The Sequence NPFXD Defines a New Class of Endocytosis Signal in *Saccharomyces cerevisiae*

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Abstract. The yeast membrane protein Kex2p uses a tyrosine-containing motif within the cytoplasmic domain for localization to a late Golgi compartment. Because Golgi membrane proteins mislocalized to the plasma membrane in yeast can undergo endocytosis, we examined whether the Golgi localization sequence or other sequences in the Kex2p cytoplasmic domain mediate endocytosis. To assess endocytic function, the Kex2p cytoplasmic domain was fused to an endocytosis-defective form of the α-factor receptor, Ste2p. Like intact Ste2p, the chimeric protein, Stex22p, undergoes rapid endocytosis that is dependent on clathrin and End3p. Uptake of Stex22p does not require the Kex2p Golgi localization motif. Instead, the sequence NPFSD, located 37 amino acids from the COOH terminus, is essential for Stex22p endocytosis. Internalization was abolished when the N, P, or F residues were converted to alanine and severely impaired upon conversion of D to A. NPFSD restored uptake when added to the COOH terminus of an endocytosis-defective Ste2p chimera lacking lysine-based endocytosis signals present in wild-type Ste2p. An NPF sequence is present in the cytoplasmic domain of the **a**-factor receptor, Ste3p. Mutation of this sequence prevented pheromone-stimulated endocytosis of a truncated form of Ste3p. Our results identify NPFSD as a clathrin-dependent endocytosis signal that is distinct from the aromatic amino acid–containing Golgi localization motif and lysine-based, ubiquitin-dependent endocytosis signals in yeast.

CLATHRIN-mediated endocytosis of plasma membrane receptors promotes the rapid and efficient uptake of receptor-bound ligands, typically nutrients and signaling molecules important for cell growth and differentiation. Plasma membrane proteins subject to efficient endocytosis contain specific, cytoplasmically disposed amino acid sequences that are necessary for uptake (for review see Trowbridge et al., 1993). Such endocytic targeting signals often contain an aromatic amino acid (tyrosine or phenylalanine) and serve to direct proteins into clathrin-coated pits. The critical importance of aromatic amino acids in the targeting sequences has been established by mutational studies (for review see Trowbridge et al., 1993). For example, mutation of tyrosine 807 in the low-density lipoprotein (LDL)[1] receptor disrupts clathrin-coated pit localization and thereby prevents uptake from the plasma membrane (Davis et al., 1986, 1987). Furthermore, introduction of a single tyrosine into the cytoplasmic domain of the influenza hemagglutinin protein dramatically increases the efficiency of internalization via clathrin-coated pits (Lazarovits and Roth, 1988). In addition to the aromatic amino acid–containing motifs, there are also endocytic targeting signals that lack aromatic amino acids but appear to mediate internalization through clathrin-coated pits. Examples of these signals include di-leucine motifs in the CD3 subunits of the T cell antigen receptor (Letourneur and Klausner, 1992) and a lysine-containing signal in the yeast α-factor receptor, Ste2p (Rohrer et al., 1993).

The location of endocytic targeting signals within the cytoplasmic domains of integral membrane proteins allows for interaction with cytosolic factors that mediate uptake. Internalization motifs containing aromatic amino acids or di-leucines serve as recognition sites for the binding of the AP-2 adaptor, a component of clathrin coats (Pearse, 1988; Glickman et al., 1989; Beltzer and Speiss, 1991; Chang et al., 1993; Nesterov et al., 1995; Ohno et al., 1995; Heilker et al., 1996). Structural analysis of aromatic amino acid–containing targeting signals suggests that they form a characteristic tight β-turn, which may provide a common structural determinant for AP-2 binding (Collawn et al., 1990; Bansal and Gierasch, 1991; Eberle et al., 1991; Backer et al., 1992). Internalization directed by other targeting signals could also involve AP-2 binding, but this has not yet been established.

Aromatic amino acid–containing targeting signals have

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1. *Abbreviations used in this paper*: CHC, clathrin heavy chain; LDL, low-density lipoprotein; M6PR, mannose-6-phosphate receptor.

also been identified in proteins that do not reside primarily at the plasma membrane. Endocytosis directed by these signals is important in the normal trafficking patterns of some of these proteins, including lysosomal acid phosphatase, the TGN proteins furin and TGN38, and the cation-independent and -dependent mannose-6-phosphate receptors (M6PR). For lysosomal acid phosphatase, endocytosis is an intermediate event in the delivery of newly synthesized protein from the Golgi complex to the organelle of residence, the lysosome (Peters et al., 1990). In the cases of M6PR, TGN38, and furin, endocytosis acts to retrieve proteins to the TGN or endosomes where they are predominantly located (Johnson et al., 1990; Canfield et al., 1991; Bos et al., 1993; Humphrey et al., 1993; Wong and Hong, 1993; Ponnambalam et al., 1994; Schäfer et al., 1995). In contrast to the aforementioned examples, an endocytosis signal was identified in a protein that does not normally reach the plasma membrane, the ER-Golgi intermediate compartment protein, ERGIC-53 (Itin et al., 1995). Endocytosis of ERGIC-53 was observed when localization was perturbed by overexpression. This finding allowed definition of an endocytic signal, KKFF, which requires the presence of aromatic amino acids in the two COOH-terminal positions and may mediate interaction with clathrin coats.

Recent studies in the yeast *Saccharomyces cerevisiae* also implicate endocytosis in the trafficking of intracellular membrane proteins that aberrantly reach the cell surface. In yeast strains with a mutation in the *VPS1* gene (*vps1*), which encodes a dynamin-like GTPase, TGN membrane proteins are mislocalized to the vacuole (Wilsbach and Payne, 1993*a*; Nothwehr et al., 1995). In *vps1* cells also carrying a mutation that blocks endocytosis, a TGN protein accumulates at the cell surface (Nothwehr et al., 1995). This finding argues that the *vps1* mutation results in routing of TGN proteins to the plasma membrane, where they are internalized and delivered to the vacuole. The same endocytosis mutation by itself does not cause accumulation of the TGN protein at the surface of cells expressing the wild-type Vps1 protein, supporting previous evidence that TGN protein localization does not normally involve retrieval from the plasma membrane (for review see Wilsbach and Payne, 1993*b*; Nothwehr and Stevens, 1994). In addition, mutation of the clathrin heavy chain gene (*CHC1*) in yeast, which also disrupts TGN protein localization, results in accumulation of the proteins at the cell surface (Payne and Schekman, 1989; Seeger and Payne, 1992), presumably because of the endocytic defect caused by the *chc1* mutation (Tan et al., 1993). These results indicate that TGN proteins are able to undergo endocytosis in yeast and raise the possibility that such proteins might contain endocytosis signals in their cytoplasmic domains.

We have used chimeric proteins to address the possibility that the yeast TGN protein, Kex2p, harbors an endocytic signal. We find that the cytoplasmic domain of Kex2p contains a novel aromatic amino acid–containing signal for clathrin-mediated endocytosis that is distinct from the previously reported Kex2p tyrosine-containing TGN localization sequence (Wilcox et al., 1992) and the previously reported lysine-based endocytosis signal identified in *S. cerevisiae* (Rohrer et al., 1993). Furthermore, the aromatic amino acid–containing endocytic sequence is present in the cytoplasmic domain of the **a**-factor pheromone receptor and is necessary for pheromone-dependent uptake of a truncated form of this receptor.

Materials and Methods

Materials

Unless noted, all reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Strains, Media, and Genetic Methods

The yeast strains and genotypes used in this work are listed in Table I. DNA transformations were performed by the lithium acetate procedure (Ito et al., 1983). The *ste2*Δ strains were produced by single-step gene replacement (Rothstein, 1994) using plasmid pAB506 (a gift from James Konopka, SUNY Stony Brook, Stony Brook, NY) cleaved with BamHI. Gene replacements were monitored by Southern blotting, immunoblotting, and the halo assay (Sprague, 1994) to verify that the *STE2* gene was disrupted. The *ste3*Δ strains were produced by single-step gene replacement using plasmid pSL1841 (a gift from George Sprague, University of Oregon, Eugene, OR) cleaved with HindIII. Immunoblotting and mating tests were used to verify gene disruption. SM1581 contains pSM219, a multicopy plasmid carrying *Mfa1* (a gift from Susan Michaelis, Johns Hopkins University, Baltimore, MD).

SD medium is 0.67% yeast nitrogen base (Difco Laboratories, Inc., Detroit, MI) and 2% dextrose with 20 μg/ml each of uracil, adenine, methionine, histidine, tryptophan, and 30 μg/ml lysine. SD CAA medium is SD containing 5 mg/ml vitamin assay casamino acid mix (Difco Laboratories, Inc.). SD−trp is SD without tryptophan and SD CAA−trp is SD CAA without tryptophan. YP medium is 1% Bacto-yeast extract (Difco Laboratories, Inc.), 2% Bactopeptone (Difco Laboratories, Inc.). YPD is YP supplemented with 2% dextrose. YPR is YP supplemented with 2% raffinose. Cell densities in liquid culture were measured in a 1-cm plastic cuvette using a spectrophotometer (model DU-62; Beckman Instruments, Fullerton, CA).

Construction of STEX22 and Mutant Derivatives

PCR and/or conventional subcloning techniques were used in plasmid constructions. PCR fragments were synthesized using vent DNA polymerase (New England Biolabs, Beverly, MA) and primers were synthesized using an ABI 391 DNA Synthesizer (Perkins-Elmer, Foster City, CA) or purchased from Operon Technologies, Inc. (Alameda, CA). PCR products were purified using the QIAquick PCR purification kit (Qiagen Inc., Chatsworth, CA) before digestion with the appropriate restriction enzymes and separation on TAE agarose gels before subcloning into vectors. All PCR products were sequenced using Sequenase (United States Biochemical Corp., Cleveland, OH) after subcloning into pBKS (Stratagene, La Jolla, CA).

To create the *STE2-KEX2* chimera, PCR fragments encoding relevant portions of *STE2* and *KEX2* were produced with the creation of a unique BglII site at the chimera junction. A 750-bp fragment of *STE2* (Nakayama et al., 1985) encoding amino acids 45–297 was amplified from pRS314-STE2 (Weiner et al., 1993) using the primers 5′-GCTTCTAGAGTTAACAGTACTGTTACTCAG-3′ (primer A) and 5′-GGAAGATCTCGTGGCCCACATTGATGA-3′. A 650-bp fragment of *KEX2* (Fuller et al., 1989) that encodes the entire cytoplasmic tail of Kex2p from amino acids 701–814 as well as roughly 300 bases of the 3′ untranslated region was amplified from pJ2B (Julius et al., 1984) with the primers 5′-GGAAGATCTTCAAGGAGAAGGATCAGA-3′ (primer B) and 5′-CGCGGATCCTTTTTAATACACCAAAGA-3′ (primer C). These PCR products were cloned into pCHC-BX8, which contains the 2-kb XbaI-BamHI fragment of *CHC1* with a unique BglII site in pUC119 (Vieira and Messing, 1987), to create pUC119-SEX2. This plasmid contains both PCR products joined at their BglII sites. pRS314-STEX2 was then created by replacing the HpaI-SacI fragment of pRS314-STE2 with the corresponding fragment of pUC119-SEX2. This construct encodes Stex2p, a protein that has the entire cytoplasmic COOH-terminal tail of Ste2p (amino acids 298–431) replaced by the entire COOH-terminal tail of Kex2p (amino acids 701–814). To create pRS314-STEX22, which encodes Stex22p, primer A was used with the primer 5′-GGAAGATCTATCTGTGGATGTTGTAAA-3′ to synthesize a 820-bp PCR fragment of *STE2* encoding amino acids 45–318

Table I. Yeast Strains Used in This Study

Strain	Genotype	Source
GPY779	*MAT***a** *ste2::LEU2 leu2-3,112 his4 or his6 trp1-289 ura3-52 sst1-3*	This study
GPY789	GPY779 pRS314	This study
GPY790	GPY779 pRS314-STE2	This study
GPY793	*MAT***a** *chc1-521* (ts) *ste2::LEU2 leu2-3,112 his6 trp1-289 ura3-52 sst1-3* pRS314-STE2	This study
GPY839	*MAT***a** *ste2::LEU2 ura3-52 leu2-3,112 his3-Δ200 his4 trp1-Δ901 sst1-1* pRS314-STE2	This study
GPY849	*MAT***a** *ste2::LEU2 end3-1 ura3-52 leu2-3,112 his3-Δ200 and/or his4 trp1-Δ901 sst1-1* pRS314-STE2	This study
GPY1016	GPY779 pRS314-Y713A,Y724A	This study
GPY1047	GPY779 pRS314-793*	This study
GPY1058	GPY779 pRS314-775*	This study
GPY1059	GPY779 pRS314-788*	This study
GPY1060	GPY779 pRS314-783-788Δ,793*	This study
GPY1061	GPY779 pRS314-776-782Δ,788*	This study
GPY1083	GPY779 pRS314-STEX22	This study
GPY1084	*MAT***a** *chc1-521* (ts) *ste2::LEU2 leu2-3,112 his4 or his6 trp1-289 ura3-52 sst1-3* pRS314-STEX22	This study
GPY1085	*MAT***a** *ste2::LEU2 ura3-52 leu2-3,112 his3-Δ200 his4 trp1-Δ901 sst1-1* pRS314-STEX22	This study
GPY1086	*MAT***a** *ste2::LEU2 end3-1 ura3-52 leu2-3,112 his3-Δ200 and/or his4 trp1-Δ901 sst1-1* pRS314-STEX22	This study
GPY1087	GPY779 pRS314-730*	This study
GPY1088	GPY779 pRS314-730-endo	This study
GPY1089	GPY779 pRS314-E776A	This study
GPY1090	GPY779 pRS314-N777A	This study
GPY1091	GPY779 pRS314-P778A	This study
GPY1092	GPY779 pRS314-S780A	This study
GPY1093	GPY779 pRS314-D781A	This study
GPY1094	GPY779 pRS314-Y713A	This study
GPY1095	GPY779 pRS314-Y724A	This study
GPY1096	GPY779 pRS314-718-730Δ	This study
GPY1097	GPY779 pRS314-702-717Δ	This study
GPY1098	GPY779 pRS314-F779A	This study
GPY1449	GPY779 pRS314-P782A	This study
GPY1474	*MATα leu2-3,112 trp1-289 ura3-52 pep4::URA3 prb1 gal2 ste3::LEU2*	This study
GPY1476	GPY1474 pRS314-Gal-Ste3Δ365	This study
GPY1477	GPY1474 pRS314-Gal-Ste3Δ365APA	This study
SM1581	*MAT***a** *ura3 leu2 his4 trp1 can1* pSM219	S. Michaelis

that was subcloned directly into pRS314-STEX2, replacing the HpaI-BglII fragment of *STEX2*. Therefore, *STEX22* contains the *STE2* upstream transcriptional regulatory region and nucleotides encoding amino acids 1–318 of Ste2p fused to the *KEX2* gene encoding amino acids 701–814 of Kex2p and 300 bases of the 3′ untranslated region, with an additional serine codon at the junction of the two genes.

To produce the tyrosine 713 mutant, primers B and C were used in a PCR reaction with pCWKX11 (Wilcox et al., 1992) as a template. The PCR product was cloned into pBKS-CEX2 (see below) using BglII and BamHI to produce pBKS-Y713A. The BglII-SacI fragment of pBKS-Y713A was then placed into pRS314-STEX22 to create pRS314-Y713A.

To facilitate production of Stex22p cytoplasmic tail mutations and deletions, pBKS-CEX2 was created by cloning the ClaI-SacI fragment of pUC119-SEX2 into pBKS. Using mutagenic primers, PCR fragments that carry either point mutations or deletions in *KEX2* were amplified from pBKS-CEX2, and the PCR fragments then were inserted into this plasmid, replacing the wild-type Kex2p cytoplasmic tail. Fragments containing the mutations were then transferred into pRS314-STEX22 using BglII and SacI, replacing the wild-type BglII-SacI fragment of *STEX22*. For example, the primer 5′-CCCGATATCATTGATACAGACTCTGAG**GC**-CGATTCTACTTTGGACAA-3′ was used with primer C to amplify DNA from pBKS-CEX2. (For each primer that produces a point mutation, residues that differ from the wild-type *KEX2* sequence appear in bold type.) The PCR fragment was then subcloned into pBKS-CEX2 with EcoRV and BamHI, replacing the corresponding fragment of pBKS-CEX2, to produce pBKS-Y724A. The BglII-SacI fragment of this plasmid was then subcloned into pRS314-STEX22 to produce pRS314-Y724A. The same PCR product was similarly subcloned into pBKS-Y713A, producing pBKS-Y713A,Y724A, and subsequently pRS314-Y713A,Y724A.

Primers used with primer C to generate internal deletions within Stex22p were 5′-GGAAGATCTATCATTGATACAGACTCT-3′ (702–717Δ) and 5′-CCCGATATCGGAACTTCCGGAATTACT-3′ (718–730Δ).

The unique HindIII site just downstream of the Kex2p termination codon in pBKS-CEX2 was used to subclone PCR fragments encoding truncations of *STEX22* from the carboxy terminus as well as internal deletions. Primers creating these deletions contain a stop codon followed by a HindIII site. Primers used with primer B in PCR reactions and the deletions produced were as follows: 5′-CCCAAGCTTAATTGTCCAAAGTA-GAATC-3′ (730*), 5′-CCCAAGCTTATGCGTTGGCGTCATTTGG-3′ (793*), 5′-CCCAAGCTTAGTTTGTTAGTACACTATC-3′ (775*), 5′-CCCAAGCTTATGGGAACTTTTGCTT-3′ (788*), 5′-CCCAAGCTTA-TGCATTAGCATCGTTAGGGTCACTAAATGG-3′ (783–788Δ,793*), and 5′-CCCAGGCTTATGGGAACTTTTGCTTTATGTTTGTTAGT-ACACT-3′ (776–782Δ,788*).

To produce pRS314-730-endo, a PCR product amplified from primer B and the primer 5′-CCCAAGCTTAGGGATCACTAAAAGGGTTCT-CGTTTGTTAACACATTGTCCAAAGTAGAATCGTA-3′ was cloned into pBKS. After sequencing, the EcoRI-HindIII fragment was removed and ligated into pBKS-CEX2 to create pBKS-730-endo. The BglII-SacI fragment was then subcloned into pRS314-STEX22.

The constructs 793*, 775*, 788*, 783–788Δ,793*, and 776–782Δ,788* contain a point mutation introduced by PCR located within the *STE2* portion of the gene that replaces methionine at position 180 with isoleucine. The mutation has no effect on endocytosis of Stex22p.

Conversion of individual residues 776–782 to alanine was carried out by oligonucleotide-directed mutagenesis. Mutagenesis was performed exactly as described in Kunkel et al. (1987). Single-stranded phage was produced by infecting the *dut*$^-$ *ung*$^-$ bacterial strain CJ236 carrying pBKS-CEX2 with the M13K07 helper phage as described in Vieira and Messing (1987). To purify the single-stranded phage DNA, the phage was precipitated from the media by incubation with 0.2 vol of ice-cold 15% PEG8000 in 3 M NaCl for 1 h on ice, followed by centrifugation at 10,000 *g* for 10 min at 4°C. The pellet was resuspended in 3 ml of 100 mM Tris, pH 7.5, 100 mM NaCl, and 25 mM EDTA, and the phage was disrupted by addition of an equal volume of 4% SDS and incubation at 70°C for 10 min. The sample was then placed on ice, 3 ml of 2.55 M KOAc was added, and

the sample was centrifuged at 15,000 *g* for 30 min at 4°C. Single-stranded DNA was purified using a Qiagen-tip 100 following the procedure provided by Qiagen, Inc. Mutagenized DNA was identified by new restriction sites introduced by the mutagenic primers. The DNA was sequenced and transferred into pRS314-STEX22 as above. Oligonucleotides used for the procedure and point mutations produced are as follows: 5′-TCACTAAATGGATTT**GC**GTTTGTTA**AC**ACACTATCAATTGTA-3′ (E776A), 5′-AGGGTCACTAAATGG**CGCC**TCGTTTGTTAGTACA-3′ (N777A), 5′-TAGGGTCACTAAATG**CA**TTTTCGTTTGTTAG-3′ (P778A), 5′-TTGCTTTATAGGGTC**G**CTA**GC**TGGATTTTCGTTTGT-3′ (F779A), 5′-CTTTTGCTTTATAGGA**TCCGC**AAATGGATTTTCGTT-3′ (S780A), and 5′-CTTTTGCTTTATAGG**AGCG**CTAAATGGATTTTCG-3′ (D781A). To introduce the P782A mutation into Stex22p, the following oligonucleotide was used with single-straded template from pBKS-CEX2 D781A: 5′-AACTTTTGCTTTATAG**CGTCA**CTAAATGGATT-3′ (P782A). Mutagenized DNA was identified by the loss of an Eco47III restriction site present in pBKS-CEX2 D781A.

For construction of pRS314-GAL STE3Δ365, partial digestion by EcoRI was used to produce a 2-kb SacI-EcoRI fragment containing *STE3*-Δ365 under control of the GAL1 promoter from pSL1922 (Davis et al., 1993). This fragment was then inserted into pRS314 (Sikorski and Hieter, 1989).

To introduce N732A and F734A mutations into Ste3-Δ365p, a 1.3-kb EcoRI-SacI fragment from pSL1590 (gift from George Sprague, University of Oregon) was inserted into pBKS to yield pBKS-STE3Δ365. Oligonucleotide mutagenesis was performed as described above using pBKS-STE3Δ365 as a template and the oligonucleotide 5′-TCAGAGTCTGTAGAA**GC**TGGG**GCTCTAGA**CTCTTTGCGTGAGGA-3′. After confirming successful mutagenesis by sequencing, a 0.3-kb NdeI-SacI fragment from the mutagenized plasmid was transferred to pRS314-GAL STE3Δ365 to generate pRS314-GAL STE3Δ365 APA.

Immunoblotting

For Ste2 and Stex22 constructs, 2×10^7 cells from mid-log phase cultures were pelleted in 13 × 100-mm glass tubes. After addition of 200 μl of 0.2-mm glass beads and 50 μl Laemmli sample buffer (Laemmli, 1970) containing 6 M urea (LSUB), the cells were lysed by vortexing at full speed for 90 s. The lysates were incubated at 37°C for 10 min and then 150 μl LSUB added. 25 μl of each lysate was loaded onto 9% SDS-PAGE gels, and after electrophoresis the proteins were transferred to nitrocellulose. Stex22p was visualized using affinity-purified antibody that recognizes the cytoplasmic tail of Kex2p (Phan et al., 1994) or an antibody raised against the amino terminus of Ste2p (R708; kindly provided by James Konopka). Ste2p was visualized using antibody R708.

For Ste3-Δ365p and Ste3-Δ365APAp, samples were pelleted and lysed as described above for Stex22p. The lysates were incubated at 70°C for 5 min and then 150 μl LSUB added. 5 μl of each lysate was loaded onto 12% SDS-PAGE gels, and after electrophoresis the proteins were analyzed by immunoblotting using affinity-purified antibodies specific for the cytoplasmic tail of Ste3p (Davis et al., 1993; provided by Nicholas Davis, Wayne State University, Detroit, MI).

Halo Assay

5×10^6 cells were evenly layered on SD−trp plates and allowed to dry. 5 μl of α-factor, serially diluted to 5, 1, and 0.2 μg/ml, was then spotted and the plates were incubated at 30°C overnight. Halo sizes for 5 μg/ml α-factor typically ranged from 17–22 mm for *STEX22* strains and 12–15 mm for *STE2* strains. Strains lacking *STEX22* or *STE2* gave no halos.

Binding and Endocytosis of Radiolabeled α-Factor

All strains were grown in SD CAA−trp at 30°C unless otherwise indicated. Binding and uptake of radiolabeled α-factor was performed as described (Tan et al., 1993) with a 5-min preshift and internalization at 30°C unless otherwise indicated. For each time point, duplicate samples were analyzed and the results averaged. All experiments were repeated at least twice and yielded the same results. A representative experiment is shown in each case.

Pronase-Sensitivity Assay for Ste3-Δ365 Endocytosis

Strains GPY 1476 and GPY 1477 were grown for >8 h at 30°C to midlog phase in YPR. Galactose was then added to 2% and cells were incubated for 1 h at 30°C to induce receptor expression. Cultures were then supplemented with 3% dextrose and incubated for an additional hour to repress further receptor expression. At this point, 10^8 cells were removed and brought to 10 mM NaN_3 and 10 mM NaF and placed on ice ($t = 0$). The remaining cells were pelleted and resuspended at 10^7 cells/ml in **a**-factor conditioned medium or YPD prewarmed to 30°C. **a**-Factor conditioned medium was prepared from a saturated culture of SM1581 by sedimenting the cells and supplementing the resulting supernatant with 2% dextrose and 0.2% Bacto-yeast extract. At the designated time intervals, 10-ml samples were removed to ice and treated with NaN_3 and NaF as described above. At the conclusion of the time course, cells were collected by sedimentation, washed once in PB (50 mM Tris-HCl, pH 7.5, 1.4 M Sorbitol, 10 mM NaN_3, 10 mM NaF, 40 mM β-mercaptoethanol, 2 mM $MgCl_2$), resuspended in 2 ml PB, and divided into two 1-ml aliquots. One aliquot was treated with pronase and the other aliquot was mock-treated (Davis et al., 1993). After a 1-h pronase treatment at 37°C, samples were placed on ice and received 4.5×10^7 SM1581 cells as carrier plus EDTA to 1 mM and a protease inhibitor cocktail (Tan et al., 1993). Cells were washed three times in PB plus protease inhibitor cocktail plus 1 mM EDTA, lysed, and analyzed for Ste3-Δ365p or Ste3-Δ365APAp by immunoblotting.

Results

Construction and Immunodetection of the Stex22p Chimera

In yeast, the type I integral membrane protein Kex2p resides in a late Golgi compartment (analogous to the mammalian TGN), where it cleaves α-factor prohormone in transit through the secretory pathway (Fuller et al., 1988; Graham and Emr, 1991). To determine whether Kex2p contains an endocytic signal, we designed a chimeric protein in which the endocytic capacity of the Kex2p cytoplasmic domain could be easily measured. In this chimeric protein, designated Stex22p, the entire 114–amino acid Kex2p cytoplasmic domain replaces the COOH-terminal cytoplasmic domain of the α-factor mating pheromone receptor, Ste2p (Fig. 1). Ste2p is a seven membrane–spanning domain receptor which mediates the clathrin-dependent endocytosis of α-factor pheromone. The NH_2-terminal 318 amino acids of Ste2p, which are included in Stex22p, form a functional domain that is sufficient to bind α-factor and activate the signal transduction pathway necessary for the mating response (Konopka et al., 1988; Reneke et al., 1988). The COOH-terminal cytoplasmic domain of Ste2p is required for endocytosis and desensitization to the effects of the ligand (Konopka et al., 1988; Reneke et al., 1988). In Stex22p, the Kex2p cytoplasmic sequences are appended to the NH_2-terminal Ste2p domain, thereby replacing the Ste2p cytoplasmic domain. Thus, the chimeric protein is designed to bind α-factor through the Ste2p sequences and rely on the Kex2p sequences for endocytosis. This design allows endocytosis of the chimeric protein to be conveniently monitored using established assays for receptor-mediated internalization of α-factor.

The *STEX22* gene, driven by the *STE2* promoter and carrying the 3′ untranslated region of *KEX2* to ensure efficient expression, was placed into a single-copy centromere-containing vector and introduced into cells carrying a deletion of the chromosomal *STE2* gene (see Materials and Methods). Expression of Stex22p was monitored by immunoblotting cell extracts with antibodies directed against the COOH terminus of Kex2p and the NH_2 terminus of Ste2p (Fig. 2). For comparison, we also examined extracts of cells with the vector alone or with the vector carrying

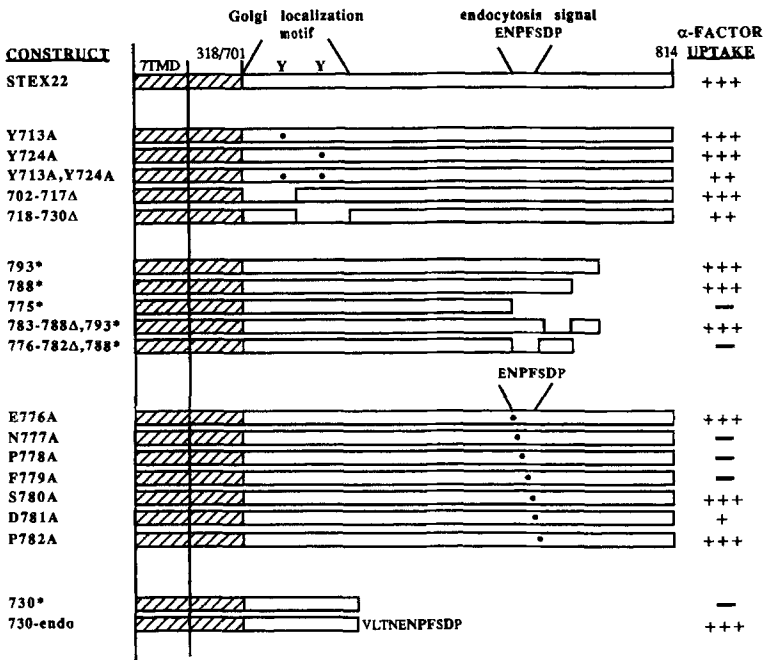

Figure 1. STEX22 constructs used in this study and summary of α-factor uptake for each construct. The seventh transmembrane domain (*7TMD*) and cytoplasmic portion of Ste2p (*hatched bars*) to amino acid 318, and the cytoplasmic domain of Kex2p from amino acids 701 to 814 (*open bars*) are represented. For STEX22 truncations and internal deletions, the * symbol follows the last remaining amino acid of a COOH-terminal truncation, and the Δ symbol follows the amino acids removed by an internal deletion. Point mutations within the open bars are indicated by the symbol •. The location of the Golgi localization motif with two tyrosine residues and amino acids in the endocytosis signal are also shown.

STE2. The Ste2p antibodies recognized a doublet of 50 and 52 kD in *STEX22* cells (Fig. 2, lane *6*), which is slightly larger than Ste2p (Fig. 2, lane *5*). Species of reduced mobility were also apparent in extracts from both *STEX22* and *STE2* cells (Fig. 2, *asterisks*) and correspond to aggregates most likely caused by the hydrophobic nature of these polytopic membrane proteins (Blumer et al., 1988; Konopka et al., 1988). No reactive proteins were detected in extracts of cells carrying the vector alone, demonstrating the specificity of the antibodies (Fig. 2, lane *4*). The Kex2p antibodies revealed a pattern of Stex22p bands identical to that produced by the Ste2p antibodies (compare Fig. 2, lanes *3* and *6*), as anticipated from the hybrid character of the protein. In cells carrying vector alone or *STE2*, the Kex2p antibodies detected only the endogenous 130-kD Kex2p (Fig. 2, lanes *1* and *2*). The steady-state amounts of Stex22p were substantially higher than that of Kex2p, reflecting in part the relative strengths of the *STE2* and *KEX2* promoters. Because of the relatively high expression levels, the low mobility forms of Stex22p masked the signal from Kex2p in *STEX22* cells (Fig. 2, lane *3*).

Stex22p Undergoes Clathrin and End3p-dependent Endocytosis

For Stex22p to be useful in endocytosis assays, it must be delivered to the plasma membrane. However, the presence of a Golgi localization signal in the Kex2p cytoplasmic domain presented a potential complication (Wilcox et al., 1992). Golgi localization of Kex2p can be overcome by overexpression, presumably because of saturation of the localization machinery (Wilcox et al., 1992). Therefore, we relied on the considerable overexpression of Stex22p compared to Kex2p (Fig. 2, lanes *1–3*) to saturate the Golgi localization machinery and allow the chimeric protein to reach the plasma membrane. Indeed, *STEX22* cells, but not cells carrying the vector alone, displayed α-factor–induced cell cycle arrest and mating, thereby providing an indication that Stex22p is present at the cell surface, where it can bind pheromone and trigger the mating response signal transduction pathway (data not shown). Furthermore, *STEX22* cells specifically bound radiolabeled α-factor with equivalent binding capacity and affinity to cells expressing Ste2p (data not shown). Thus, significant levels of Stex22p are delivered to the cell surface, where it displays α-factor receptor properties similar to the native Ste2p receptor.

Internalization of Stex22p was assessed by monitoring the uptake of prebound radiolabeled α-factor (Tan et al., 1993). Radiolabeled α-factor was allowed to bind cells on ice in the absence of glucose, which provides an energy source necessary for endocytosis. After removing unbound pheromone, the temperature was elevated for 5 min, and then glucose was added to initiate endocytosis. Uptake was determined at 5- and 20-min time intervals by subjecting cells to a low pH wash to remove α-factor remaining at the surface. We chose these time points because uptake in wild-type *STE2* cells is linear for at least 5 min and approaches a plateau after 20 min (Tan et al., 1993). Interestingly, the levels of α-factor internalization by *STEX22* cells and *STE2* cells were the same at both time points (Fig. 3), showing that both the rate and extent of Stex22p endocytosis are comparable to Ste2p. Some variation in the relative endocytosis rates of Stex22p and Ste2p was observed in different genetic backgrounds (for example, see Fig. 5). Nevertheless, Stex22p uptake was never less that 50% as efficient as Ste2p uptake.

Mutations in a number of genes interfere with Ste2p endocytosis, including *CHC1* and the *END* genes (Raths et al., 1993; Tan et al., 1993; Munn and Riezman, 1994). To determine whether internalization of Stex22p and Ste2p proceeds through similar pathways, we compared internalization of the two proteins in cells carrying a temperature-sensitive allele of *CHC1* (*chc1-ts*) or in cells with a defective *END3* gene (*end3-1*). Plasmids carrying *STEX22* or *STE2* were introduced into mutant and wild-type cells

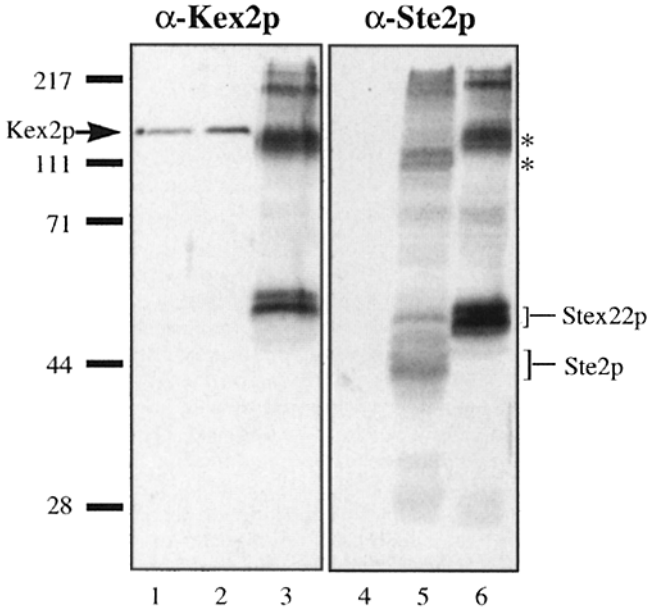

Figure 2. Expression of Stex22p. Extracts were prepared from GPY789 (pRS314; lanes *1* and *4*), GPY 790 (pRS314-STE2; lanes *2* and *5*), and GPY 1083 (pRS314-STEX22; lanes *3* and *6*). Samples were subjected to SDS-PAGE and transferred to nitrocellulose. The nitrocellulose was then divided and one half probed with antibodies to the COOH terminus of Kex2p (lanes *1–3*) and the other half probed with antibodies to the NH_2 terminus of Ste2p (lanes *4–6*). The positions of Stex22p, Ste2p, and Kex2p are indicated. Asterisks denote forms of Stex22p and Ste2p with reduced mobility.

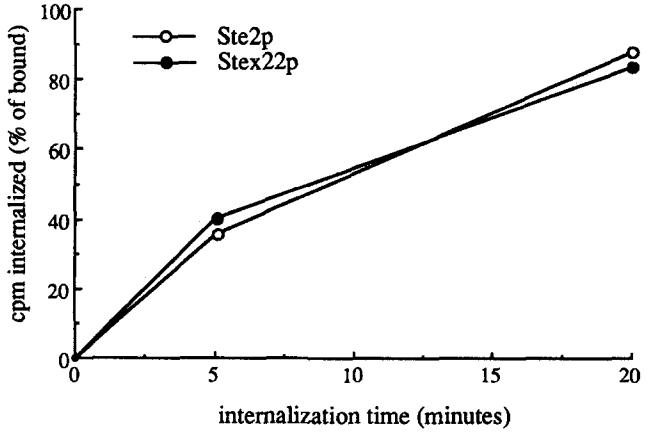

Figure 3. Ligand-induced endocytosis of Stex22p is rapid and equivalent to that of Ste2p. Uptake of radiolabeled α-factor was measured in cells expressing Ste2p (GPY 790; *open symbols*) and Stex22p (GPY 1083; *closed symbols*). Radiolabeled α-factor was bound to cells on ice. After removal of the unbound pheromone, cells were incubated at 30°C for 5 min, and then glucose was added to a 10% final concentration to initiate endocytosis. Internalized α-factor was measured after the indicated internalization time periods.

lacking the endogenous *STE2* gene. With *chc1-ts* cells, endocytosis of Stex22p and Ste2p was determined at permissive (24°C) and nonpermissive temperatures (37°C) by measuring uptake of radiolabeled α-factor. Ste2p internalization was equivalent in wild-type (*CHC1*) and *chc1-ts* cells at 24°C (Fig. 4 *A*, *open symbols*). As reported previously (Tan et al., 1993), inactivation of the temperature-sensitive clathrin heavy chain in *chc1-ts* cells at 37°C immediately reduced uptake two- to threefold relative to the wild-type cells (Fig. 4 *A*, *closed symbols*). Similar results were obtained with Stex22p (Fig. 4 *B*). At the permissive temperature, uptake of bound ligand was equivalent in *CHC1* and *chc1-ts* cells, whereas at the nonpermissive temperature, internalization was reduced five- to sixfold. It should be noted that we consistently observed a slightly more severe effect of *chc1-ts* on Stex22p (five- to sixfold) than on Ste2p (two- to threefold). The *end3-1* allele blocks Ste2p uptake almost completely at all temperatures (Raths et al., 1993; Bénédetti et al., 1994). In cells carrying the *end3-1* allele, Stex22p endocytosis was blocked to the same degree as Ste2p (Fig. 5, *closed symbols*). The results described in this section indicate that the Kex2p cytoplasmic domain has the potential to mediate efficient endocytosis through a clathrin and *END3*-dependent pathway.

The Kex2p Golgi Localization Motif Is Not Required for Endocytosis of Stex22p

Previous studies show that mutation of the Kex2p cytoplasmic sequences adjacent to the membrane spanning domain severely disrupt Golgi localization (Wilcox et al., 1992). This work defined a tyrosine-based signal for Golgi localization. The similarity of the Kex2p Golgi localization signal to tyrosine-based clathrin-coated pit targeting signals in mammalian proteins, combined with the involvement of clathrin in uptake of Stex22p, led us to address whether the Golgi localization motif functions as an endocytosis signal in Stex22p. For this purpose, mutations were generated in the cytoplasmic domain of Stex22p (Fig. 1) that were modeled on mutations shown to cause defects in Golgi localization of Kex2p (Wilcox et al., 1992). In particular, two deletions were produced, 702–717Δ and 718–730Δ, as well as a single amino acid conversion of tyrosine 713 to alanine (Y713A). (All numbering refers to the amino acid positions within the full-length Kex2p sequence. Stex22p contains Kex2p residues 701–814.) Considering the importance of tyrosines in endocytic signals, tyrosine 724 was also converted to alanine (Y724A), although this residue is not important for Kex2p Golgi localization. Finally, we produced a double tyrosine point mutant (Y713A, Y724A), leaving no other tyrosines in the Stex22p cytoplasmic domain.

Cells carrying each of these mutant forms of Stex22p were subjected to endocytosis assays (Fig. 6). Individual point mutants Y713A and Y724A, as well as the 702–717Δ internal deletion, had no effect on endocytosis compared to wild-type Stex22p. The double tyrosine point mutant Y713A,Y724A and the 718–730Δ internal deletion displayed slightly reduced uptake compared to wild-type Stex22p. After 5 min of endocytosis, the Y713A,Y724A and 718–730Δ mutants internalized 18 and 13% bound pheromone, respectively, compared to 31% uptake for Stex22p. After 20 min, the mutants internalized 46% (Y713A,Y724A) and 39% (718–730Δ), whereas the wild-type internalized 64% of the bound ligand. This corresponds to a 1.5–2-fold reduction in uptake. The absence of

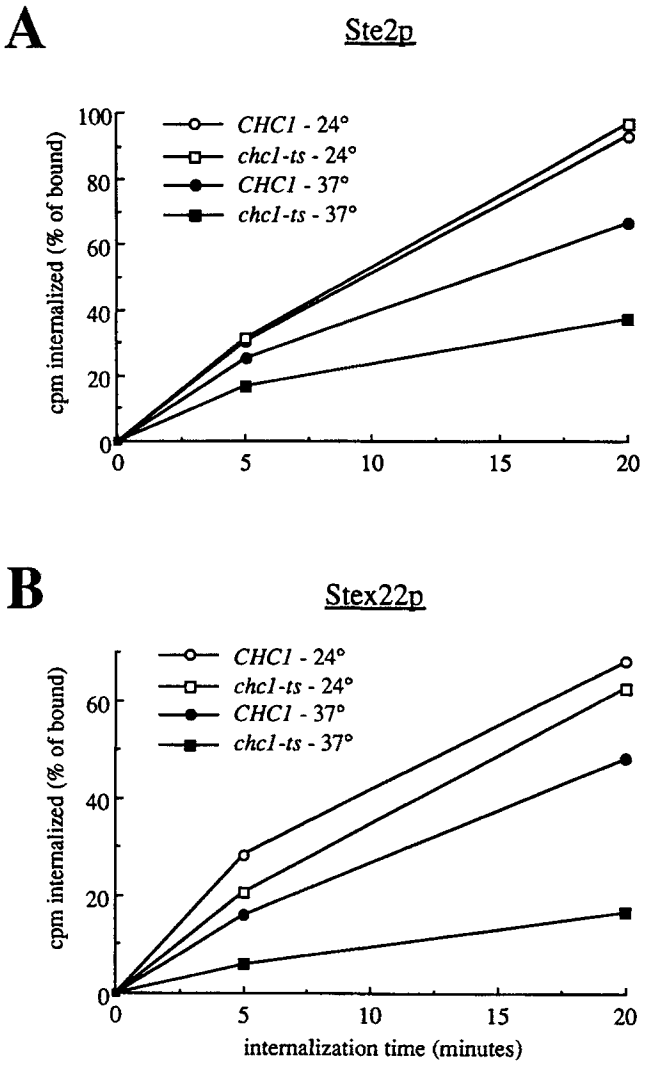

Figure 4. Rapid endocytosis of Stex22p is dependent on functional clathrin heavy chain. Uptake of radiolabeled α-factor was measured in isogenic *CHC1* (*circles*) and *chc1-ts* (*squares*) strains expressing Ste2p (*A*) or Stex22p (*B*). Strains used were: GPY 790 (*CHC1*, Ste2p), GPY 1083 (*CHC1*, Stex22p), GPY 793 (*chc1-ts*, Ste2p), and GPY 1084 (*chc1-ts*, Stex22p). After binding and removal of unbound pheromone as described in the legend for Fig. 3, cells were incubated for 5 min at the permissive temperature of 24°C (*open symbols*) or for 5 min at the nonpermissive temperature of 37°C (*closed symbols*). Uptake of radiolabeled α-factor at 24° and 37°C was measured as described in the legend for Fig. 3 after the indicated internalization time periods.

strong endocytic defects exhibited by these mutants argues that the Golgi localization sequence does not also function as an endocytic signal. Further mutagenesis studies presented below confirm this interpretation.

Identification of a Sequence Necessary for Endocytosis of Stex22p

A series of COOH-terminal truncations were constructed to define sequences necessary for endocytosis (Fig. 1). Initially, we analyzed two truncations, one which removes 21 amino acids (793*), and one which removes 84 amino acids, leaving just the first 30 amino acids of the Kex2p cytoplasmic domain (730*). The 793* mutant exhibited wild-type uptake of pheromone but the 730* mutant was completely defective (Fig. 7 *A*). After 20 min, only 3% of the bound ligand was internalized by the 730* mutant. The results from these truncations indicated that the sequences necessary for endocytosis occur between residues 731 and 793 of the Kex2p cytoplasmic domain. The endocytic defect of 730* further strengthens the conclusion that the Golgi localization signal located between residues 702 and 730 does not function as an endocytic signal.

A deletion removing 25 residues from the Stex22p COOH terminus (788*) had no effect on endocytosis while a deletion removing 38 residues (775*) abolished up-

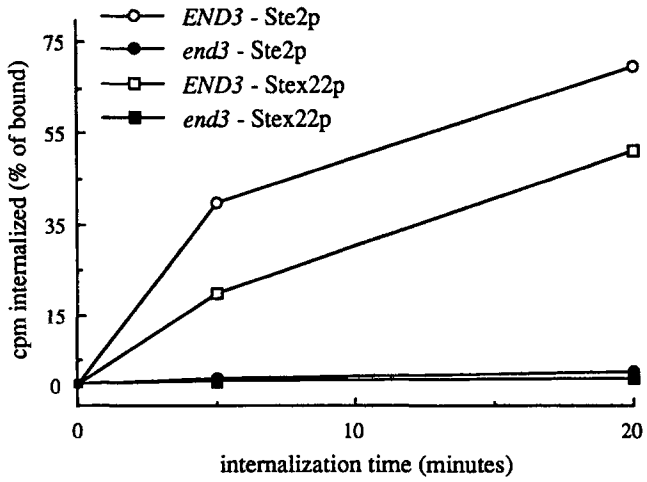

Figure 5. Endocytosis of Stex22p is dependent on End3p. Uptake of radiolabeled α-factor was measured in wild-type (*END3*; *open symbols*) and *end3-1* mutant (*end3*; *closed symbols*) cells expressing Ste2p (*circles*) or Stex22p (*squares*). The experiment was done as described in legend for Fig. 3. Strains used were: GPY 839 (*END3*, Ste2p), GPY 1085 (*END3*, Stex22p), GPY 849 (*end3*, Ste2p), and GPY 1086 (*end3*, Stex22p).

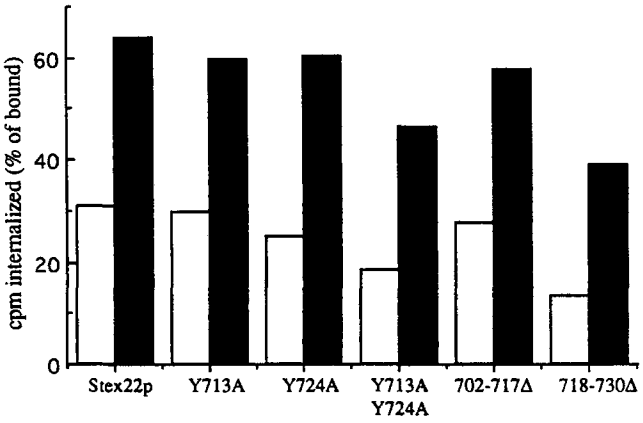

Figure 6. The Kex2p localization motif is not required for endocytosis of Stex22p. Uptake of radiolabeled α-factor after 5 min (*open bars*) and after 20 min (*closed bars*) was performed for the indicated strains as described in the legend for Fig. 3. Strains used were: GPY 1083 (Stex22p), GPY 1094 (Y713A), GPY 1095 (Y724A), GPY 1016 (Y713A, Y724A), GPY 1097 (702–717Δ), and GPY 1096 (718–730Δ).

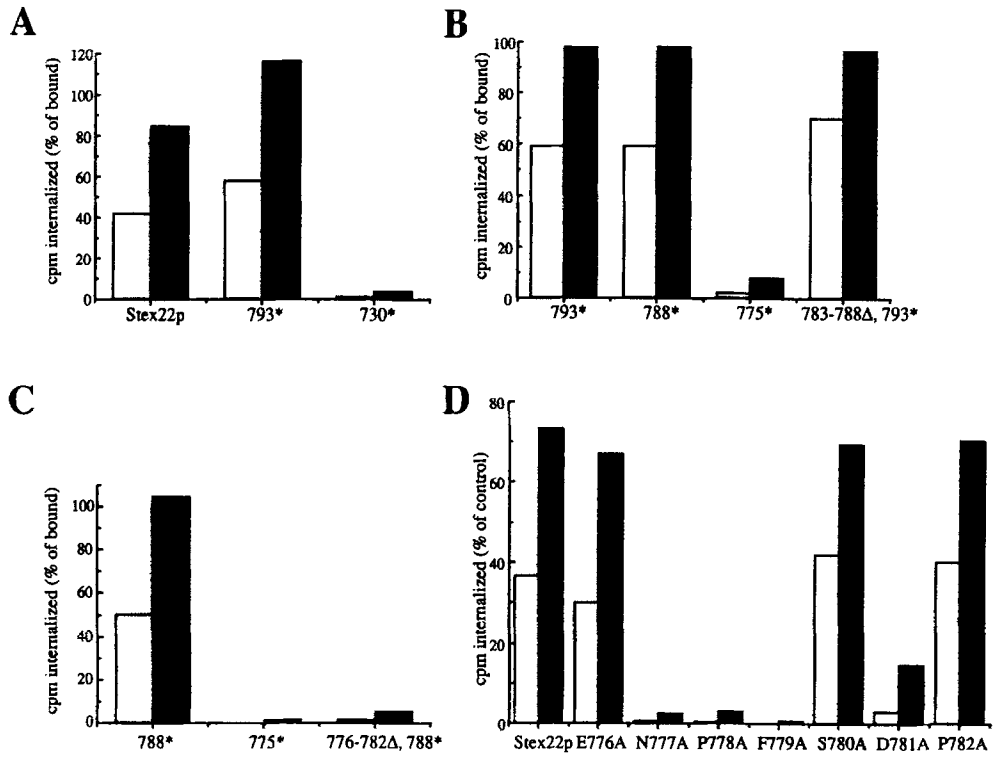

Figure 7. Uptake of radiolabeled α-factor in *STEX22* mutant strain defines a sequence necessary for endocytosis. Experiments were performed as described in the legend for Fig. 3. Uptake in each indicated strain was determined after 5 min (*open bars*) and after 20 min (*closed bars*). Strains used were: (*A*) GPY 1083 (*Stex22p*), GPY 1047 (*793**), and GPY 1087 (*730**); (*B*) GPY 1047 (*793**), GPY 1058 (*775**), GPY 1059 (*788**), and GPY 1060 (*783–788Δ*, 793*); (*C*) GPY 1047 (*793**), GPY 1058 (*775**), and GPY 1061 (*776–782Δ*, *788**); (*D*) GPY 1083 (*Stex22p*), GPY 1089 (*E776A*), GPY 1090 (*N777A*), GPY 1091 (*P778A*), GPY 1098 (*F779A*), GPY 1092 (*S780A*), GPY 1093 (*D781A*), and GPY 1449 (*P782A*).

take (Fig. 7 *B*). Two internal deletions between residues 775 and 788 further defined the endocytic signal. For convenience, these deletions were engineered in the endocytically competent COOH-terminal truncations 793* and 788*. Deletion of amino acids 783–788 within the truncated 793* mutant did not affect endocytosis (783–788Δ, 793*, Fig. 7 *B*). In contrast, removal of amino acids 776–782 from 788* resulted in a severe endocytic defect comparable to the 775* and 730* mutants (776–782Δ, 788*, Fig. 7 *C*). This analysis revealed that residues 776–782 are critical for endocytosis of Stex22p.

The amino acid sequence corresponding to residues 776 to 782 is ENPFSDP (Fig. 1). We converted each of these residues individually to alanine within the full-length Kex2p cytoplasmic domain and measured internalization of each mutant (Fig. 7 *D*). Strikingly, mutation of asparagine 777, proline 778, or phenylalanine 779 eliminated endocytosis of the chimera, with only 1–3% uptake of radiolabeled α-factor after the 20-min time point (Fig. 7 *D*, *N777A*, *P778A*, *F779A*). Mutation of aspartate 781 to alanine impaired but did not abolish uptake (Fig. 7 *D*, *D781A*). After 5 min of endocytosis, the D781A mutant internalized just 3% of the bound pheromone relative to 37% uptake for the wild-type Stex22p chimera, and after 20 min the mutant internalized 15% of the bound ligand compared to 73% for Stex22p. These values reveal a five- to tenfold reduction in the rate of internalization for the D781A mutant. In contrast, conversion of glutamate 776, serine 780, or proline 782 to alanine had no effect on endocytosis (Fig. 7 *D*, *E776A*, *S780A*, *P782A*). Therefore, we conclude that asparagine 777, proline 778, and phenylalanine 779 are absolutely required and that aspartate 781 is very important but not required for endocytosis. These point mutations define a signal, NPFXD, that is necessary for uptake of Stex22p. Because these point mutations have been generated in the full-length chimera, it is likely that the NPFXD sequence is the only signal for endocytosis of Stex22p.

The NPFXD Endocytosis Signal Completely Restores Uptake in a Truncated Chimera

To determine whether the endocytosis signal is sufficient for internalization and can function in other sequence contexts, we placed 11 amino acids spanning the signal, VLTNE**NPFSD**P, at the end of the endocytosis-defective truncation 730* to produce 730-endo (Fig. 1). Addition of the signal to this endocytosis-deficient mutant completely restored uptake of radiolabeled α-factor to levels equivalent to the wild-type Stex22p (Fig. 8). Thus, the NPFSD sequence acts as an autonomous signal for endocytosis.

NPF Functions in Endocytosis of a Truncated, Pheromone-dependent Form of the a-Factor Mating Pheromone Receptor

Since the pheromone receptors are internalized as a regular part of their intracellular transport pattern, we sought similar sequences in the cytoplasmic domains of these proteins. The cytoplasmic domain of Ste2p does not contain a sequence with the three critical residues, NPF. In contrast, the **a**-factor receptor Ste3p contains a sequence NPFSTD beginning at residue 332 in its cytoplasmic domain.

To facilitate examination of the role of NPFSTD in Ste3p endocytosis, we took advantage of a truncated form of the receptor, Ste3-Δ365p (Davis et al., 1993). Ste3-Δ365p is efficiently transported to the cell surface but, unlike the full-length Ste3p, is not internalized unless pheromone is present. This property allowed us to monitor synchronized endocytosis of receptors initiated by the addition of **a**-factor. The putative NPF endocytosis signal

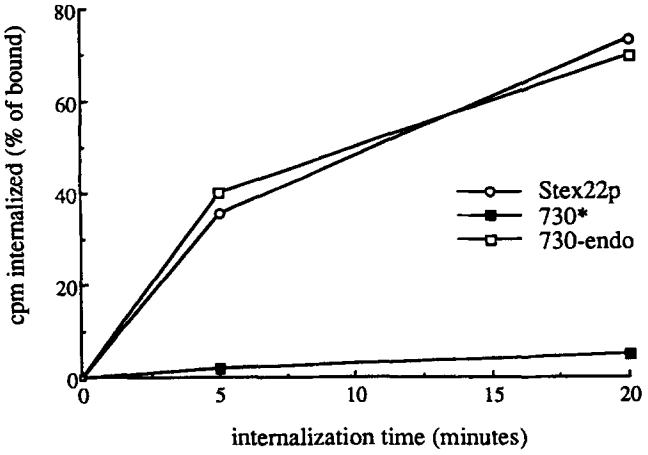

Figure 8. The NPFXD sequence completely restores internalization in an endocytosis-deficient construct. Uptake of radiolabeled α-factor was measured as described in the legend for Fig. 3 in strains expressing Stex22p (GPY 1083; *open squares*), 730* (GPY 1087; *closed squares*) and 730-endo (GPY 1088; *open circles*).

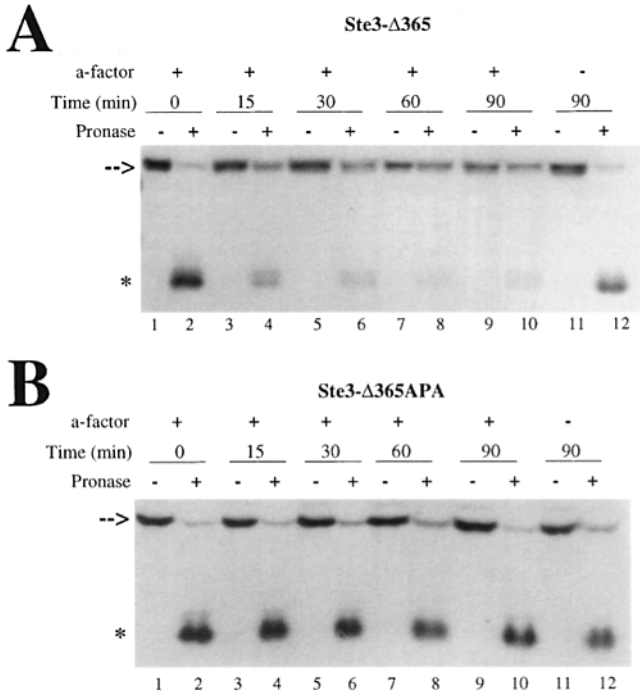

Figure 9. NPF is necessary for endocytosis of Ste3-Δ365p. Strains expressing either *STE3-Δ365* (GPY1476) or *STE3-365APA* (GPY1477) under control of the *GAL1* promoter were grown for 1 h in galactose medium to induce receptor expression. After addition of glucose to repress expression and a 1 h incubation to allow accumulation of receptors at the cell surface, **a**-factor was added to stimulate endocytosis (***a-factor***, +). At the designated times, cells were harvested and divided into two aliquots. One aliquot was treated with pronase (*Pronase*, +) while the second aliquot was mock treated (*Pronase*, −). Cells were then lysed, the extracts were subjected to SDS-PAGE, and receptors were detected by immunoblotting (*arrows*). As a control for the ligand-dependence of receptor endocytosis, one sample was incubated for 90 min in the absence of **a**-factor (lanes *11* and *12*, ***a-factor***, −). The asterisks mark pronase-resistant COOH-terminal receptor fragments.

in Ste3-Δ365 was mutated to APA (Ste3-Δ365APAp), and then both wild-type and mutant genes were placed under control of the inducible *GAL1* promoter. The resulting plasmids were introduced into cells carrying a disruption of the chromosomal *STE3* locus.

Ligand-stimulated endocytosis of Ste3-Δ365p and Ste3-Δ365APAp was compared using a protease protection assay. Receptor expression was induced by incubating cells in galactose-containing media and then repressed by addition of glucose. After a 1-h incubation to ensure that all receptors reached the cell surface, **a**-factor was added to stimulate endocytosis. The extent of receptor endocytosis was determined at various times by treatment of cells with pronase, which cleaves receptors present at the cell surface. Before pheromone addition (Fig. 9, *A* and *B*, lanes *1* and *2*), both the mutant and wild-type receptors were almost completely sensitive to pronase, indicating that the bulk of both receptor populations resided at the plasma membrane. Degradation of the receptors was accompanied by the appearance of a proteolytically resistant fragment that is derived from the cytoplasmic domain (Fig. 9, *asterisk*; Davis et al., 1993; Tan et al., 1993). After pheromone addition, a distinct difference was apparent in the pronase sensitivity of Ste3-Δ365p and Ste3-Δ365APAp. Ste3-Δ365p became resistant to protease, reflecting the sequestration of receptor from the external pronase by endocytosis (Fig. 9 *A*, lanes *1–10*). Uptake was pheromone-dependent as judged by the degradation of the receptor in cells incubated for 90 min in the absence of pheromone (Fig. 9 *A*, lanes *11* and *12*). In contrast, the Ste3-Δ365APA receptor remained pronase-sensitive throughout the course of the experiment, revealing a defect in endocytosis (Fig. 9 *B*, lanes *1–10*). These experiments show that the NPF sequence is necessary for the ligand-induced endocytosis of the Ste3-Δ365 receptor.

Discussion

We have identified a novel yeast internalization signal, NPFXD, through the analysis of a chimeric protein. The amino acid sequence derives from the cytoplasmic domain of the late Golgi membrane protein Kex2p and mediates rapid End3p- and clathrin-dependent endocytosis of the Stex22p chimera. Endocytosis is abolished when the asparagine, proline, and phenylalanine are individually converted to alanine, and uptake is severely impaired when the aspartate is changed to alanine. Furthermore, endocytosis is fully restored upon addition of the sequence to an endocytosis-deficient construct. The complete block in uptake when the sequence is mutated and the complete restoration of uptake when the sequence is present argue that the NPFXD sequence is the only signal that mediates uptake of Stex22p.

The NPFXD sequence is only the second internalization signal identified in yeast to our knowledge. The first, DAKSS, was uncovered in the cytoplasmic domain of Ste2p and participates in ligand-induced endocytosis of the receptor (Rohrer et al., 1993). In the context of a truncated form of Ste2p, the lysine in DAKSS is specifically required for uptake, and recent results indicate that phero-

mone-stimulated ubiquitination of this residue plays a critical role in the internalization process (Hicke and Riezman, 1996). Mutation of the DAKSS lysine eliminates ligand-induced ubiquitination and endocytosis of the truncated Ste2p (Hicke and Riezman, 1996). We attempted to determine whether uptake of Stex22p, like Ste2p, is ligand inducible, but the intracellular pool of Stex22p precluded unequivocal interpretation of experiments designed to measure receptor clearance from the cell surface (Tan, P., unpublished results). Regardless of the role of ligand in Stex22p uptake, two considerations suggest that ubiquitination may not be necessary for NPFXD-mediated endocytosis. First, the sequence contains no lysine residues for ubiquitination. Second, the 730-endo construct is efficiently internalized. The only lysines in 730-endo are contained in the Ste2p portion of the molecule. A truncated Ste2p that contains these lysines but lacks the DAKSS lysine is not subject to ligand-induced ubiquitination or endocytosis (Hicke and Riezman, 1996). Consequently, the cytoplasmically disposed lysines within the first 318 Ste2p residues do not act as ubiquitin acceptors after pheromone binding. Because the 730-endo protein contains no other lysines, internalization of this receptor occurs in the absence of sites that serve as ubiquitin acceptors. Therefore, endocytosis mediated by NPFXD is likely to be mechanistically distinct, to some degree, from that mediated by DAKSS. Based on this argument, we suggest that at least two classes of endocytic targeting signals exist in *S. cerevisiae*: one class contains lysine residues and requires ubiquitination for uptake, and the second class contains a critical aromatic amino acid and may not require ubiquitination.

Analysis of Ste3-Δ365p revealed that the NPF-based endocytic signal is necessary for ligand-dependent internalization of this form of the **a**-factor receptor. This finding demonstrates that the NPF endocytic signal is not a feature peculiar to Kex2p but also is found in a protein that normally undergoes endocytosis. Endocytosis of Ste3-Δ365p differs from full-length Ste3p in the extent of pheromone dependence. Full-length Ste3p is rapidly internalized in the absence of pheromone, while Ste3-Δ365p requires ligand binding for uptake (Davis et al., 1993). The basis for this difference is not clear. Preliminary experiments suggest that mutation of the NPF sequence in the full-length receptor does not impede uptake in the absence of ligand (Howard, J.P., and G. Payne, unpublished results), arguing that some other signal, presumably between residue 365 and the COOH terminus, mediates ligand-independent endocytosis of Ste3p. A recent study indicates that ubiquitination may be involved in endocytosis of both Ste3p and Ste3-Δ365p (Roth and Davis, 1996). With the identification of NPF as an endocytic targeting signal in Ste3-Δ365p, it should now be possible to address the relationship between the NPF signal and ubiquitination in endocytosis of this receptor.

The NPFSD and NPFSTD internalization signals resemble the NPXY internalization signal of the LDL receptor and other plasma membrane proteins that mediates clustering into clathrin-coated pits and uptake into clathrin-coated vesicles (Davis et al., 1986; Chen et al., 1990). Since mutations in clathrin heavy chain interfere with Stex22p and Ste3-Δ365p uptake (Tan et al., 1993), it is possible that NPF also functions as a signal for clathrin-mediated endocytosis. In view of this proposal, it may be noteworthy that Stex22p endocytosis is more severely impaired than Ste2p in *chc1-ts* cells. Conversion of the asparagine, proline, or tyrosine in the NPXY sequence severely impairs endocytosis of the LDL receptor (Davis et al., 1986, 1987), a result similar to the corresponding mutations within the NPFXD signal of Stex22p. Although a phenylalanine rather than a tyrosine is present in the Stex22p sequence, phenylalanine in place of the tyrosine in NPXY results in normal uptake (Davis et al., 1986, 1987). The NPXY sequence adopts a tight β-turn conformation, which is implicated as a structural determinant of the endocytic signal (Collawn et al., 1990; Bansal and Gierasch, 1991; Eberle et al., 1991). It is therefore possible that the NPFXD signal also forms this structural conformation involving a tight turn. Consistent with this idea, the combination of asparagine and proline is a statistically favored pair in type I β-turns, as well as in the first turn of an α helix (Wilmot and Thornton, 1988; Richardson and Richardson, 1988). Further experiments will be needed to establish the conformation of the NPFXD signal.

The sequence in the Kex2p cytoplasmic domain that mediates Golgi localization of Kex2p is also based on an essential aromatic amino acid (tyrosine 713) and resembles clathrin-coated pit targeting sequences in mammalian proteins (Wilcox et al., 1992). Mutations of this sequence in Kex2p result in Golgi localization defects, but the same mutations in Stex22p have little or no effect on internalization. Conversely, mutations that remove the NPFXD endocytosis signal from the cytoplasmic domain of native Kex2p do not cause Golgi localization defects (Wilcox et al., 1992). Thus, the cytoplasmic domain of Kex2p carries two functionally distinct, and physically separate, aromatic amino acid–based targeting signals, one for Golgi localization and one for endocytosis.

Since both Golgi localization of Kex2p and endocytosis of Stex22p are dependent on clathrin function (Payne and Schekman, 1989; Seeger and Payne, 1992; this work), it is tempting to speculate that the distinct targeting signals serve as recognition sites for clathrin coat components that differ between the endocytic pathway and the Golgi localization pathway. In mammalian cells, the clathrin-associated protein (AP) complexes differ between plasma membrane clathrin coats (AP-2) and TGN clathrin coats (AP-1) (for reviews see Robinson, 1992; Kirchhausen, 1993). AP-2 interacts with the cytoplasmic tails of plasma membrane receptors (Pearse, 1988; Chang et al., 1993), whereas AP-1 specifically binds to the cytoplasmic domains of cation-independent and -dependent M6PR that are sorted in the TGN (Glickman et al., 1989; Sosa et al., 1993). In yeast, genes encoding proteins homologous to AP-1 subunits have been identified and genetic analysis suggests that they interact with clathrin and Kex2p (Phan et al., 1994; Rad et al., 1995; Stepp et al., 1995). Thus, Golgi localization of Kex2p may involve interaction between the yeast AP-1 complex and the Kex2p Golgi localization motif. However, although other yeast genes may encode subunits of a plasma membrane AP-2 complex, there is no functional evidence linking these proteins to clathrin-mediated endocytosis (Phan et al., 1994; Rad et al., 1995; Stepp et al., 1995; Tan, P., H. Phan, and G. Payne, unpublished). Con-

sequently, there are no promising candidates for NPFXD-recognizing yeast proteins at present.

Why is there an endocytosis targeting signal in the cytoplasmic domain of the Golgi-localized Kex2p? By analogy with the mammalian TGN proteins such as M6PR, TGN38, and furin, the endocytosis signal could function in retrieval from the plasma membrane. However, unlike the mammalian TGN proteins that cycle between the TGN, plasma membrane, and endosomes (for reviews see Nilsson and Warren, 1994; Sandoval and Bakke, 1994), the current evidence supports a model for Kex2p localization in wild-type cells that involves direct cycling between endosomes and the TGN without transport to the plasma membrane (for reviews see Wilsbach and Payne, 1993b; Nothwehr and Stevens, 1994). For example, significant levels of Kex2p do not reach the cell surface in wild-type cells even when Kex2p is mislocalized by overexpression or by mutation of the Golgi localization signal (Wilcox et al., 1992). In these cases, mislocalized Kex2p reaches the vacuole without traveling via the plasma membrane. Thus, endocytic retrieval is not expected to be an important aspect of Kex2p localization in wild-type cells. In support of this model, removal of the endocytosis signal from Kex2p in wild-type cells does not result in the appearance of the protein at the plasma membrane, even if the Golgi localization signal is also absent (Wilcox et al., 1992). We speculate that yeast cells growing in the wild may experience conditions that result in a degree of Kex2p mislocalization to the cell surface. Under these conditions, retrieval of Kex2p from the surface could provide the selective pressure for evolution of a functional endocytic targeting sequence.

In conclusion, we have identified a novel yeast endocytosis targeting signal, NPFXD, that is unrelated to the previously identified signal in the Ste2p cytoplasmic domain. Although both sequences direct internalization through End3p- and clathrin-dependent pathways, the Ste2p signal requires ubiquitination while the NPFXD sequence may act independently of the ubiquitination process. We suggest that the NPFXD sequence serves as a specific recognition site for the endocytic apparatus. The availability of endocytosis-defective mutants of this signal with single amino acid changes will greatly facilitate efforts to identify endocytosis apparatus components that recognize the signal.

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References

Backer, J.M., S.E. Shoelson, M.A. Weiss, Q.X. Hua, R.B. Cheatham, E. Haring, D.C. Cahill, and M.F. White. 1992. The insulin receptor juxtamembrane region contains two independent tyrosine/β-turn internalization signals. *J. Cell Biol.* 118:831–839.

Bansal, A., and L.M. Gierasch. 1991. The NPXY internalization signal of the LDL receptor adopts a reverse-turn conformation. *Cell.* 67:1195–1201.

Beltzer, J.P., and M. Speiss. 1991. *In vitro* binding of the asialoglycoprotein receptor to the β adaptin of plasma membrane coated vesicles. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:3735–3742.

Bénédetti, H., S. Raths, F. Crausaz, and H. Riezman. 1994. The *END3* gene encodes a protein that is required for the internalization step of endocytosis and for actin cytoskeleton organization in yeast. *Mol. Biol. Cell.* 5:1023–1037.

Blumer, K.S., J.E. Reneke, and J. Thorner. 1988. The *STE2* gene product is the ligand binding component of the α-factor receptor of *Saccharomyces cerevisiae*. *J. Biol. Chem.* 263:10836–10842.

Bos, K., C. Wraight, and K. Stanley. 1993. TGN38 is maintained in the *trans*-Golgi network by a tyrosine-containing motif in the cytoplasmic domain. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:2219–2228.

Canfield, W.M., K.F. Johnson, R.D. Ye, W. Gregory, and S. Kornfeld. 1991. Localization of the signal for rapid internalization of the bovine cation-independent mannose 6-phosphate/insulin-like growth factor-II receptor to amino acids 24–29 of the cytoplasmic tail. *J. Biol. Chem.* 266:5682–5688.

Chang, M.P., W.G. Mallet, K.E. Mostov, and F.M. Brodsky. 1993. Adaptor self-aggregation, adaptor-receptor recognition and binding of α-adaptin subunits to the plasma membrane contribute to recruitment of adaptor (AP2) components of clathrin-coated pits. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:2169–2180.

Chen, W.J., J.L. Goldstein, and M.S. Brown. 1990. NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor. *J. Biol. Chem.* 265:3116–3123.

Collawn, J.F., M. Stangel, L.A. Kuhn, V. Esekowgwu, S. Jing, I.S. Trowbridge, and J.A. Tainer. 1990. Transferrin receptor internalization sequence YXRF implicates a tight turn as the structural recognition motif for endocytosis. *Cell.* 63:1061–1072.

Davis, C.G., M.A. Lehrman, D.W. Russell, R.G.W. Anderson, M.S. Brown, and J.L. Goldstein. 1986. The J.D. mutation in familial hypercholesterolemia: amino acid substitution in cytoplasmic domain impedes internalization of LDL receptors. *Cell.* 45:15–24.

Davis, C.G., I.R. van Driel, D.W. Russell, M.S. Brown, and J.L. Goldstein. 1987. The low density lipoprotein receptor: identification of amino acids in the cytoplasmic domain required for rapid endocytosis. *J. Biol. Chem.* 262: 4075–4082.

Davis, N.G., J.L. Horecka, and G.F. Sprague. 1993. *Cis* and *trans*-acting functions required for endocytosis of the yeast pheromone receptors. *J. Cell Biol.* 122:53–65.

Eberle, W., C. Sander, W. Klaus, B. Schmidt, K. von Figura, and C. Peters. 1991. The essential tyrosine of the internalization signal in lysosomal acid phosphatase is part of a β turn. *Cell.* 67:1203–1209.

Fuller, R.S., R.E. Sterne, and J. Thorner. 1988. Enzymes required for yeast prohormone processing. *Annu. Rev. Physiol.* 50:345–362.

Fuller, R.S., A. Brake, and J. Thorner. 1989. Yeast prohormone processing enzyme (*KEX2* gene product) is a Ca^{2+}-dependent serine protease. *Proc. Natl. Acad. Sci. USA.* 86:1434–1438.

Glickman, J.N., E. Conibear, and B.M.F. Pearse. 1989. Specificity of binding of clathrin adaptors to signals on the mannose-6-phosphate/insulin-like growth factor II receptor. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:1041–1047.

Graham, T.R., and S.D. Emr. 1991. Compartmental organization of Golgi-specific protein modification and vacuolar protein sorting events defined in a yeast *sec18* (NSF) mutant. *J. Cell Biol.* 114:207–218.

Heilker, R., U. Manning-Krieg, J.-F. Zuber, and M. Speiss. 1996. *In vitro* binding of clathrin adaptors to sorting signals correlates with endocytosis and basolateral sorting. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:2893–2899.

Hicke, L., and H. Riezman. 1996. Ubiquitination of a yeast plasma membrane receptor signals its ligand-stimulated endocytosis. *Cell.* 84:277–287.

Humphrey, J.S., P.J. Peters, L.C. Yuan, and J.S. Bonifacino. 1993. Localization of TGN38 to the *trans*-Golgi network: involvement of a cytoplasmic tyrosine-containing sequence. *J. Cell Biol.* 120:1123–1135.

Itin, C., F. Kappeler, A.D. Linstedt, and H.-P. Hauri. 1995. A novel endocytosis signal related to the KKXX ER-retrieval signal. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:2250–2256.

Ito, H., K. Fukuda, K. Murata, and K. Kimura. 1982. Transformation of intact yeast cells with alkali cations. *J. Bacteriol.* 153:163–168.

Johnson, K.F., W. Chan, and S. Kornfeld. 1990. Cation-dependent mannose 6-phosphate receptor contains two internalization signals in its cytoplasmic domain. *Proc. Natl. Acad. Sci. USA.* 87:10010–10014.

Julius, D., A. Brake, L. Blair, R. Kunisawa, and J. Thorner. 1984. Isolation of the putative structural gene for the lysine-arginine-cleaving endopeptidase required for processing of yeast prepro-α-factor. *Cell.* 37:1075–1089.

Kirchhausen, T. 1993. Coated pits and coated vesicles—sorting it all out. *Curr. Opin. Struct. Biol.* 3:182–188.

Konopka, J.B., D.D. Jenness, and L.H. Hartwell. 1988. The C-terminus of the S. cerevisiae α-pheromone receptor mediates an adaptive response to pheromone. *Cell.* 54:609–620.

Kunkel, T.A., J.D. Roberts, and R.A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* 154: 367–381.

Laemmli, U.K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Lazarovits, J., and M.G. Roth. 1988. A single amino acid change in the cytoplasmic domain allows the influenza virus hemagglutinin to be endocytosed through coated pits. *Cell.* 53:743–752.

Letourneur, F., and R.D. Klausner. 1992. A novel di-leucine motif and a tyrosine-based motif independently mediate lysosomal targeting and endocytosis of CD3 chains. *Cell.* 69:1143–1157.

Munn, A.L., and H. Riezman. 1994. Endocytosis is required for the growth of vacuolar H(+)-defective yeast: identification of six new *END* genes. *J. Cell Biol.* 127:373–386.

Nakayama, N., A. Miyajima, and K. Arai. 1985. Nucleotide sequences of *STE2* and *STE3*, cell type-specific sterile genes from *Saccharomyces cerevisiae*. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:2643–2648.

Nesterov, A., R.C. Kurten, and G.N. Gill. 1995. Association of epidermal growth factor receptors with coated pit adaptins via a tyrosine phosphorylation-regulated mechanism. *J. Biol. Chem.* 270:1–8.

Nilsson, T., and G. Warren. 1994. Retention and retrieval in the endoplasmic reticulum and the Golgi apparatus. *Curr. Opin. Cell Biol.* 6:517–521.

Nothwehr, S.F., and T.H. Stevens. 1994. Sorting of membrane proteins in the yeast secretory pathway. *J. Biol. Chem.* 269:10185–10188.

Nothwehr, S.F., E. Conibear, and T.H Stevens. 1995. Golgi and vacuolar membrane proteins reach the vacuole in *vps1* mutant yeast cells via the plasma membrane. *J. Cell Biol.* 129:35–46.

Ohno, H., J. Stewart, M.C. Fournier, H. Bosshart, I. Rhee, S. Miyatake, T. Saito, A. Gallusser, T. Kirchhausen, and J.S. Bonifacino. 1995. Interaction of tyrosine-based sorting signals with clathrin-associated proteins. *Science (Wash. DC).* 269:1872–1875.

Payne, G.S., and R.S. Schekman. 1989. Clathrin: a role in the intracellular retention of a Golgi membrane protein. *Science (Wash. DC).* 44:1358–1365.

Pearse, B.M.F. 1988. Receptors compete for adaptins found in plasma membrane coated pits. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:3331–3336.

Peters, C., M. Braun, B. Weber, M. Wendland, B. Schmidt, R. Pohlmann, A. Waheed, and K. von Figura. 1990. Targeting of a lysosomal membrane protein: a tyrosine-containing edocytosis signal in the cytoplasmic tail of lysosomal acid phosphatase is necessary and sufficient for targeting to lysosomes. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:3497–3506.

Phan, H.L., J.A. Finlay, D.S. Chu, P.K. Tan, T. Kirchhausen, and G.S. Payne. 1994. The *S. cerevisiae APS1* gene encodes a homologue of the small subunit of the mammalian clathrin AP-1 complex: evidence for functional interaction with clathrin at the Golgi complex. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:1706–1717.

Ponnambalam, S., C. Rabouille, P. Luzio, T. Nilsson, and G. Warren. 1994. The TGN38 glycoprotein contains two non-overlapping signals that mediate localization to the *trans*-Golgi network. *J. Cell Biol.* 125:253–268.

Rad, M.R., H.L. Phan, L. Kirchrath, P.K. Tan, T. Kirchhausen, C.P. Hollenberg, and G.S. Payne. 1995. *Saccharomyces cerevisiae* Apl2p, a homologue of the mammalian clathrin AP β subunit, plays a role in clathrin-dependent Golgi functions. *J. Cell Sci.* 108:1605–1615.

Raths, S., J. Rohrer, F. Crausaz, and H. Riezman. 1993. *end3* and *end4*: two mutants defective in receptor-mediated and fluid-phase endocytosis in *Saccharomyces cerevisiae*. *J. Cell Biol.* 120:55–66.

Reneke, J.E., K.J. Blumer, W.E. Courchesne, and J. Thorner. 1988. The carboxy-terminal segment of the yeast α-factor receptor is a regulatory domain. *Cell.* 55:221–234.

Richardson, J.A., and D.C. Richardson. 1988. Principles and patterns of protein conformation. *In* Prediction of Protein Structure and the Principles of Protein Conformation. G.D. Fasman, editor. Plenum Publishing Corp., New York. 1–98.

Robinson, M.S. 1992. Adaptins. *Trends Cell Biol.* 2:293–297.

Rohrer, J., H. Bénédetti, B. Zanolari, and H. Riezman. 1993. Identification of a novel sequence mediating regulated endocytosis of the G-protein-coupled α-pheromone receptor in yeast. *Mol. Biol. Cell* 4:511–521.

Roth, A.F., and N.G. Davis. 1996. Ubiquitination of the yeast a-factor receptor. *J. Cell Biol.* 134:661–674.

Rothstein, R. 1994. Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. *Methods Enzymol.* 194:281–301.

Sandoval, I.V., and O. Bakke. 1994. Targeting of membrane proteins to endosomes and lysosomes. *Trends Cell Biol.* 4:292–297.

Schäfer, W., A. Stroh, S. Berghöfer, J. Seiler, M. Vey, M.-L. Kruse, H.-F. Kern, H.-D. Klenk, and W. Garten. 1995. Two independent targeting signals in the cytoplasmic domain determine *trans*-Golgi network localization and endosomal trafficking of the proprotein convertase furin. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:2424–2435.

Seeger, M., and G.S. Payne. 1992. Selective and immediate effects of clathrin heavy chain mutations on Golgi membrane protein retention in *Saccharomyces cerevisiae*. *J. Cell Biol.* 118:531–540.

Sikorski, R.S., and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics.* 122:19–27.

Sosa, M.A., B. Schmidt, K. von Figura, and A. Hille-Rehfeld. 1993. *In vitro* binding of plasma membrane coated vesicle adaptors to the cytoplasmic domain of lysosomal acid phosphatase. *J. Biol. Chem.* 268:12537–12543.

Sprague, G.F., Jr. 1994. Assay of yeast meting reaction. *Methods Enzymol.* 194: 77–93.

Stepp, J.D., A. Pellicena-Palle, S. Hamilton, T. Kirchhausen, and S.K. Lemmon. 1995. A late Golgi sorting function for *Saccharomyces cerevisiae* Apm1p, but not for Apm2p, a second yeast clathrin AP medium chain-related protein. *Mol. Biol. Cell.* 6:41–58.

Tan, P.K., N.G. Davis, G.F Sprague, and G.S Payne. 1993. Clathrin facilitates the internalization of seven transmembrane segment receptors for mating pheromones in yeast. *J. Cell Biol.* 123:1707–1716.

Trowbridge, I.S., J.F. Collawn, and C.R. Hopkins. 1993. Signal-dependent membrane protein targeting in the endocytic pathway. *Annu. Rev. Cell Biol.* 9:129–161.

Vieira, J., and J. Messing. 1987. Production of single-stranded plasmid DNA. *Methods Enzymol.* 153:3–11.

Weiner, J.L., C. Guttierez-Steil, and K.J. Blumer. 1993. Disruption of receptor-G protein coupling in yeast promotes the function of an SST2-dependent adaptation pathway. *J. Biol. Chem.* 268:8070–8077.

Wilcox, C.A., K. Redding, R. Wright, and R.S. Fuller. 1992. Mutation of a tyrosine localization signal in the cytosolic tail of the yeast Kex2 protease disrupts Golgi retention and results in default transport to the vacuole. *Mol. Biol. Cell.* 3:1353–1371.

Wilmot, C.M., and J.M. Thornton. 1988. Analysis and prediction of the different types of β-turn in proteins. *J. Mol. Biol.* 203:221–232.

Wilsbach, K., and G.S. Payne. 1993*a*. Vps1p, a member of the dynamin GTPase family, is necessary for Golgi membrane protein retention in *Saccharomyces cerevisiae*. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:3049–3059.

Wilsbach, K., and G.S. Payne. 1993*b*. Dynamic retention of *trans*-Golgi network membrane proteins in *Saccharomyces cerevisiae*. *Trends Cell Biol.* 3: 426–432.

Wong, S.H., and W. Hong. 1993. The SXYQRL sequence in the cytoplasmic domain of TGN38 plays a major role in *trans*-Golgi network localization. *J. Biol. Chem.* 268:22853–22862.