Control of Ste6 Recycling by Ubiquitination in the Early Endocytic Pathway in Yeast

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We present evidence that ubiquitination controls sorting of the ABC-transporter Ste6 in the early endocytic pathway. The intracellular distribution of Ste6 variants with reduced ubiquitination was examined. In contrast to wild-type Ste6, which was mainly localized to internal structures, these variants accumulated at the cell surface in a polar manner. When endocytic recycling was blocked by Ypt6 inactivation, the ubiquitination deficient variants were trapped inside the cell. This indicates that the polar distribution is maintained dynamically through endocytic recycling and localized exocytosis ("kinetic polarization"). Ste6 does not appear to recycle through late endosomes, because recycling was not blocked in class E *vps* (vacuolar protein sorting) mutants ($\Delta vps4$, $\Delta vps27$), which are affected in late endosome function and in the retromer mutant $\Delta vps35$. Instead, recycling was partially affected in the sorting nexin mutant $\Delta snx4$, which serves as an indication that Ste6 recycles through early endosomes. Enhanced recycling of wild-type Ste6 was observed in class D *vps* mutants ($\Delta pep12$, $\Delta vps8$, and $\Delta vps21$). The identification of putative recycling signals in Ste6 suggests that recycling is a signal-mediated process. Endocytic recycling and localized exocytosis could be important for Ste6 polarization during the mating process.

INTRODUCTION

In the yeast endocytic pathway, two distinct endosomal compartments, early and late endosomes, can be distinguished morphologically (Hicke *et al.*, 1997; Prescianotto-Baschong and Riezman, 2002) and biochemically (Singer and Riezman, 1990). Internalized cell surface proteins pass through these compartments on their way to the lysosome-like vacuole where they are degraded. Some proteins, however, escape degradation and are recycled back to the cell surface. Recycling in yeast has been demonstrated for several proteins, such as Chs3, the catalytic subunit of chitin synthase III (Ziman *et al.*, 1996), the α -factor receptor Ste3 (Chen and Davis, 2000), and the v-SNARE Snc1 (Lewis *et al.*, 2000). The mechanism of docking and fusion of endosome-derived vesicles with the trans-Golgi has been examined in detail. A multisubunit tethering complex, the VFT (Vps fifty-three) or GARP (Golgi-associated retrograde protein) complex, is required for the initial docking of endosomal vesicles to the Golgi membrane (Conibear and Stevens, 2000). This complex interacts with the Rab protein Ypt6 on the Golgi membrane through its subunit Vps52 and with the SNARE protein Tlg1 through its subunit Vps51 (Siniosoglou and Pelham, 2002; Conibear *et al.*, 2003). After tethering, fusion between endosomal vesicles and the Golgi is mediated by a SNARE complex consisting of the SNAREs Tlg1, Tlg2, Vti1, and Snc1 (Paumet *et al.*, 2001; Lewis and Pelham, 2002). Another factor involved in recycling of Snc1 is the F-box protein Rcy1, which forms a non-SCF complex with Skp1

(Galan *et al.*, 2001). After retrieval to the Golgi, recycling proteins like Snc1 are again packaged into secretory vesicles that travel along actin filaments to the site of polarized growth, the bud.

In addition to proteins internalized from the cell surface, the Golgi resident proteins Kex2 and Ste13 (DPAP A) functioning in the processing of the mating pheromone α -factor and the carboxypeptidase Y (CPY) sorting receptor Vps10 are also retrieved to the Golgi by endosome-derived carriers (Voos and Stevens, 1998). Retrieval from late endosomes is mediated by the retromer complex (Seaman *et al.*, 1998; Nothwehr *et al.*, 2000). The mechanisms governing sorting from early endosomes to the Golgi are less well defined. A role in the retrieval of Snc1 from early endosomes to the Golgi has been suggested for the sorting nexins *snx4/41/42* (Hettema *et al.*, 2003). Sorting of proteins from Golgi to endosomes is mediated by clathrin coats in combination with distinct adapter complexes specific for early and late endosome sorting (Black and Pelham, 2000; Costaguta *et al.*, 2001; Deloche *et al.*, 2001; Mullins and Bonifacio, 2001). Cargo destined for early endosomes appears to be packaged into clathrin-coated vesicles in combination with the AP-1 adapter complex while the Gga (Golgi-localized, gamma-ear-containing, ARF-binding) proteins appear to be responsible for sorting to late endosomes.

We are studying the sorting of the ABC (ATP-binding-cassette) transporter Ste6, which is required for the secretion of the mating-pheromone α -factor (Kuchler *et al.*, 1989; McGrath and Varshavsky, 1989). Ste6 is transported to the cell surface but does not accumulate there to a considerable degree due to efficient endocytosis. After internalization from the plasma membrane, Ste6 is transported to the vacuole for degradation (Berkower *et al.*, 1994; Kölling and Hollenberg, 1994). Transport to the vacuole is regulated by ubiquitination (Kölling and Hollenberg, 1994), which appears to be important for sorting of Ste6 into the multivesicular bodies (MVB) pathway (Losko *et al.*, 2001). Here, we

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Table 1. Yeast strains

Strain	Genotype	Reference
HMSF169	<i>MATa sec14-3</i>	Yeast Genetic Stock Center
JD52	<i>MATa ura3-52 his3-Δ200 leu2-3112 trp1-Δ63 lys2-801</i>	J. Dohmen, Köln
JD53	<i>MATα ura3-52 his3-Δ200 leu2-3112 trp1-Δ63 lys2-801</i>	J. Dohmen, Köln
RKY592	<i>MATa ura3 his3 leu2 trp1 Δste6::HIS3 end4</i>	this study
RKY959	<i>MATa ura3-52 his3-Δ200 leu2-3112 trp1-Δ63 lys2-801 Δste6::LEU2</i>	Losko <i>et al.</i> , 2001
RKY1510	<i>MATa ura3-52 his3-Δ200 leu2-3112 trp1-Δ63 lys2-801 Δsnf7::HIS3</i>	Kranz <i>et al.</i> , 2001
RKY1511	<i>MATa ura3-52 his3-Δ200 leu2-3112 trp1-Δ63 lys2-801 Δvps4::HIS3</i>	Kranz <i>et al.</i> , 2001
RKY1634	<i>MATa ura3-52 his3-Δ200 leu2-3112 trp1-Δ63 lys2-801 Δsnx4::HIS3</i>	this study
RKY1718	<i>MATa his3-Δ200 ura3-52 trp1-Δ63 cdc12-6</i>	this study
RKY1875	<i>MATa ura3-52 his3-Δ200 leu2-3112 trp1-Δ63 lys2-801 Δvps8::HIS3</i>	this study
RKY1876	<i>MATa ura3-52 his3-Δ200 leu2-3112 trp1-Δ63 lys2-801 Δvps27::kanMX6</i>	this study
RKY1920	<i>MATa ura3-52 his3-Δ200 leu2-3112 trp1-Δ63 lys2-801 Δvps21::LEU2</i>	this study
RKY1921	<i>MATa ura3-52 his3-Δ200 leu2-3112 trp1-Δ63 lys2-801 Δpep12::kanMX6</i>	this study
RKY1922	<i>MATa ura3-52 his3-Δ200 leu2-3112 trp1-Δ63 lys2-801 Δbro1::kanMX6</i>	this study
RKY1926	<i>MATa ura3-52 his3-Δ200 leu2-3112 trp1-Δ63 lys2-801 Δvps8::HIS3 Δvps21::LEU2</i>	this study
RKY1927	<i>MATa ura3-52 his3-Δ200 leu2-3112 trp1-Δ63 lys2-801 Δvps8::HIS3 Δsnf7::HIS3</i>	this study
RKY1928	<i>MATa ura3-52 his3-Δ200 leu2-3112 trp1-Δ63 lys2-801 Δvps8::HIS3 Δbro1::kanMX6</i>	this study
RKY1930	<i>MATa ura3-52 his3-Δ200 leu2-3112 trp1-Δ63 lys2-801 Δvps8::HIS3 Δpep12::kanMX6</i>	this study
RKY2057	<i>MATa ura3 his3 leu2 trp1 lys2 ypt6-2::URA3 (ts)</i>	this study
RKY2074	<i>MATa ura3-52 his3-Δ200 leu2-3112 trp1-Δ63 lys2-801 Δvps35::HIS3</i>	this study
RKY2113	<i>MATa ura3-52 leu2-3112 trp1-Δ63 lys2-801 sec14-3</i>	this study
RKY2116	<i>MATa ura3 his3 leu2 trp1 Δste6::HIS3 end4 Δsnx4::kanMX6</i>	this study
RKY2118	<i>MATa ura3 his3 leu2 trp1 lys2 end4 ypt6-2::URA3</i>	this study
ZLY2	<i>MATα ura3 his3 leu2 trp1 ade8 ypt6-2::URA3 (ts)</i>	Luo and Gallwitz, 2003

present evidence for an additional role of Ste6 ubiquitination in the early endocytic pathway. We show that Ste6 variants with reduced ubiquitination accumulate at the cell surface in a polar manner. This polar distribution appears to be maintained by endocytic recycling and localized exocytosis.

MATERIALS AND METHODS

Yeast Strains and Plasmids

The yeast strains used are listed in Table 1. Deletion strains are derived from the wild-type strain JD52. They were constructed by one-step gene replacement with PCR-generated cassettes (Longtine *et al.*, 1998). The deletions were verified by PCR. Site-directed mutagenesis of *STE6* was performed with the Bio-Rad Muta-Gene kit (Richmond, CA) based on the method of (Kunkel *et al.*, 1987). A 1.2-kb internal *Pst*I *STE6* fragment cloned into the phagemid pUC218 was mutagenized with mutagenic primers as summarized in Table 2. The *Pst*I fragments were subcloned into the 2 μ -plasmid pYKS2 (Kuchler *et al.*, 1993) coding for a c-myc tagged Ste6 variant (Table 2). To construct pRK845, the 5.3-kb *Sac*I/*Hind*III *STE6* fragment of pYKS2 was transferred to YEplac112 (Gietz and Sugino, 1988). To construct the plasmids pRK264 and pRK873, the

1.2-kb *Pst*I *STE6* fragment carrying the Δ A-box deletion (Kölling and Losko, 1997) was cloned into pYKS2 and pRK845, respectively. Plasmid pRK69 contains a 6.2-kb *Bgl*II/*Sal*I chromosomal *STE6* fragment cloned into the 2 μ -vector YEpl429 (Ma *et al.*, 1987). Plasmid pRK814 was constructed by inserting the 3.77-kb internal *Bam*HI *STE6* fragment of pRK658 into pRK69. Plasmid pRK278 contains the 6.2-kb *Bgl*II/*Sal*I *STE6* fragment cloned into the CEN/ARS vector YCplac33 (Gietz and Sugino, 1988; with deleted *Pst*I site). Based on this plasmid, several single-copy *STE6* variants were constructed. In pRK658 and pRK909, the 1.2-kb *Pst*I *STE6* fragment of pRK278 was replaced by the corresponding fragment of pRK657 (Table 2) and pRK891. In pRK939 and pRK940, a 4-kb *Bam*HI/*Hind*III fragment of pYKS2 and pRK891 coding for the C-terminal part of Ste6 was inserted into pRK278. Plasmid pRK656 is based on the 2 μ -vector YEplac195 (Gietz and Sugino, 1988) and contains a c-myc tagged version of *STE6*, derived from pYKS2, fused in frame with a 250-base pair ubiquitin PCR-fragment. To construct pRK1043, the 1.2-kb internal *STE6* *Pst*I fragment of pRK656 was replaced by the corresponding fragment of pRK658.

Immunofluorescence

The immunofluorescence experiments were essentially performed as described (Pringle *et al.*, 1989). Cells were grown to exponential phase ($OD_{600} = 0.5$ – 0.8 , 3 – 4×10^7 /ml) and fixed directly for 4 h with formaldehyde (final

Table 2. Plasmids generated by site-directed mutagenesis

Phagemid	pYKS2 subclones	Mutations	Primer no.	Primer sequence
pRK298	pRK311	K612R	98	GAATGACTACTCTGATGCGAGAACGATCGTAGATACAGAGACTGAAG
pRK334	pRK338	K622R	113	GATACAGAGACTGAAGAAAGATCTATACACACTGTGG
pRK343	pRK352	K638R	115	AACCTCAATTGGAAACACCTAGGCTTGGATCATGCTTAAG
pRK657	pRK659	K663R	418	TATCAAAGAAGATCGAACGTTAGA
		K673R	419	ACAAGAAGGGTTAGAGTTGAAGAG
		K684R, K687R	420	GCACTAAGACAACAAAGGAACACC
		K705R, K709R	421	ATTATCAGAAGAATGATTAGAAGCATA
		K714R, K715R	422	AGATACAGAAGAATTCTAATC
pRK883	pRK888	F630L	937	ACTGTGGAAAGCCTGAATTCTCAATTGGAA
pRK884	pRK889	Y648L	938	AGTAATCTGGGTCTAGATGAGACAGATCAG
pRK885	pRK890	F656L, Y657L, Y661L	939	GATCAGTTAAGCTTATTGGAAGCAATCTTACAAAAAAGATCG
pRK886	pRK891	Y681L	940	GAAAATATTGGGCTAGCACTAAAAACAACAA
pRK887	pRK892	Y713L	941	AGCATAAGATTAAAAAAGATCTTAATCTTGGGA

concentration, 5%). The fixed cells were spheroplasted and extracted with 0.1% Triton X-100 for 5 min and then attached to a multiwell slide treated with 0.1% poly-lysine (Sigma, Seelz, Germany). The cells were first incubated with the anti-c-myc mouse monoclonal primary antibody (9E10, Covance, Madison, WI; 1:200 dilution in phosphate-buffered saline (PBS) + 1 mg/ml bovine serum albumin) for 90 min and then another 90 min with FITC-conjugated anti-mouse secondary antibodies (Dianova, Hamburg, Germany; 1:300 dilution in PBS/bovine serum albumin). Fluorescence was visualized with a Zeiss Axioskop fluorescence microscope using the FITC-filter set. Images were acquired with a CCD camera (AxioCam, Zeiss, Oberkochen, Germany).

Other Methods

Pulse-chase and immunoprecipitation experiments were performed as described previously (Losko *et al.*, 2001).

RESULTS

Polar Distribution of Ste6 Δ A-box through Continuous Recycling

After transport to the cell surface, the a-factor transporter Ste6 is quickly internalized by endocytosis and transported to the yeast vacuole for degradation (Berkower *et al.*, 1994; Kölling and Hollenberg, 1994). Internalization and rapid degradation of Ste6 is mediated by a signal in the linker region, which connects the two homologous halves of Ste6 (Kölling and Losko, 1997). In contrast to wild-type Ste6, which is mainly found associated with internal, presumably endosomal structures, a Ste6 variant with a deletion in the linker region (Ste6 Δ A-box) accumulates at the cell surface. Most interestingly, the Ste6 Δ A-box variant is not evenly distributed over the whole yeast cell surface. As can be seen in Figure 1A, it is mainly concentrated at the surface of the newly emerging daughter cell, the bud. We were interested to know, how this polar distribution is achieved and maintained. Secretion in yeast is polarized, i.e., newly synthesized material bound for the cell surface is directed toward the growing bud (Novick and Botstein, 1985). Thus, initially, membrane proteins that travel to the cell surface via the secretory pathway are asymmetrically deposited at the cell surface. However, this initial, asymmetric distribution should quickly be dissipated by lateral diffusion. One explanation for the persistent asymmetry of Ste6 Δ A-box could be that the septin ring that surrounds the bud-neck constitutes a diffusion barrier for the Ste6 Δ A-box protein. There is precedent for such a mechanism (Takizawa *et al.*, 2000). Therefore, the distribution of Ste6 Δ A-box was examined in the septin ring mutant *cdc12-6* by immunofluorescence microscopy. Already at permissive temperature (25°C), the *cdc12-6* mutant gave rise to elongated, distorted buds (Figure 1B), due to defective cytokinesis. This was even more pronounced at nonpermissive temperature (37°C). Although the morphology of the cells was severely distorted, the polar distribution of Ste6 Δ A-box was unaffected in the mutant. Ste6 Δ A-box displayed a striking polar localization at the tips of the elongated buds. Thus, the septin ring does not appear to play a role in restricting Ste6 Δ A-box to the bud cell surface.

Cell surface proteins may also be polarized kinetically by localized exocytosis and endocytic recycling, as has been suggested for the yeast v-SNARE protein Snc1 (Valdez-Taubas and Pelham, 2003). If the polar distribution of Ste6 Δ A-box is maintained through continuous endocytic recycling, the protein should accumulate inside the cell when the recycling pathway is blocked. To block the recycling pathway, which leads from endosomes via the Golgi to the cell surface, we used the conditional *ypt6-2* mutant, which is defective for the trans-Golgi Rab protein Ypt6 (Luo and Gallwitz, 2003). Ypt6 is required for the docking of endosome-derived vesicles to Golgi membranes (Siniosoglou

and Pelham, 2001). Because *ypt6-2* is a conditional mutant, it is possible to observe the immediate consequences of Ypt6 inactivation by shifting cells from permissive (25°C) to nonpermissive temperature (37°C). At permissive temperature, Ste6 Δ A-box showed the same polar distribution in the *ypt6-2* mutant as in wild type (Figure 1C). However, after a short exposure to high temperature (20 min), Ste6 Δ A-box was completely found in internal patch-like structures. This immediate redistribution upon Ypt6 inactivation demonstrates that the polar distribution of Ste6 Δ A-box is achieved through a dynamic process, i.e., through continuous recycling. The same redistribution from cell surface to internal structures was also observed with another mutant (*sec14*) that affects Golgi function (Figure 1D).

To prove that the internal patches indeed result from internalization of surface-localized Ste6 protein, the Ste6 Δ A-box distribution was examined in an *end4 ypt6-2* double mutant. Because internalization of cell surface proteins is blocked in the *end4* (*ts*) mutant, no internal patches should be detectable in the double mutant at nonpermissive temperature. And this is exactly what we observed (Figure 1E). This confirms that the internally localized Ste6 Δ A-box protein in the *ypt6-2* mutant is derived from the cell surface. In the *end4 ypt6-2* mutant, Ste6 Δ A-box is no longer polarized. This supports our notion that continuous endocytosis and recycling is required for polarization of Ste6 Δ A-box.

Reduced Ste6 Ubiquitination Leads to Enhanced Recycling

Sorting of Ste6 into the vacuolar degradation pathway is regulated by ubiquitination (Kölling and Hollenberg, 1994). In contrast to wild-type Ste6, the Δ A-box variant is no longer ubiquitinated (Kölling and Losko, 1997). We were interested to know whether this lack of ubiquitination is responsible for the enhanced recycling observed with the Ste6 Δ A-box variant. Ste6 Δ A-box is mutated in the linker region, which connects the two homologous halves of Ste6. Based on the distribution of charged amino acids, the 100 amino acid long linker region can be divided into a region containing predominantly acidic amino acids (A-box) and a region containing predominantly basic amino acids (B-box; Figure 2A). In Ste6 Δ A-box, the complete A-box (~50 amino acids) had been deleted. Because this deletion is relatively large, it is possible that other sorting signals in addition to the signal controlling ubiquitination were removed. To exclude this possibility, we wanted to eliminate Ste6 ubiquitination selectively by mutating potential ubiquitination target sites in the linker region. Ubiquitin is attached to lysine residues in the substrate protein via an isopeptide bond. Previously, we have shown that mutating the three A-box lysine residues to arginine had no effect on Ste6 ubiquitination (Kölling and Losko, 1997). But, this does not necessarily exclude a function of these lysine residues as ubiquitin acceptor sites. Apparently, the ubiquitination machinery is able to use other lysine residues in the vicinity of the original acceptor site when the site is no longer available (Kornitzer *et al.*, 1994). Therefore, we decided to mutate all 11 lysine residues in the linker region to arginine (Ste6 R¹¹ mutant) by site-directed mutagenesis. The Ste6 R¹¹ mutant was fully functional, as determined in a mating assay (unpublished data).

To test for ubiquitination, Ste6 was coexpressed with HA-tagged ubiquitin. Ste6 was immunoprecipitated from cell extracts with anti-Ste6 antibodies and the immunoprecipitates were examined for the presence of HA-tagged ubiquitin by Western blotting. A ubiquitin signal can only be detected, if ubiquitin is covalently attached to Ste6. As described previously (Kölling and Hollenberg, 1994), a dif-

Figure 1. Polar cell surface localization of Ste6 Δ A-box. Different yeast strains were transformed with pRK873 coding for the c-myc-tagged Ste6 Δ A-box variant. The intracellular distribution of Ste6 Δ A-box was examined by immunofluorescence microscopy with anti-myc primary antibodies (9E10) and FITC-conjugated anti-mouse secondary antibodies in strains grown at 25°C (left columns) or shifted to 37°C (right columns) for 20 min (JD52, RKY2057, RKY2116, RKY2118) or 60 min (RKY1718, RKY2113). (A) JD52 (WT), (B) RKY1718 (*cdc12-6*), (C) RKY2057 (*ypt6-2*), (D) RKY2113 (*sec14*), (E) RKY2118 (*end4 ypt6-2*), and (F) RKY2116 (*end4 Δ snx4*). Alternating: FITC fluorescence and phase contrast images.

fusely migrating high-molecular-weight signal was detected for wild-type Ste6 with anti-HA antibodies (Figure 2B). The ubiquitin signal was much weaker for the Ste6 R¹¹ variant (10% of wild-type intensity, normalized to the Ste6 signal). Also, distinct bands were discernible corresponding to

mono- and di-ubiquitinated Ste6, as judged from their calculated molecular weights. In comparison to wild type, the ubiquitination pattern appeared to be shifted from higher molecular weight species down to faster migrating (i.e., less ubiquitinated) species. Thus although ubiquitination could

Figure 3. Fusion to ubiquitin suppresses the Ste6^{R11} recycling phenotype. (A) The *STE6* deletion strain RKY959 was transformed with different Ste6 encoding plasmids; from top to bottom: pYKS2 (WT Ste6), pRK659 (Ste6^{R11}), pRK1043 (Ste6^{R11}-Ub). The distribution of the Ste6 variants was examined by immunofluorescence microscopy with anti-myc primary antibodies (9E10) and FITC-conjugated anti-mouse secondary antibodies; left, FITC fluorescence; right, phase contrast image. (B) Equal amounts of cell extract were examined for the presence of the different Ste6 variants by Western blotting with anti-Ste6 antibodies: (1) pYKS2 (WT Ste6), (2) pRK659 (Ste6^{R11}), and (3) pRK1043 (Ste6^{R11}-Ub).

determined. If Ste6 trafficking to the vacuole is affected, Ste6 should be stabilized. The Ste6 half-life was determined by a pulse-chase experiment. As reported previously (Kölling and Hollenberg, 1994), wild-type Ste6 was quickly degraded with a half-life of 14 min (Figure 2C). In contrast, the Ste6 R¹¹ variant was four times more stable than wild type (half-life: 56 min). Thus, as observed before for Ste6 Δ A-box, reduction in ubiquitination leads to stabilization of Ste6. The intracellular distribution of the Ste6 R¹¹ variant (Figure 3A), as determined by immunofluorescence microscopy, resembled the distribution of the Ste6 Δ A-box protein. Like Ste6 Δ A-box, the Ste6 R¹¹ variant stained the surface of the bud. Similar to Ste6 Δ A-box, it was redistributed to internal structures in the *ypt6-2* mutant upon shift to nonpermissive temperature (unpublished data). However, the phenotypes of

To study the consequences of the ubiquitination defect on Ste6 trafficking, the half-life of the Ste6 R¹¹ protein was

the A-box deletion and the R¹¹ mutation were not completely identical. In contrast to Ste6 ΔA-box, where no internal staining was obvious, staining of the vacuolar membrane was observed for Ste6 R¹¹ in addition to the polar cell surface staining. Thus, a fraction of Ste6 R¹¹ escapes recycling and progresses further down the endocytic pathway.

The similar phenotypes of the ΔA-box and R¹¹ mutants suggest that enhanced recycling is indeed the result of reduced ubiquitination and not due to deletion of some other signal in the linker region. If enhanced recycling solely results from loss of ubiquitination, it should be possible to restore internal localization by addition of a foreign ubiquitination signal. It is well established that in-frame fusion of ubiquitin to endocytic cargo proteins can substitute for missing ubiquitination signals (Terrell *et al.*, 1998). We therefore fused the Ste6 R¹¹ variant at its C-terminus with ubiquitin and examined the intracellular localization of the fusion protein by immunofluorescence. In contrast to Ste6 R¹¹, the Ste6 R¹¹-Ub variant was no longer detected at the cell surface but instead localized to internal structures (Figure 3A), similar to wild type. Also, the intracellular concentration of the fusion protein was lower than the concentration of Ste6 R¹¹ (Figure 3B), indicative of enhanced turnover. Thus, these experiments further substantiate the view that loss of ubiquitination is responsible for enhanced Ste6 recycling.

Ste6 Recycles from Early Endosomes

Membrane proteins that have escaped from the Golgi or proteins taken up by endocytosis can travel back to the Golgi from early or late endosomes. To determine which pathway is used by Ste6 ΔA-box and Ste6 R¹¹, the distribution of these proteins was examined by immunofluorescence microscopy in different protein sorting mutants of the endocytic pathway (Figure 4). Class E *vps* (vacuolar protein sorting) mutants are useful tools to decide whether a protein travels through late endosomes (Raymond *et al.*, 1992). Late endosome function is disrupted in these mutants resulting in the formation of an exaggerated late endosomal structure located close to the vacuole ("class E compartment"). Membrane proteins that travel through late endosomes are trapped in this dot-like structure. For wild-type Ste6, a typical class E staining was observed in the class E mutant Δ*vps4*. The distribution of the Ste6 ΔA-box variant, however, was unaffected by the Δ*vps4* mutation. This suggests that the Ste6 ΔA-box protein does not cycle through late endosomes. The Ste6 R¹¹ variant showed an intermediate staining pattern with polar cell surface staining and some class E staining. This is consistent with the "leaky" recycling phenotype of the Ste6 R¹¹ mutant observed in the wild-type background. A slightly different result was obtained with another class E mutant, Δ*vps27*. Although wild-type Ste6 and Ste6 ΔA-box showed the same staining pattern as in Δ*vps4*, only cell surface staining and no internal class E-like staining was observed for Ste6 R¹¹. Apparently, progression of Ste6 R¹¹ from early to late endosomes is blocked in the Δ*vps27* mutant. This suggests that Vps27 could have a function at early endosomes in addition to its late endosome function.

Retrieval of proteins, like Vps10 and Pep12, from late endosomes to the Golgi requires a multimeric protein complex, called the retromer complex (Seaman *et al.*, 1998). We examined the distribution of our Ste6 variants in the retromer mutant Δ*vps35*. Wild-type Ste6 accumulated in internal dot-like structures that surrounded the vacuole. Like in the class E mutants, the cell surface staining of Ste6 ΔA-box and Ste6 R¹¹ was unaffected in this mutant. This serves as another hint that these variants do not recycle through late endosomes. The machinery required for recycling from early

endosomes is less well characterized. It has been reported that Snx4 (sorting nexin 4) is involved in recycling of Sncl from early endosomes to the Golgi (Hettema *et al.*, 2003). The Δ*snx4* deletion had no significant effect on the distribution of wild-type Ste6 and Ste6 R¹¹. However for Ste6 ΔA-box, we observed vacuolar membrane staining in addition to the polar cell surface staining. Because internal staining was not visible in a wild-type strain, this suggests that recycling of Ste6 ΔA-box is partially affected in the Δ*snx4* mutant. Because Snx4 has been implicated in recycling from early endosomes to the Golgi, this partial recycling defect indicates that Ste6 also recycles through early endosomes. Accumulation of internal structures was not observed in an *end4* Δ*snx4* double mutant (Figure 1F), again demonstrating that the internal Ste6 ΔA-box protein is derived from the cell surface.

Enhanced Recycling of Wild-type Ste6 in Class D *vps* Mutants

Several different *vps* mutants were analyzed for their effect on Ste6 localization. When we examined the Δ*vps8* mutant, a striking change in the localization pattern was observed (Figure 4). In this mutant, wild-type Ste6 accumulated at the cell surface in a polar manner, similar to Ste6 ΔA-box in a wild-type strain. This suggests that recycling of wild-type Ste6 is enhanced in the Δ*vps8* mutant. The same localization pattern was observed for the other two Ste6 variants, which are already polarized in a wild-type strain. Interestingly, no internal staining was observed for Ste6 R¹¹, suggesting that Vps8 is required for progression of Ste6 R¹¹ from early to late endosomes. Vps8 is one of the so-called class D Vps functions (Raymond *et al.*, 1992) that are thought to be involved in docking and fusion of transport vesicles with late endosomes (Gerrard *et al.*, 2000). It has been reported that Vps8 interacts with another class D protein, the late endosomal Rab protein Vps21 (Horazdovsky *et al.*, 1996). Its exact function, however, is unclear. To see whether the observed effect is specific for Δ*vps8*, other class D *vps* mutants were examined (Figure 5A). The other class D *vps* mutants tested (Δ*vps21* and Δ*pep12*) showed the same polar localization pattern as Δ*vps8*. This suggests that enhanced Ste6 recycling is a general feature of all class D *vps* mutants. Enhanced endosome-to-plasma membrane trafficking in a *vps8* mutant has also been noted for a mutant plasma membrane ATPase (Pma1; Luo and Chang, 2000).

To narrow down the point in the endocytic pathway where Vps8 functions, an epistasis analysis was performed (Figure 5A). To this end, double mutants were constructed. Combinations of Δ*vps8* with other class D mutants (Δ*pep12*, Δ*vps21*) showed the same phenotype as the Δ*vps8* single mutant. This finding corroborates the classification of Vps8 as a class D Vps function. Also, when the class E *vps* mutants Δ*bro1* and Δ*snf7* were combined with Δ*vps8*, the double mutants displayed the Δ*vps8* phenotype. From this result, it can be concluded that Vps8 acts upstream of Snf7 and Bro1.

The class D *vps* mutants could exert their effects on Ste6 sorting by somehow affecting Ste6 ubiquitination. Therefore, Ste6 ubiquitination was examined in the Δ*vps8* mutant (Figure 5B). However, no reduction in Ste6 ubiquitination could be detected in the Δ*vps8* mutant. Thus, the class D *vps* mutants affect Ste6 sorting by a mechanism independent of ubiquitination.

Identification of a Recycling Signal in Ste6

It may be expected that sorting of Ste6 into the recycling pathway is a signal-mediated process. For other yeast pro-

Figure 4. Ste6 distribution in different protein sorting mutants. Different mutant strains were transformed with pYKS2 (WT Ste6, left row), pRK264 (Ste6 ΔA-box, middle row), or pRK659 (Ste6 R¹¹, right row). The Ste6 distribution was examined by immunofluorescence microscopy with anti-myc primary antibodies (9E10) and FITC-conjugated anti-mouse secondary antibodies. The strains used are (from top to bottom): JD52 (WT), RKY1511 (Δvps4), RKY1876 (Δvps27), RKY2074 (Δvps35), RKY1634 (Δsnx4), and RKY1875 (Δvps8).

teins that are retrieved from endosomes to the Golgi, such as Kex2, Ste13 and Vps10, aromatic amino acid-based retrieval signals have been identified (Wilcox *et al.*, 1992; Nothwehr *et al.*, 1993; Cooper and Stevens, 1996). To identify putative retrieval signals, we therefore decided to focus on tyrosine and phenylalanine residues in the linker region. Because our previous work has highlighted the importance of this region for Ste6 trafficking, the linker-region presents itself as a prime target for this analysis. Seven tyrosine and phenylalanine residues are present in the linker region and were mutagenized to leucine by site-directed mutagenesis, either singly or in combination (Figure 2A). The five mutant pro-

teins obtained were examined for Ste6 localization by immunofluorescence microscopy in a Δvps8 background. As described above, Ste6 shows a polar cell surface localization in this mutant due to enhanced recycling. If a recycling signal is disrupted, cell surface localization should be lost and Ste6 should accumulate in intracellular compartments. As can be seen in Figure 6, four of the mutants (F630L, Y648L, F656L/Y657L/Y661L, Y713L) showed polar cell surface staining indistinguishable from wild-type Ste6. However, one mutant (Y681L) had the expected phenotype with internal dot-like staining. Thus, tyrosine 681 could be part of a recycling signal. Further analysis of the Ste6 Y681L mutant

Figure 5. Effect of class D *vps* mutants on Ste6 trafficking. (A) Epistasis analysis: single (left row) or double mutants (right row) were transformed with pYKS2 (WT Ste6) and the Ste6 distribution was determined by immunofluorescence microscopy with anti-myc primary antibodies (9E10) and FITC-conjugated anti-mouse secondary antibodies. The following strains were used (from top to bottom): RKY1920 ($\Delta vps21$)/RKY1926 ($\Delta vps8 \Delta vps21$), RKY1921 ($\Delta pep12$)/RKY1930 ($\Delta vps8 \Delta pep12$), RKY1922 ($\Delta bro1$)/RKY1928 ($\Delta vps8 \Delta bro1$), and RKY1510 ($\Delta snf7$)/RKY1927 ($\Delta vps8 \Delta snf7$). (B) Ste6 was immunoprecipitated from cell extracts of strains transformed with a plasmid expressing HA-tagged ubiquitin (YEpl12; Hochstrasser *et al.*, 1991) and YEplac195 (1) or pRK69 (2 μ -STE6; 2 and 3). The following strains were used: (1) RKY959 ($\Delta ste6$), (2) JD52 (WT), and (3) RKY1875 ($\Delta vps8$). The immunoprecipitates were analyzed by Western blotting with anti-Ste6 antibodies (left) and anti-HA antibodies (right). Expression of HA-ubiquitin from the *CUP1* promoter of YEpl12 was induced with 0.5 mM CuSO_4 3 h before extract preparation. A background band is marked by an asterisk; ubiquitinated Ste6 forms are marked by arrows.

suggested that there are two redundant recycling signals in Ste6. The original mutagenesis was performed with the plasmid pYKS2 (Kuchler *et al.*, 1993) that codes for a Ste6 variant with a slightly altered C-terminus (... LFSRSRN instead of

... IVSNQSS). This Ste6 variant behaves in every respect (mating activity, half-life, ubiquitination, localization) like wild-type Ste6. But, upon subcloning, we noticed that the recycling defect was only expressed in combination with the

Figure 6. Identification of putative recycling signals in Ste6. Aromatic amino acids in the Ste6 linker-region were mutated to leucine (Figure 2A, Table 2). The mutated plasmids were transformed into the $\Delta vps8$ strain RKY1875. The following plasmids were used (from top to bottom): pYKS2 (WT Ste6), pRK888 (Ste6 F630L), pRK889 (Ste6 Y648L), pRK890 (Ste6 F656L/Y657L/Y661L), pRK891 (Ste6 Y681L), and pRK892 (Ste6 Y713L). Left row, FITC fluorescence; right row, phase contrast image.

altered C-terminus. This suggests that there are two redundant recycling signals one each in the two homologous halves of Ste6. In the following, the variant with the altered C-terminus is designated Ste6* to distinguish it from wild-type Ste6.

Figure 7. Trafficking of the Ste6* Y681L mutant. Different strains were transformed with pRK891 (Ste6* Y681L). The Ste6 distribution was examined by immunofluorescence microscopy with anti-myc primary antibodies (9E10) and FITC-conjugated anti-mouse secondary antibodies. The following strains were used (from top to bottom): JD52 (WT), RKY592 (*end4*), and RKY1876 ($\Delta vps27$).

Although the internal localization of Ste6* Y681L is suggestive of a recycling defect, the internal localization could also be due to a block in the initial exocytic delivery to the cell surface. To see whether Ste6* Y681L is transported to the cell surface, we examined its localization in the endocytosis mutant *end4*. If Ste6* Y681L ever reaches the cell surface, it should be trapped there in the *end4* mutant at nonpermissive temperature (37°C). As can be seen in Figure 7, cell surface staining was observed for Ste6* Y681L in the *end4* mutant, demonstrating that Ste6* Y681L is properly transported to the cell surface. In addition, we examined the localization of Ste6* Y681L in the class E *vps* mutant $\Delta vps27$ to see whether the protein is transported further along the endocytic pathway. In the $\Delta vps27$ mutant, Ste6* Y681L showed a typical class E staining pattern (Figure 7) demonstrating that it travels through late endosomes. These experiments show that Ste6* Y681L trafficking is pretty normal except for its recycling defect.

During mating, several proteins become polarized to the mating-projection, the so-called shmoo-tip. To see whether Ste6 accumulates at the shmoo-tip, the Ste6 distribution in α -factor treated cells was examined by immunofluorescence microscopy. As noted previously (Kuchler *et al.*, 1993), Ste6* accumulated at the shmoo-tip upon α -factor exposure (Figure 8A). In contrast, the Ste6* Y681L mutant was localized to internal patches. Thus, cell surface localization of this variant is lost not only in the $\Delta vps8$ mutant, but also in pheromone-treated wild-type cells. The effect of this altered localization on mating was tested by a mating-assay. A *MATa* $\Delta ste6$ strain was transformed with single-copy plasmids expressing different Ste6 variants. In a serial dilution patch test, *MATa* cultures were mated to a lawn of *MAT α* cells. Zygotes were selected by replica-plating onto selective me-

A

B

dia (Figure 8B). With wild-type Ste6, zygotes could be detected down to a dilution of 10^{-3} , whereas no zygotes could be detected with the vector control. In this assay, the Ste6* Y681L mutant did not show any mating activity, whereas the other variants, Ste6 Y681L (with wild-type C-terminus) and Ste6* (with altered C-terminus) mated normally. Thus, there is a correlation between loss of cell surface localization and loss of mating activity. All different variants were expressed to the same level and had about the same turnover rate (unpublished data).

The reason for this lack of mating activity of the Ste6* Y681L strain could be a general defect in a-factor secretion or a loss in polarity of a-factor secretion. In the latter case, a-factor activity should still be detectable in culture supernatants probably in amounts comparable to wild type. To test for a-factor secretion, culture supernatants were analyzed for the presence of a-factor by a halo-assay (Figure 8B). Serial dilutions of culture supernatants were spotted onto a lawn of a-factor supersensitive *MAT α sst2* cells. These cells are arrested in their division cycle by a-factor and therefore do not grow when exposed to a-factor (visible as dark spots in the lawn of *sst2* cells). In this experiment, detection of a-factor activity in the culture supernatants always correlated with mating activity. In particular, no a-factor activity could be detected in culture supernatants of the Ste6* Y681L strain. This suggests that this strain is completely defective for a-factor secretion.

DISCUSSION

Here we show that Ste6 variants with mutations in the linker region accumulate at the cell surface in a polar manner. The polar distribution is maintained by endocytic recycling and localized exocytosis and is controlled by ubiquitination.

Figure 8. Effect of the Y681L mutation on mating. (A) Cultures of strain JD52 transformed with pYKS2 (Ste6*, top panels) or pRK891 (Ste6* Y681L, bottom panels) were treated with α -factor (5 μ M) for 2 h. Then the Ste6 distribution was determined by immunofluorescence microscopy with anti-myc primary antibodies (9E10) and FITC-conjugated anti-mouse secondary antibodies. Left, FITC fluorescence; right, phase-contrast images. (B) To examine the effect of the Y681L mutation on mating activity, the Δ ste6 strain RKY959 was transformed with single-copy plasmids expressing different Ste6 variants. Tenfold serial dilutions of the different cultures were spotted onto a lawn of a *MAT α* strain. The cells were allowed to mate for 12 h and were then replica plated to a selective plate (SD minimal medium) to select for zygotes. Left panel, cell spotted on rich medium plate (YPD); middle panel, SD plate; right panel, halo-assay, culture supernatants (2-fold serial dilutions) were spotted onto a lawn of a-factor supersensitive *MAT α* cells. Plates were incubated for 3 d at 30°C. The following plasmids were used (from top to bottom): YEplac195 (vector, Δ ste6), pRK278 (WT Ste6), pRK909 (Ste6 Y681L), pRK940 (Ste6*), and pRK939 (Ste6* Y681L).

Polar Localization of Ste6 through Endocytic Recycling

How is the polar localization of the Ste6 Δ A-box and the Ste6 R¹¹ variants maintained? For the v-SNARE Snc1, which has a localization similar to our Ste6 variants, it has been proposed that it is polarized to the bud cell surface by endocytic recycling and localized exocytosis ("kinetic polarization"; Valdez-Taubas and Pelham, 2003). The immediate redistribution to internal structures upon inactivation of Ypt6, which is an essential component of the endocytic recycling loop (Siniosoglou *et al.*, 2000), indicates that the Ste6 variants are polarized by a similar mechanism. Alternatively, it has been proposed that proteins are polarized to the shmoo-tip by a lipid-based sorting mechanism (Bagnat and Simons, 2002). According to this model, polarized membrane proteins partition into lipid rafts that are concentrated at the shmoo-tip. But, this mechanism has been questioned (Valdez-Taubas and Pelham, 2003). How this lipid asymmetry could be established is unclear. Also, the effect of Ypt6 inactivation on Ste6 polarization would have to be incorporated into this model. One would have to postulate that Ypt6 and thus probably endocytic recycling plays a central role in establishing lipid asymmetry. Thus, in any case, endocytic recycling would be important for protein polarization, either directly or indirectly.

Control of Recycling by Ubiquitination

Enhanced recycling was observed with the Ste6 Δ A-box and Ste6 R¹¹ variants. In both variants, ubiquitination was reduced compared with wild type. The magnitude of the recycling phenotype correlated with the degree of ubiquitination. The strongest effect was seen with the Ste6 Δ A-box variant, which did not show any ubiquitination (Kölling and Losko, 1997). The Ste6 R¹¹ variant, which displayed some

Figure 9. Sorting in the early endocytic pathway. A model for ubiquitination-dependent sorting of Ste6 in the early endocytic pathway is presented (modified from Maxfield and McGraw, 2004). Nonubiquitinated Ste6 ($\diamond$) is sorted into tubules that pinch off from sorting endosomes giving rise to recycling endosomes. From there it can be transported to the cell surface. Ubiquitinated Ste6 ($\blacklozenge$) is retained in the vacuolar part of the sorting endosome and directed into the multivesicular bodies (MVB) degradation pathway.

residual ubiquitination, had a somewhat “leaky” recycling phenotype, i.e., a certain fraction of the protein escaped recycling and was transported further down the endocytic pathway. In the Ste6 R^{11} variant only putative ubiquitin acceptor sites had been mutated. Although, we cannot definitely exclude that some other signal is affected by the mutations, the most likely interpretation of our data are that lack of ubiquitination is responsible for the observed recycling phenotype.

Analysis of Ste6 recycling in different mutants of the endocytic pathway suggested that the ubiquitination-deficient Ste6 variants recycle through early endosomes. To explain how ubiquitination could interfere with Ste6 sorting, we like to propose the following model which is largely based on information gathered from mammalian cells about sorting events in the early endocytic pathway (Figure 9) (Gruenberg and Stenmark, 2004; Maxfield and McGraw, 2004). The early endosome/sorting endosome constitutes a central sorting station in the early endocytic pathway. It consists of a vacuolar part from which tubular extension emanate. Because of the high surface-area-to-volume ratio of the tubules, most membrane proteins will end up in the tubules by default unless they are specifically retained in the vacuolar part of the sorting endosome (“geometry based sorting”). The tubules pinch off and develop into recycling endosomes. According to our model, ubiquitinated Ste6 will be retained in the vacuolar part of the sorting endosome, whereas nonubiquitinated Ste6 is distributed into the tubules and is thus funneled into the recycling pathway. In mammalian cells, there is evidence that planar clathrin coats on sorting endosomes are involved in retention of cargo proteins that are destined for lysosomal degradation (Raiborg *et al.*, 2001; Sachse *et al.*, 2002). Ubiquitinated cargo proteins have been detected in these coat structures (Raiborg *et al.*, 2002). After recruitment to the planar coat structure, ubiquitinated cargo proteins are sorted into vesicles that bud off into the interior of the sorting endosome

It is thought that sorting into the recycling pathway is not a signal-mediated process, but instead occurs by default (Maxfield and McGraw, 2004). We have obtained evidence for the existence of redundant signals in Ste6 that are required for directing Ste6 into the recycling pathway. This is consistent with the identification of similar signals in other yeast proteins such as Kex2, Ste13, and Vps10 that are retrieved from endosomes back to the Golgi (Wilcox *et al.*, 1992; Nothwehr *et al.*, 1993; Cooper and Stevens, 1996). It is conceivable that the initial sorting step, i.e., sorting into tubules, occurs by default, but that later steps require specific signals.

Two Distinct Ubiquitination-dependent Events in Endosomal Sorting?

The results reported in this study and previous observations suggest that two distinct ubiquitination-dependent events could be involved in endosomal sorting. Previously, we and others have used the *doa4* mutant as a tool to reduce ubiquitination. In this mutant, ubiquitination-dependent processes are affected because the free ubiquitin level is lowered (Swaminathan *et al.*, 1999). With this mutant, we observed an accumulation of Ste6 at the vacuolar membrane, but not at the cell surface (Losko *et al.*, 2001). Similar results have been reported for other proteins (Katzmann *et al.*, 2001; Reggiori and Pelham, 2001; Urbanowski and Piper, 2001). Apparently, sorting of cargo into internal MVB vesicles is defective in the *doa4* mutant. So, after fusion of late endosomes with the vacuole the proteins end up at the vacuolar membrane. This contrasts with the localization of our ubiquitination-deficient Ste6 variants reported in this study. What could be the reason for these different effects of reduced ubiquitination on the localization of Ste6? One possibility is that retention of cargo proteins and incorporation into MVB vesicles are two distinct events, which differ in their requirements with respect to ubiquitination. These two events could require a different degree or different kind of ubiquitination. The requirements could be more stringent for MVB vesicle sorting. Thus, although a somewhat reduced ubiquitination could still be sufficient for retention in the vacuolar part of the sorting endosome, it may not be sufficient for incorporation into MVB vesicles. This could be the situation in the *doa4* mutant. In contrast, strongly reduced ubiquitination, as in the Ste6 ΔA -box and R^{11} variants, would affect both retention and MVB vesicle sorting. Indeed, for the Ste6 R^{11} variant with an intermediate degree of ubiquitination, we observe both accumulation at the cell surface and at the vacuolar membrane.

Physiological Role of Recycling?

So far, endocytic recycling has only been demonstrated for mutated Ste6 variants. Does wild-type Ste6 recycle as well under normal conditions? There are indications that it does. For Ste6, a half-life of ~15–20 min has been determined. Although this looks like a pretty short half-life, it is still substantially longer than the half-life of another endocytic protein, the α -factor receptor Ste2, which is around 5 min (Hicke and Riezman, 1996; our unpublished observations). Thus, in principle, degradation of cell surface proteins via the endocytic pathway appears to be a very rapid process. Also, earlier we noticed in pulse-chase experiments that there is a delay of ~20 min before the start of Ste6 degradation (Kölling and Hollenberg, 1994). Such a delay is not observed, e.g., for the transport of carboxypeptidase Y (CPY) from the endoplasmic reticulum to the vacuole. The delay in degradation and the relatively “long” half-life of Ste6 is compatible with Ste6 cycling a few times before degrada-

tion. In addition, we obtained evidence for Ste6 recycling from cell fractionation experiments (Losko *et al.*, 2001).

What could be the function of Ste6 recycling? The function of endocytic recycling could be to polarize Ste6 to the mating projection during mating. Polarized localization of a number of proteins to the shmoo-tip appears to be important for ordered cell fusion during mating. A Ste6 mutant with reduced activity has been isolated that is specifically blocked at the fusion step (Elia and Marsh, 1996). Because this mutant provides enough a-factor activity to complete the earlier steps in the mating cascade, this suggests that the demand for Ste6 activity is especially high at the fusion step. Thus, it may be necessary to concentrate the available Ste6 protein at the shmoo-tip.

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