degradation (*13*, *22*−*24*). In this study, we demonstrate that the glycosylation machinery utilizes two of the five potential consensus sites. Although the wild-type receptor protein does not appear to require this modification for proper function or localization, the misfolded Ste2-3p receptor is degraded less rapidly and shows improved plasma membrane localization when glycosylation is eliminated.

MATERIALS AND METHODS

Yeast Strains and Media. The yeast strains used in this study are described in Table 1. Cells were grown in synthetic media containing amino acid additives and adenine but lacking uracil to select for the plasmid. To construct PMY5, plasmid pDJ225 (*22*) was digested with *Sac*I and *Spe*I to release the *stp22::TRP1* fragment and then yeast strain YLG123 was transformed with the resulting linear DNA. Trp$^+$ colonies were tested for proper integration of *stp22::TRP1* at the *STP22* locus by PCR analysis of genomic DNA. To construct PMY6, a *cne1::kan^R* disruption allele was amplified by PCR of genomic DNA from yeast strain YAL058W BY4741**,** obtained from the American Type Culture Collection (ATCC). Yeast strain YLG123 was transformed with the resulting DNA, and we selected for kanamycin-resistant transformants via growth in the presence of G418 (200 μg/mL). Positive clones were confirmed by PCR analysis of the *CNE1* locus.

Plasmids. Plasmid pDB02, which contains the *STE2* gene inserted into the low-copy number vector YCplac33, was described previously (*25*). Site-directed mutations to change the Asn codons to Gln codons in the *STE2* gene were constructed using a Quick Change site-directed mutagenesis kit (Stratagene). The mutant *STE2* plasmids constructed in this study are summarized in Table 2. The C-terminal truncation mutants were constructed by subcloning an *Eco*RI−*Aat*II fragment from pLG66 either into pDB02 to create *ste2-T326* or into pPK6 to create *ste2-T326/3NQ*. The temperature-sensitive *ste2-3* mutants were constructed by inserting an *Hpa*I−*Aat*II fragment, containing the A52T substitution, from pJBK079 into pDB02 and pPK4 to yield plasmids pPK15 and pPK16, respectively. Chimeric receptors in which a GFP tag was fused to the C-terminus were constructed by subcloning an *Eco*RI−*Cla*I fragment from pMD209, which contains a *STE2-GFP* gene on a high-copy number YEp vector, into the corresponding sites of pDB02, pPK6, pPK4, pPK15, and pPK16 to create pPK12, pPK13, pPK14, pPK17, and pPK18, respectively. An *Eco*RI−*Hin*dIII fragment that contains the coding region of *STE2* was cloned downstream of the T7 RNA polymerase promoter in pGEM1 to yield pJBK-019. The *STE2* coding sequence was also placed in pGEM2, in the antisense orientation, to create plasmid pJBK-020.

α*-Factor-Induced Responses*. Patch mating assays were performed by replica-plating *MAT***a** cells harboring a plasmid bearing either wild-type *STE2* or a mutant receptor onto a lawn of *MAT*α cells (strain lys1α) on a YPD plate. After incubation at the indicated temperature for 4 h to allow for mating to occur, the cells were then replica-plated to a minimal medium plate to select for growth of diploid cells. Quantitative mating assays were performed by spreading a series of 10-fold dilutions from 1×10^6 *MAT***a** cells onto a plate that had been spread with 10^7 *MAT*α cells and recording the number of diploid cells divided by the number of input *MAT***a** cells after growth for 2−3 days on minimal media. Halo assays for α-factor-induced cell division arrest were carried out by spreading 4×10^5 cells from an overnight culture onto solid media lacking uracil. The indicated amounts of α-factor were spotted onto sterile filter disks that were placed onto the lawns of cells that were then incubated at 30 °C for 2 days. Induction of *FUS1-lacZ* was assessed in logarithmic phase cells grown overnight in selective medium, diluted to 4×10^6 cells/mL in YPD medium, and then incubated with 10^{-7} M α-factor for 2 h. β-Galactosidase assays were performed in duplicate using the colorimetric substrate ONPG as described previously (*25*).

α*-Factor Receptor Analysis*. For Western blot analysis, approximately 2.5×10^8 cells were harvested and lysed with glass beads in 250 μL of lysis buffer [50 mM Tris (pH 7.5), 1 mM EDTA, 100 μg/mL PMSF, and 2 μg/mL pepstatin]. Membranes were harvested from the lysate by centrifugation in a microfuge (14000*g*) for 60 min and were then resuspended in 100 μL of TE. An equal amount of loading buffer [3% SDS, 50 mM Tris (pH 6.8), 8 M urea, and 0.01% bromophenol blue] was added, and 20 μL was separated by electrophoresis in an 8% SDS−polyacrylamide gel, transferred to nitrocellulose, and then probed with rabbit anti-Ste2p antibodies (*26*).

For endo H and λProtein Phosphatase (New England Biolabs) digestion analysis, logarithmically growing cells were incubated with 20 μg/mL cycloheximide for 10 min and, where indicated, α-factor was added (10^{-7} M). Cells were harvested after 10 min, and membranes were prepared as described above except that the pellet was resuspended in either endo H buffer [100 mM NaCl, 50 mM Tris (pH 7),

and 1 mM EDTA] or λProtein Phosphatase buffer supplied by NEB and supplemented with 2 mM $MnCl_2$. Endo H (3 μL) or λProtein Phosphatase (200 units) was then added to 10 μL of membranes, and then the endo H (3 μL) or λProtein Phosphatase samples were incubated for 2 h at 37 °C or for 1 h at 30 °C, respectively. For analysis of protein stability, cycloheximide was added to logarithmically growing cells, aliquots were removed at the indicated times, and the samples were processed for Western blot analysis as described above.

In vitro transcription of *STE2* utilized the T7 RNA polymerase promoter, and the resulting RNA was then translated in vitro using a rabbit reticulocyte lysate and [^{35}S]methionine. The effects of tunicamycin on Ste2p glycosylation were analyzed by treating 3×10^7 log phase cells with tunicamycin at a final concentration of 10 μg/mL for 30 min in synthetic medium lacking methionine. The cells were then pulse-labeled with 100 μCi of [^{35}S]methionine for 10 min, resuspended in 1% SDS, and lysed by agitation with glass beads. The extract was diluted into 1 mL of a buffer containing 1% Triton X-100, 10 mM Tris (pH 7.5), 1 mM EDTA, and 150 mM NaCl and centrifuged in a microfuge (11000*g*) for 15 min at 4 °C. Equal portions of the supernatants were then immunoprecipitated with either preimmune serum or anti-Ste2p antibodies (*26*). A parallel sample of radiolabeled Ste2p was diluted 10-fold into 50 mM sodium citrate (pH 5.5), split into two aliquots, and incubated in the presence or absence of endo H at 37 °C for 8 h. Samples were resolved by PAGE and visualized by fluorography.

Microscopy. GFP analysis was performed with cells that were grown overnight to log phase. The cells were then adjusted to a density of $\sim 2 \times 10^6$ cells/mL in YPD medium and grown for 4 h. A 1.0 mL sample of cells was harvested, washed with H_2O, resuspended in 100 μL of H_2O, and examined by fluorescence microscopy. Photographs were taken with an Olympus BH2 fluorescence microscope equipped with a Zeiss Axiocam camera.

RESULTS

Mutation of Consensus Sites for N-Linked Glycosylation in Ste2p. Five potential sites of N-linked glycosylation (Asn-X-Ser/Thr) were identified by inspection of the extracellular domains of Ste2p (Figure 1). To determine which of these sites are utilized, the Asn residues of the consensus sites were mutated. The Asn residues at positions 25, 32, and 105 were mutated to the conservative amino acid Gln by site-directed mutagenesis (see Materials and Methods). In the case of the Asn residues at positions 46 and 205, the N46S and N205K substitutions were analyzed since these mutations, as well as a N105S mutation, were isolated previously as part of random genetic screens (*27*; W. Parrish and J. B. Konopka, in preparation).

The degree to which these receptors were glycosylated was analyzed by comparing the electrophoretic mobility of the mutant receptor proteins to that of wild-type Ste2p. Protein extracts from *ste2*Δ cells carrying either a wild-type *STE2* plasmid or one of the single-mutant receptor genes (N25Q, N32Q, N46S, N105Q, or N205K) on a plasmid were analyzed on Western immunoblots (Figure 2A). As expected, wild-type Ste2p was detected as a heterogeneous set of bands due at least in part to glycosylation. Two major bands (p53 and p48) were detected that are thought to correspond to

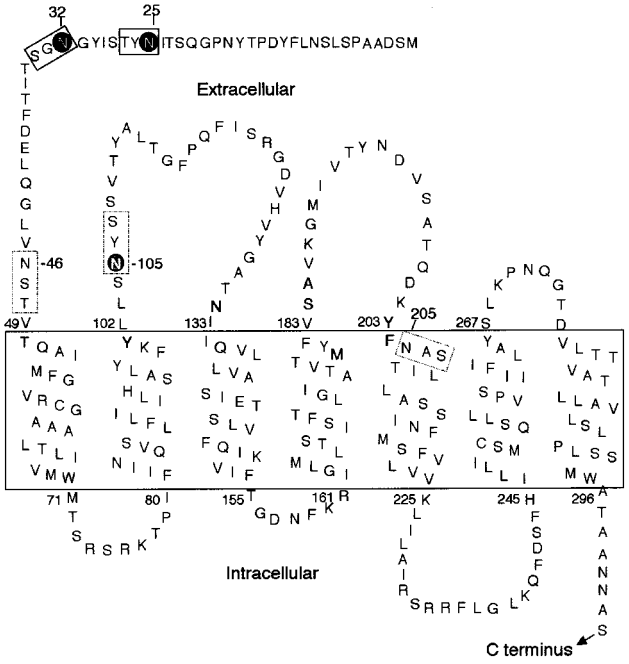

FIGURE 1: Topological structure of the α-factor receptor (Ste2p). The amino acid residues indicated by the one-letter code are displayed in the predicted positions of the seven transmembrane domains of the α-factor receptor. Residues 1−49 comprise the N-terminal domain, and residues 296−431 comprise the cytoplasmic C-terminus, of which only the juxtamembrane portion is shown. The consensus sequences for N-linked glycosylation are boxed. Black circles indicate the Asn residues at positions 25 and 32, which are glycosylated, and Asn^{105} is shown in a gray circle.

different levels of N-glycosylation. The N46S and N205K substituted receptors resembled wild-type Ste2p, indicating that these sites are not glycosylated in vivo. In contrast, receptors containing the N25Q and N32Q substitutions displayed a decrease in the magnitudes of the higher-molecular weight (MW) bands and a corresponding increase in the magnitudes of the lower-MW bands, suggesting some loss of glycosylation on these receptor proteins. The N105Q substituted receptor gave variable results such that in some gels less of the upper (p53) band was detected but in other gels the N105Q receptor protein was detected as a series of bands similar to those for wild-type Ste2p. Further analysis described below indicates that the Asn^{105} site is not utilized to any detectable level. Thus, of the five possible sites for N-linked glycosylation in Ste2p, only Asn^{25} and Asn^{32} appear to be utilized.

Receptors with Multiple Glycosylation-Site Mutations. Since the single-mutant receptors in which one glycosylation site was eliminated still appeared to be glycosylated to some extent, mutants with multiple substitutions were constructed. In particular, the N25/32Q double substitution was constructed because receptors with single mutations at these sites lead to proteins with altered gel mobilities. Mutant combinations involving the N105Q substitution (N25/105Q and N32/105Q) were also constructed to further assess the variable results observed for the N105Q substituted single-mutant receptor. The N25/32Q/105Q triple mutant in which all three residues were mutated was also constructed. To simplify the nomenclature, the N25/32Q double substitution mutant will be called 2NQ and the N25/32Q/105Q triple-substitution mutant 3NQ.

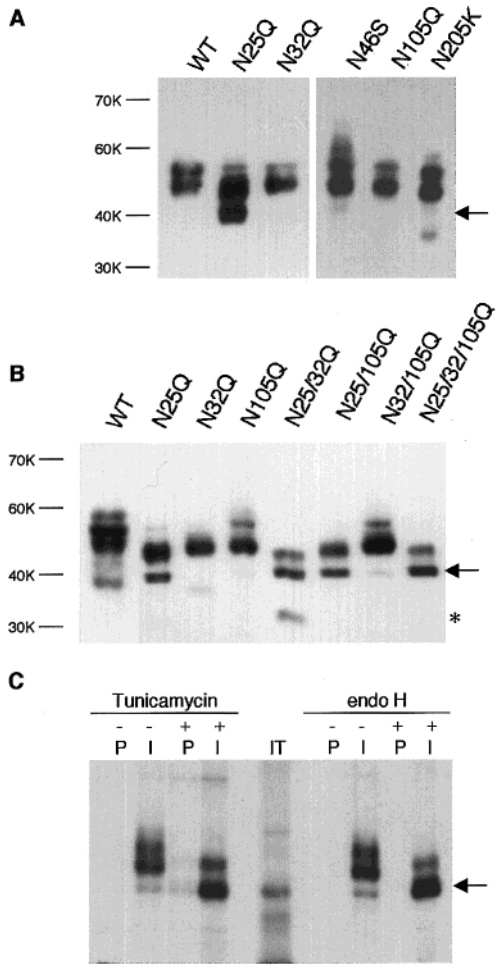

FIGURE 2: Analysis of Ste2p mutants with single and multiple glycosylation site mutations. (A) Western blot analysis of membrane fractions from *ste2*Δ cells (YLG123) carrying a wild-type *STE2* plasmid (pDB02) or a mutated version in which the indicated Asn residue in a consensus site for N-linked glycosylation was substituted as indicated at the top of the gel. Ste2 proteins were detected by anti-N-terminal Ste2p antibodies. The molecular weights of marker proteins are given at the left. An arrow on the right specifies the position of the unmodified form of Ste2p (p43). (B) The indicated single-, double-, and triple-glycosylation site mutant combinations indicated at the top of the gel were analyzed as described for panel A. An asterisk indicates the position of a band that is thought to be due to degradation of Ste2p as its occurrence varies. (C) Immunoprecipitation analysis of *STE2* cells that were incubated either in the presence (+) or in the absence (−) of tunicamycin and then pulse-labeled with [^{35}S]methionine. Cell lysates were immunoprecipitated with preimmune (P) and immune (I) anti-Ste2p antibodies as indicated at the top of the gel. A parallel set of immune complexes was incubated with (+) or without (−) endo H prior to electrophoresis. Ste2p produced in an in vitro translation assay and labeled with [^{35}S]methionine is shown in the lane labeled IT.

The effects of the double- and triple-mutant combinations on receptor glycosylation were analyzed by comparing the mobility of the mutant receptor proteins on Western blots (Figure 2B). *STE2-2NQ* mutant cells produced receptor proteins with greatly decreased heterogeneity due to the absence of higher-MW bands. One major receptor protein was detected that comigrated with the lowest-MW form of the wild-type receptor protein (~43000). This band is proposed to be the unmodified form since it comigrates with the Ste2p synthesized in an in vitro translation system in which N-linked glycosylation cannot occur (Figure 2C, lane 5). (A minor band of slightly higher MW was also detected that will be analyzed below.) In contrast, cells carrying multiple-mutant combinations involving the N105Q substitution did not show a further decrease in apparent MW as compared to the corresponding single mutants or the 2NQ mutant, providing additional evidence that Asn^{105} is not detectably glycosylated. Altogether, these results indicate that the heterogeneity of Ste2p when resolved by PAGE is due at least in part to differential glycosylation at two sites rather than a variable degree of mannosylation at one site.

Interestingly, a minor band of higher MW was also detected in the *STE2-2NQ* cells, suggesting that a portion of this receptor protein still undergoes some form of modification. This modification was not due to glycosylation of Asn^{105} since similar results were observed for the *STE2-3NQ* triple mutant. The possibility that the remaining modification may not be due to N-linked glycosylation was examined by comparing Ste2p that was translated in vitro to Ste2p produced in cells exposed to tunicamycin to block N-linked glycosylation (Figure 2C). For this analysis, cells were incubated in the presence or absence of tunicamycin, and then the proteins were metabolically labeled with a pulse of [^{35}S]methionine. Although Ste2p extracted from cells treated with tunicamycin primarily comigrated with in vitro translated Ste2p, it was interesting that an additional band was detected that was similar to the extra band detected in the *STE2-2NQ* cells (Figure 2C). Similar results were also obtained when endo H was used to enzymatically deglycosylate the radiolabeled Ste2p. These results suggest that the upper band detected in these cells is due to a different type of modification, and also confirm that Asn^{25} and Asn^{32} are the only two sites that are detectably glycosylated in Ste2p.

Basal Phosphorylation of Ste2p. The *STE2-2NQ* cells were analyzed further to determine the nature of the modification that resulted in the minor band with a higher MW. Treatment of membrane fractions with endo H showed that the Ste2-2NQ receptors were unaffected (data not shown), confirming that this band was due to a different type of modification. Since Ste2p is also known to be phosphorylated in its basal state and hyperphosphorylated in response to α-factor (*9*, *10*, *28*), the Ste2p, 2NQp, and 3NQp receptors were treated with λProtein Phosphatase. Interestingly, the higher-MW form of the nonglycosylated receptors was sensitive to treatment with λProtein Phosphatase, indicating that the additional modification that was detected was due to phosphorylation (Figure 3A).

Further evidence that this extra modification was due to phosphorylation was obtained by examining the effects of deleting target sites for phosphorylation that are present in the cytoplasmic C-terminal domain. To carry out this analysis, the *STE2-3NQ* mutation was combined with the *ste2-T326* truncation mutation. This C-terminally truncated receptor protein is not detectably phosphorylated in vivo as the target sites for phosphorylation are absent (*10*). The *ste2-T326* receptor protein gave the expected result in that it was of lower MW than wild-type Ste2p and resolved as a heterogeneous set of bands due to N-glycosylation (Figure 3B). In contrast, cells carrying a mutant form of *ste2-T326* in which the glycosylation sites were also mutated (*ste2-3NQ/T326*) produced only a single band corresponding to the lowest-MW form of Ste2-T326p. These results confirm that the extra band seen for the mutant receptors is due to

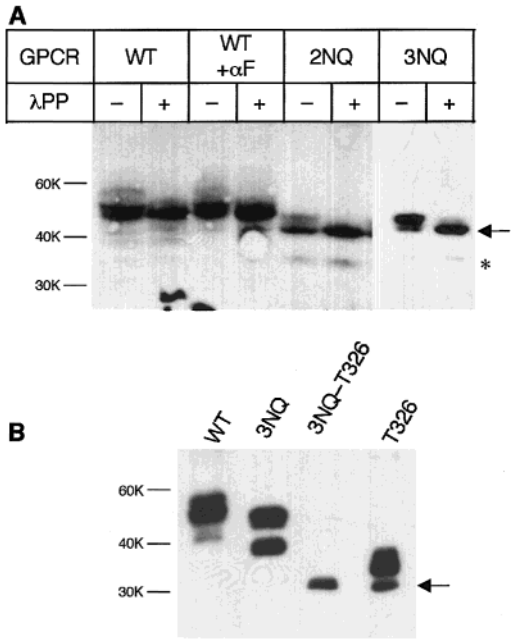

FIGURE 3: Identification of basal phosphorylation of α-factor receptors. (A) Membrane fractions from *ste2*Δ cells (YLG123) carrying the indicated receptor gene on a plasmid were treated either with (+) or without (−) λProtein Phosphatase (λPP) and analyzed on a Western immunoblot. The additional high-molecular weight band detected for the mutant receptors disappears upon phosphatase treatment. (B) Western blot analysis of cells producing mutant receptors carrying the indicated substitution of Gln for Asn at positions 25, 32, and 105 together with truncation of the C-terminal tail that resulted in a completely unmodified receptor protein that resolved as a single band. The molecular weights of the marker proteins are indicated at the left. An arrow on the right specifies the position of the completely unmodified form of Ste2p.

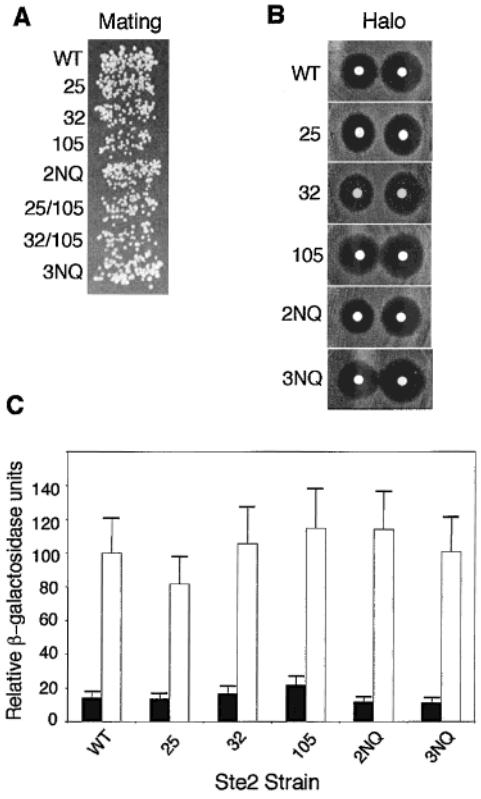

FIGURE 4: Analysis of receptor activity. (A) The *ste2*Δ strain YLG123 carrying a wild-type *STE2* plasmid or a mutant receptor gene with Gln substituted for Asn at the indicated positions was assayed for the ability to mate with lys1α cells and produce diploids on selective medium. (B) Halo assays in which a filter disk containing either 900 or 300 ng of α-factor was placed on a lawn of the indicated wild-type and mutant *STE2* strains and incubated at 30 °C. (C) Cells producing the indicated receptors were assayed for their ability to induce the *FUS1-lacZ* reporter gene in the absence (black bars) or presence (white bars) of α-factor. The *β*-galactosidase activity for the wild type was normalized to 100 units.

phosphorylation. Interestingly, these experiments also revealed that only a subpopulation of the Ste2p present in the cell is phosphorylated prior to addition of α-factor.

Functional Analysis of Glycosylation-Deficient Receptor Mutants. The *STE2* mutant strains were analyzed to determine whether N-linked glycosylation is necessary for the production of functional cell-surface α-factor receptors. All of the mutant receptor strains were able to mate, indicating that the mutant receptors could respond to pheromone and activate the downstream signal pathway (Figure 4A). The glycosylation-defective receptor mutants were then examined for their ability to promote α-factor-induced cell division arrest since this is a more sensitive test of receptor function. This response was quantitated by measuring the zone of growth inhibition (halo) surrounding filter disks containing α-factor that were placed on a lawn of cells expressing the indicated mutant receptors. All of the mutant cells produced halos that were similar to that of the wild type. The diameters of the halos were of approximately the same size, indicating a similar degree of sensitivity to α-factor. It was also significant that the zones of growth inhibition stayed clear and did not fill in as often occurs in strains which produce receptors that are not properly targeted to the plasma membrane (*22*, *29*) (Figure 4B). Similar results were obtained over a temperature range of 23−36 °C (data not shown). Cells containing the mutant receptors also responded to α-factor in essentially the same manner as the wild type when assayed for their ability to activate the pheromone-responsive *FUS1-lacZ* reporter gene (Figure 4C), and to undergo the characteristic pheromone-induced morphogenesis known as shmoo formation (data not shown). Thus, glycosylation does not appear to be critical for α-factor receptor function.

The results described above suggest that the mutant receptors are targeted efficiently to the plasma membrane. However, a partial defect in receptor trafficking may not be detected by these assays, and therefore, the subcellular localization of the 2NQ and 3NQ mutant receptors was examined. This analysis was facilitated by fusing the green fluorescent protein (GFP) to the C-terminus of the receptors, allowing visualization by fluorescence microscopy. In agreement with the results of other investigators (*22*, *30*), Ste2-GFP fluorescence appeared as a ring surrounding the cell as expected for a protein that is localized efficiently to the plasma membrane (Figure 5). The 2NQ and 3NQ mutant receptors exhibited a similar pattern of fluorescence in the absence of α-factor. In addition, α-factor caused the fluorescence of the wild-type and mutant receptors to relocalize to intracellular compartments as expected for receptors undergoing ligand-induced endocytosis (data not shown), indicating that N-linked glycosylation does not have a detectable effect on the trafficking of wild-type α-factor receptors.

Suppression of Mislocalized Ste2-3p Receptors. Mutation of the glycosylation sites was also used to determine whether N-linked glycosylation was involved in the observed phe-

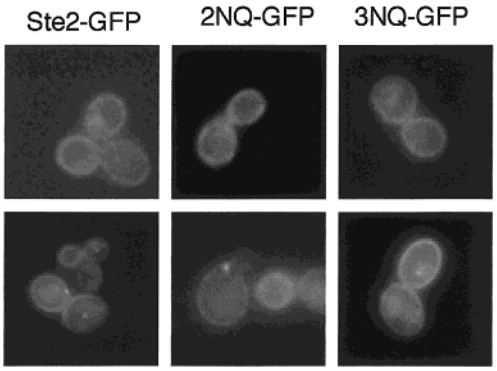

FIGURE 5: Subcellular localization of GFP-tagged receptors. The *ste2*Δ yeast strain YLG123 harboring a plasmid containing either *STE2-GFP*, *2NQ-GFP*, or *3NQ-GFP* was examined microscopically. The majority of fluorescence for all chimeric receptors was detected in the plasma membrane.

notype of Ste2-3p, a temperature-sensitive mutant form of the α-factor receptor that has been used previously as a model for studying the trafficking of integral membrane proteins (*13*). The mutant properties of this receptor are caused by a substitution of Ala^{52} with Thr (*13*). At restrictive temperatures, the Ste2-3p mutant receptors are still functional, but are depleted from the plasma membrane due to trafficking of the receptors to the vacuole for degradation. This is caused by a combination of events, including newly synthesized Ste2-3p being diverted from the Golgi apparatus to the vacuole and receptors at the plasma membrane being rapidly endocytosed (*22*).

As expected, the *ste2-3* strain mated poorly at both 30 and 36 °C in patch mating assays (Figure 6A). Interestingly, this mating defect was partially suppressed when the two glycosylation consensus sites at Asn^{25} and Asn^{32} were mutated to Gln in *ste2-3* (resulting in the *ste2-3/2NQ* strain). The degree of suppression was quantitated in assays for receptor function carried out at 30 °C, which proved to be a sensitive temperature for detecting changes in the signaling activity of *ste2-3* cells. Quantitative mating analysis demonstrated that *ste2-3* cells mated with an efficiency of only 12.5% compared to that of the wild type, but the *ste2-3/2NQ* cells mated at essentially wild-type levels (115%) (Figure 6B). Analysis of *FUS1-lacZ* reporter gene activity showed that α-factor-induced *ste2-3* cells signaled at a level that was approximately 20% of that of wild-type cells, whereas the *STE2-3/2NQ* cells showed a significant improvement in signaling to 60% of the wild-type level (Figure 6C). Thus, although N-glycosylation did not seem to be important for wild-type Ste2p function, these results suggested that this modification influenced the function of the mutant Ste2-3p.

The improved function of the *ste2-3/2NQ* mutant suggested that the mutation of the glycosylation sites improved delivery to the plasma membrane. To address this possibility, the subcellular localization of the receptor proteins was examined (Figure 7). Cells producing Ste2-3-GFP contained almost no detectable fluorescence at the plasma membrane, in agreement with previous results (*22*). The majority of fluorescence was detected as intracellular punctate spots in contrast to Ste2-3/2NQ-GFP which was detected primarily at the plasma membrane (Figure 7). To more accurately assess this improvement in localization, the number of punctate GFP

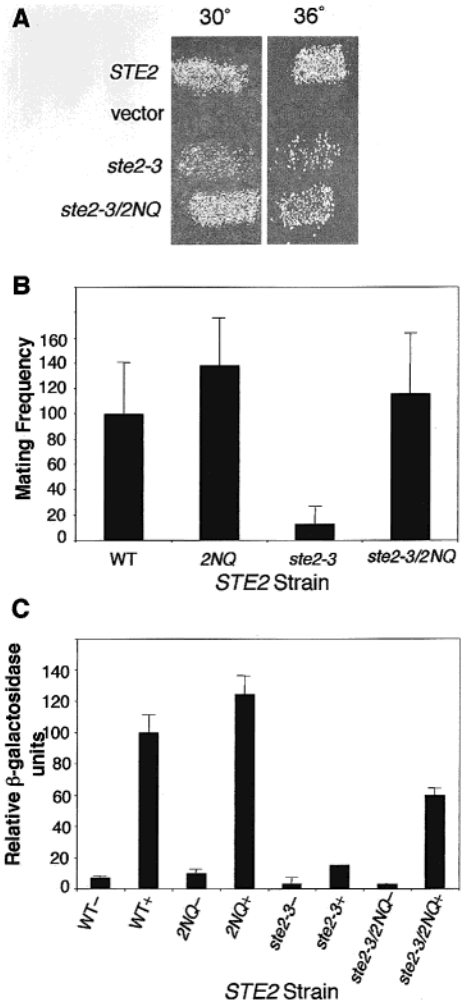

FIGURE 6: Glycosylation-site mutations suppress the temperature-sensitive phenotype of *ste2-3*. (A) Patch mating assays at both 30 and 36 °C showed a decreased level of mating for the *ste2-3* strain that was suppressed by mutation of the N-linked glycosylation sites (*ste2-3/2NQ*). The mating frequency for these strains was quantitated and is shown in panel B. (C) The *ste2*Δ strain YJL7 producing the indicated receptor mutant was assayed for β-galactosidase activity to assess the *FUS1-lacZ* reporter gene in the presence (+) and absence (−) of 10^{-7} M α-factor.

spots inside the cells was counted. Most *ste2-3-GFP* cells displayed either weak diffuse intracellular GFP fluorescence or multiple intracellular GFP spots (more than three). In contrast, the majority of cells expressing *ste2-3/2NQ-GFP* receptors displayed fluorescence mainly in the plasma membrane localization, and typically, only one to three intracellular GFP spots were observed (Figure 7). Although plasma membrane localization of Ste2-3p was greatly improved by mutation of the glycosylation sites, it did not lead to a complete recovery of the trafficking defect. The Ste2-3/2NQ mutant receptors did not show the same high degree of plasma membrane localization as wild-type Ste2-GFP. The results of these subcellular localization studies are therefore consistent with the partial suppression of *ste2-3* in halo and *FUS1-lacZ* induction assays caused by mutation of the glycosylation sites.

Extragenic Suppression of ste2-3. The *stp22*Δ and *cne1*Δ mutations have been reported previously to act as extragenic suppressors of *ste2-3* (*22*, *31*). Mutation of *STP22* (*VPS23*) apparently improved the plasma membrane localization and

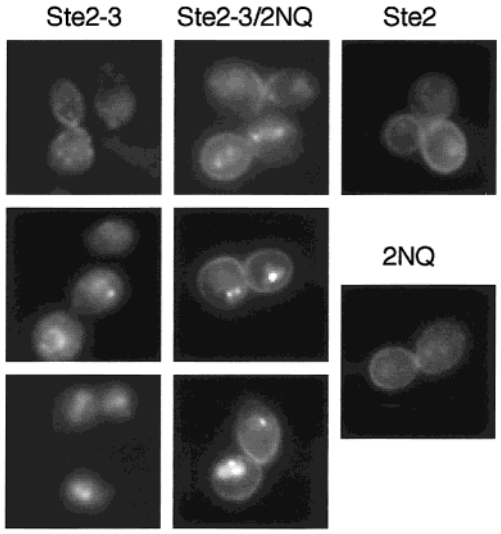

FIGURE 7: Subcellular localization of GFP-tagged Ste2-3p mutant receptors. Yeast strain YLG123 harboring plasmids containing either *Ste2-3-GFP*, *Ste2-3/2NQ-GFP*, *STE2-GFP*, or *2NQ-GFP* examined by fluorescence microscopy.

stability of Ste2-3p by preventing the trafficking of mutant receptors to the vacuole for degradation. Stp22p was proposed to be a component of a prevacuolar compartment and to be involved in a quality control pathway that recognizes misfolded proteins in the Golgi apparatus and reroutes their trafficking to the vacuole (*22*). The *cne1*Δ mutation was also reported to suppress the *ste2-3* mutation, albeit more weakly (*31*). *CNE1* is interesting because its sequence is similar to those of mammalian calnexin and calreticulin genes that are involved in the recognition of misfolded glycoproteins in the endoplasmic reticulum. However, Cne1p does not appear to carry out the identical function in yeast cells (*32*).

To determine the relationship between N-linked glycosylation and the *STP22*-dependent protein sorting pathway, the *STE2*, *ste2-3*, and *ste2-3/2NQ* receptors were expressed in a *ste2*Δ *stp22*Δ strain. As expected, the mating defect of *ste2-3* was partially suppressed in this strain at both 30 and 36 °C (Figure 8A). The additional mutation of the glycosylation sites at Asn^{25} and Asn^{32} in *ste2-3* (*ste2-3/2NQ*) caused a slight improvement in mating efficiency from 57 to 74% relative to the wild-type level at 30 °C (data not shown). *stp22*Δ also partially suppressed the *ste2-3* defect in halo assays for cell division arrest (note the clearer zone of growth arrest; Figure 8B). However, there was no obvious further improvement in the quality of the halo assays for *stp22*Δ cells expressing *ste2-3/2NQ*.

It was difficult to determine whether the mutation of the glycosylation sites significantly improved the suppression of *ste2-3* in *stp22*Δ cells in pheromone-response assays, in part because *stp22*Δ alone was already a fairly strong suppressor of *ste2-3*. Therefore, we also examined whether glycosylation affected the localization of the Ste2-3p receptors in the *stp22*Δ strain. Consistent with previous reports, Ste2-3-GFP exhibited slightly improved plasma membrane localization in the *stp22*Δ strain (ref *22* and data not shown). However, no significant improvement in localization was detected by mutating the glycosylation sites as Ste2-3/2NQ-GFP exhibited a degree of plasma membrane localization similar to that of Ste2-3-GFP in the *stp22*Δ strain (data not shown).

The relationship between N-linked glycosylation and the suppression of *ste2-3* by mutation of *CNE1*, a yeast gene homologous to mammalian calnexin, was analyzed by expressing *ste2-3* in a *cne1*Δ strain. The ability of this strain to respond to α-factor was analyzed by halo assays designed to assess α-factor-induced cell division arrest (Figure 8B). In contrast to the results described above for suppression by *stp22*Δ, *ste2-3* was not detectably suppressed by *cne1*Δ in halo assays for cell division arrest, even at a semipermissive temperature. This was not too surprising as *cne1*Δ was reported to cause a only relatively weak suppression of the *ste2-3* mating defect at 37 °C (*31*) as opposed to the stronger suppression observed with *stp22*Δ (*22*). However, it was interesting that mutation of the glycosylation sites (*ste2-3/2NQ*) suppressed *ste2-3* to a similar degree in a *cne1*Δ strain and in a *CNE1* strain. These results indicate that suppression of *ste2-3* by mutation of the N-linked glycosylation sites does not occur by preventing Cne1p function.

Glycosylation Site Mutations Improve Mutant Receptor Stability. The improved plasma membrane localization detected microscopically did not directly address whether the stability of Ste2-3p was also improved by mutation of the glycosylation sites. Therefore, the stability of Ste2-3/2NQp was analyzed by methods similar to those used previously to examine the stability of Ste2-3p (*22*). For this analysis, cells were incubated with cycloheximide to block new receptor synthesis and then aliquots were removed at the indicated time points and membrane extracts analyzed on Western immunoblots. In agreement with previous results (*22*), Ste2p was fairly stable with a half-life in excess of 30 min whereas Ste2-3p was relatively unstable and decayed rapidly with a half-life of approximately 5 min (compare panels A and B of Figure 9). Interestingly, this assay indicated that the stability of Ste2-3/2NQp was greatly improved (15−20 min half-life). These results demonstrate that mutation of the glycosylation sites increases the stability of Ste2-3p.

DISCUSSION

Post-Translational Modifications of Ste2p. The α-factor pheromone receptor (Ste2p) produced by haploid *S. cerevisiae MAT***a** cells is a highly regulated protein that carries out a crucial role in mating. Previous studies have shown that post-translational modification of Ste2p by phosphorylation and ubiquitination plays an important role in regulating the signaling activity and membrane trafficking of the receptors. Ste2p is also known to be modified by N-linked glycosylation, but the precise sites and role of this modification in receptor function had not been examined. Glycosylation of other GPCRs has been observed to influence a number of properties, including protein folding, membrane trafficking, functional properties, and the recognition of misfolded proteins. We therefore sought to assess the significance of N-linked glycosylation on the cell surface localization and function of the α-factor receptor.

The function of N-glycosylation in α-factor receptor function was examined by substitution of a Gln for the Asn in the five consensus sites for N-linked glycosylation (Asn-X-Thr/Ser). Asn^{25} and Asn^{32} are the only sites whose

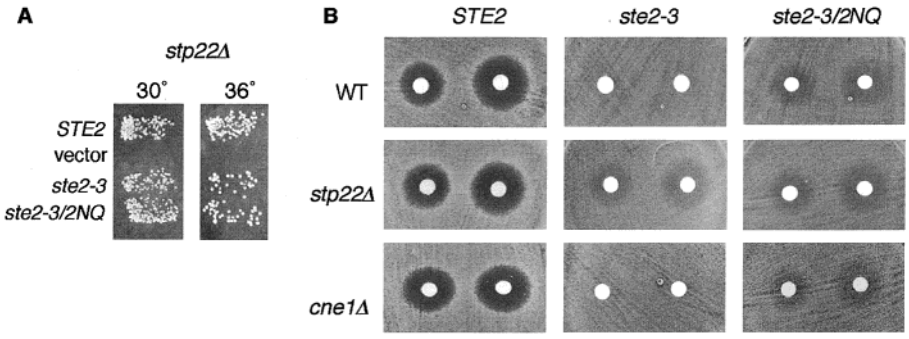

FIGURE 8: Relationship of glycosylation-site mutations to extragenic suppressors of *ste2-3*. (A) Patch mating assays for strain PMY5 (*stp22*Δ) carrying the indicated receptor gene on a plasmid. Assays were carried out at 30 and 36 °C. (B) Halo assays demonstrating the α-factor sensitivity of cells expressing either *STE2*, *ste2-3*, or *ste2-3/2NQ* receptor genes in wild-type (YLG123), *stp22*Δ (PMY5), or *cne1*Δ (PMY6) yeast strains as indicated.

substitution with Gln either individually or in combination led to a change in gel mobility, indicating that these residues serve as acceptor sites for oligosaccharide. In agreement with this, Asn^{25} and Asn^{32} are the only N-linked glycosylation consensus sites that are predicted to be more than 15 residues away from the plasma membrane. This distance is thought to be necessary for recognition by the glycosylation machinery since studies on the mechanisms of N-linked glycosylation indicate a consensus site must be located at least 12−14 residues away from the plasma membrane to be utilized (*33*). Apparently, the oligosaccharyltransferase active site is positioned 30−40 Å above and parallel to the membrane surface, which therefore limits its access to those acceptor sites that are at a suitable distance (*33*). In contrast, Asn^{46}, Asn^{105}, and Asn^{205} are all predicted to be fewer than five residues away from the plasma membrane.

Our studies also indicate that glycosylation occurs more efficiently at Asn^{25} than at Asn^{32}. This is inferred from results showing that mutation of Asn^{25} (N25Q) leads to a preponderance of nonglycosylated receptors in the cell, while mutation of Asn^{32} (N32Q) resulted mainly in monoglycosylated receptors. The most obvious difference between these two acceptor sites is the hydroxy amino acid at position 3 of the consensus site (Ser/Thr). A threonine is located at residue 27, but a serine is present at residue 34. Interestingly, this is consistent with experimental evidence obtained in a wide range of cell types that suggests that consensus sites containing threonine are utilized more frequently than those containing serine (*34*, *35*). Thus, the presence in Ste2p of two naturally occurring variations of the consensus site further demonstrates that, similar to other organisms, a threonine in the consensus site increases glycosylation efficiency in yeast.

In addition to identifying the sites of N-linked glycosylation, mutation of the consensus sites also revealed the relative degree of basal phosphorylation of Ste2p. When the nonglycosylated receptors were analyzed on Western immunoblots, the phosphorylated form usually corresponded to a relatively minor band of slightly higher molecular weight than the unmodified receptors. Previous studies carried out by radiolabeling with $^{32}PO_4$ demonstrated that the receptors were phosphorylated in the basal state. However, it was difficult to quantify the proportion of receptors that are phosphorylated in these metabolic labeling approaches due to the added heterogeneity caused by glycosylation (*9*, *10*, *28*). Mutation of the glycosylation sites alleviated these

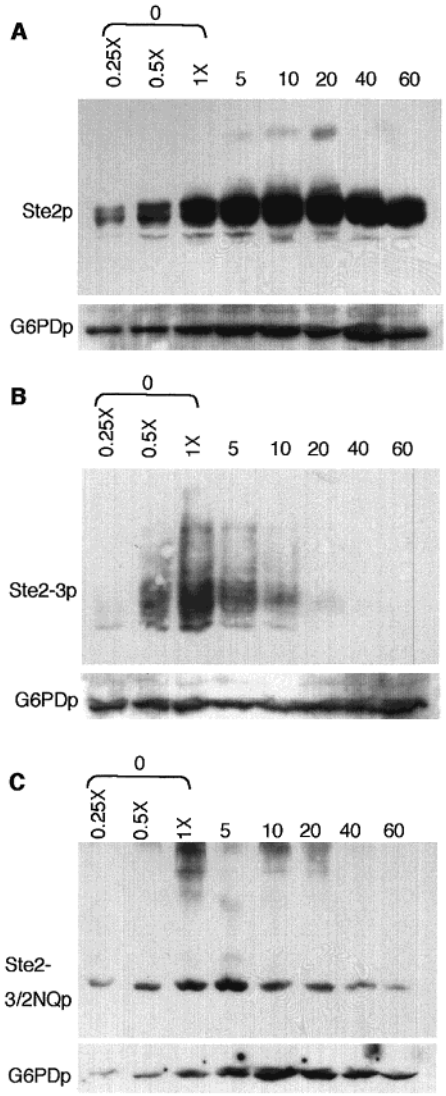

FIGURE 9: Mutation of glycosylation sites improves Ste2-3p stability. Log phase cultures of YLG123 carrying a plasmid encoding Ste2p (A), Ste2-3p (B), and Ste2-3/2NQp (C) were treated with cycloheximide at time zero, and then aliquots were removed at the indicated times (in minutes) and processed for immunoblotting. To facilitate the estimation of the half-life, different dilutions of the aliquot taken at time zero (0.25-, 0.5-, and 1-fold) are included. Receptor proteins were detected with anti-N-terminal Ste2p antibodies. As a control, the blots were subsequently reprobed with anti-glucose-6-phosphate dehydrogenase (G6PDp).

problems and simplified the examination of Ste2p phosphorylation. The nonglycosylated mutant receptor, Ste2-2NQp, therefore provides a useful new tool with which to study biochemical modifications of Ste2p, including phosphorylation and ubiquitination.

Role of Glycosylation in the Trafficking of Ste2-3p Mutant Receptors. Although N-glycosylation was not necessary for the cell surface expression or activity of wild-type Ste2p, it was interesting that mutation of the Asn residues in the two N-glycosylation sites partially rescued the trafficking defect of the temperature-sensitive Ste2-3p receptors. The increased level of signaling of *ste2-3/2NQ* mutant cells relative to *ste2-3* cells was most likely due to the improved plasma membrane localization and longer half-life of Ste2-3/2NQ receptors. This improvement may be a direct or indirect result of the loss of glycosylation. It is possible that the attached carbohydrate is recognized directly by the sorting pathway that diverts misfolded receptors from the Golgi apparatus to the vacuole for degradation. In this case, the nonglycosylated Ste2-3p would no longer be recognized and thus proceed to the plasma membrane. Alternatively, carbohydrate modification may be involved indirectly in this post-ER quality control process such that elimination of glycosylation may alter the overall structure of Ste2-3p and prevent it from being recognized as misfolded. However, glycosylation has been found to have a stabilizing effect on protein structure (*15*, *16*). Therefore, the loss of this modification in the Ste2-2NQ and Ste2-3/2NQ proteins would be expected to have a further destabilizing effect on these temperature-sensitive proteins, which was not observed.

Proteins of the post-ER quality control process that directly recognize misfolded receptor proteins have not yet been identified in yeast, making the investigation of the precise role of Ste2p glycosylation in this process difficult. However, it is interesting that mutations in two genes, *CNE1* and *STP22*, both of which are homologous to mammalian components of protein trafficking pathways, were identified as suppressors of *ste2-3* (*22*, *31*). The mammalian homologue of *CNE1*, calnexin, is thought to act as a chaperone that retains misfolded glycoproteins in the ER. *cne1*Δ only weakly suppressed the trafficking defect of *ste2-3* receptors (*22*, *31*), and mutation of the glycosylation sites in *ste2-3* caused the same degree of suppression in both *CNE1* and *cne1*Δ yeast strains. This indicates that the suppression of the *ste2-3* defect by mutating the glycosylation sites was not caused by escape from *CNE1* function. This is not too surprising since *CNE1* does not appear to carry out the same role in yeast as calnexin does in mammalian cells (*17*, *32*). The temperature-sensitive phenotype of *ste2-3* was significantly suppressed in a *stp22*Δ strain as reported previously (*22*). Mutation of the glycosylation sites did not consistently lead to a further suppression of *ste2-3* in the *stp22*Δ strain. This could suggest that glycosylation and *STP22* act in the same pathway, but it does not necessarily imply a direct relationship. *STP22* (*VPS23*) appears to encode a protein that is localized to a prevacuolar compartment and is therefore more likely to be involved in the later steps of trafficking to the vacuole rather than in the initial steps where misfolded proteins are recognized in the ER or Golgi apparatus (*22*, *36*).

Functional Properties of Nonglycosylated Receptors. The observation that glycosylation is not necessary for wild-type Ste2p function is similar to results obtained with a subset of the other members of the GPCR family. For example, nonglycosylated forms of the histamine H2, muscarinic, V2 vasopressin, and *N*-formyl peptide receptors were fully functional and properly localized at the plasma membrane (*21*, *37*, *38*). In addition, the wild-type forms of the adenosine A_2 and α_{2B}-adrenergic receptors are not glycosylated in vivo (*21*).

However, the global significance of N-linked glycosylation for GPCRs has been elusive due to the conflicting results obtained with other GPCRs. For example, N-linked glycosylation was found to be important for both the cell surface expression and activity of the human thromboxane A_2 and PTH/PTHrP receptors (*19*, *39*). Elimination of the consensus sequences for N-linked glycosylation of the human calcium and rat AT_{1a} angiotensin receptors demonstrated that glycosylation was necessary for cell surface expression and receptor stability, but had no significant effect on the signaling properties of the receptors (*18*, *20*, *40*).

In view of the suppression of *ste2-3* by mutation of glycosylation sites observed in this work, it would be interesting to perform a similar analysis on mutant mammalian GPCRs that are defective in trafficking to the plasma membrane. To our knowledge, the role of glycosylation in mammalian GPCRs has been analyzed primarily within the context of otherwise wild-type receptors. Analysis of whether glycosylation site mutations would suppress the phenotypes of trafficking-defective receptor mutants in other systems may be particularly useful in that it would permit direct analysis of the signaling properties of a broader range of mutant receptors at the plasma membrane. This approach may also be applicable in the study of certain human diseases that are caused by mutations that alter the membrane trafficking of glycoproteins (*41*). For example, the majority of mutations in the human G protein-coupled vasopressin-2 receptor that are associated with nephrogenic diabetes insipidus appear to cause the retention of the mutant receptor proteins within the cell, rather than causing a specific defect in signaling [*42*; NDI mutation database (http//www.medconmcgill.ca/nephros/)]. Since N-linked glycosylation is not required for the function of the wild-type vasopressin-2 receptors (*21*), compounds that modulate the glycosylation machinery may have therapeutic value by preventing the glycosylation of the mutant vasopressin-2 receptors. If these receptors behave like the Ste2-3p mutant receptors, then blocking the glycosylation of the mutant vasopressin-2 receptors may prevent the recognition and intracellular retention of the mutant receptors and thereby allow more receptors to reach the cell surface. This approach may complement existing strategies aimed at searching for small compounds that act as molecular chaperones to assist the proper folding of mutant proteins (*41*).

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