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Mutations affecting phosphorylation, ubiquitination and turnover of the ABC-transporter Ste6

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Abstract In this report, the role of phosphorylation in the regulation of ubiquitination and turnover of the ABC-transporter Ste6 was investigated. We demonstrate that Ste6 is phosphorylated in vivo and that this phosphorylation is dependent on the presence of an acidic stretch ('A-box') in the linker region previously shown to be important for ubiquitination and fast turnover of Ste6. By mutagenesis, two serine/threonine residues were identified in the A-box region that are crucial for ubiquitination and trafficking to the yeast vacuole. In the mutants there was no simple correlation between phosphorylation and ubiquitination levels, suggesting that the two events may not be coupled.

Key words: Ubiquitination; Phosphorylation; Endocytosis; Turnover; ABC-transporter

1. Introduction

Information about substrate features that specify recognition by the ubiquitination machinery is still limited. The N-terminal amino acid has been recognized as a feature which may specify the half-life of a protein ('N-end rule') [1]. Other sequence motifs shown to be important for ubiquitin-mediated degradation are the cyclin destruction box, first identified in mitotic cyclins [2] and stretches rich in Pro, Glu/Asp, Ser and Thr residues, so-called 'PEST-regions' [3,4]. Another ubiquitination signal is contained in the α-factor receptor Ste2. It could be shown that the nine amino acid sequence SINNDAKSS, which is required for the endocytosis of the liganded receptor [5], serves as an attachment site for ubiquitin [6]. Ubiquitination at the SINNDAKSS sequence appears to be regulated by phosphorylation [7].

We are interested in identifying the signals that mediate ubiquitination and rapid turnover of the ABC-transporter Ste6. Ste6 accumulates at the plasma membrane in a ubiquitinated form upon block of endocytosis [8]. This ubiquitination is mediated by a signal contained in the 100-amino acid long linker region ('D-box' for degradation box) which connects the two homologous halves of Ste6 [9]. A Ste6 mutant where part of the D-box is deleted ('A-box deletion') is no longer ubiquitinated and strongly stabilized. In this report, we investigated whether Ste6 ubiquitination is regulated by phosphorylation. We found that Ste6 is indeed phosphorylated, as reported previously [10] and that this phosphorylation is abolished by the A-box deletion. To identify the phosphorylation sites in the A-box region, we mutagenized potential target sites for serine/threonine phosphorylation. Two serine/threonine exchanges were identified which in combination led to a stabilization and a reduction in ubiquitination comparable to the A-box deletion. However, there was no clear-cut correlation between the degree of phosphorylation and ubiquitination, suggesting that these two events may not be coupled.

2. Materials and methods

2.1. Strains, plasmids and growth conditions

The *Saccharomyces cerevisiae* strain used for all experiments was RKY959 *MAT**a** his3-Δ200 leu2-3,112 lys2-801 Δste6::LEU2 trp1-Δ63 ura3-52*. The parent single-copy plasmid was pRK278, which contains a 6.2 kb *Bgl*II/*Sal*I chromosomal *STE6* fragment cloned into the centromer vector YCplac33 [11] (with deleted *Pst*I site). As parent 2μ-vector the plasmid pYKS2 was used, which is derived from YEp352 and contains a c-myc-tagged *STE6* gene [10]. Plasmid pRK182 contains the same *STE6* fragment as pRK278 cloned into the centromer plasmid YCp50 [12]. The cells were grown in SD-medium with 1% casamino acids [13]. HA-tagged ubiquitin was expressed from the 2μ-plasmid YEp112 [14] under the control of the *CUP1* promoter by the addition of 0.5 mM $CuSO_4$ for 30 min before extract preparation.

2.2. Construction of mutants

Site-directed mutagenesis was performed with the Bio-Rad Muta-Gene™ kit based on the method of Kunkel et al. [15]. A 1.2 kb internal *Pst*I *STE6* fragment (pos. 1620–2830), cloned into the phagemid pUC218, was mutagenized with the following mutagenic primers (exchanges marked in bold print). T613A (primer #98): 5′-G (pos. 2283) AATGACTACTCTGATGCGAAA**GC**G**AT**CGTAGA-TACAGAGACTGAAG (new *Pvu*I site); S623L (primer #114): 5′-A (pos. 2317) CAGAGACTGAAGAAAA**GCT**TATACACACTG-TGGAAAG (new *Hin*dIII site). All mutations were confirmed by sequencing.

2.3. In vivo labeling of cells and immunoprecipitation

For the phosphorylation experiments, cells were grown overnight in SD-low phosphate medium [16] (30 μM KH_2PO_4) to an $A_{600} \leq 0.8$ (4×10^7 cells/ml). 5×10^7 cells were labeled with 200 μCi [^{32}P]orthophosphate (Amersham, PBS11) for 30 min at 30°C in 0.5 ml of SD-low phosphate medium. The cells were washed in 10 mM NaN_3, resuspended in 110 μl lysis-buffer (50 mM HEPES, 0.3 M sorbitol, 10 mM NaN_3, pH 7.5+ protease inhibitor cocktail and 50 μg/ml RNase A) and vortexed for 3 min with 400 mg of glass beads. Ste6 was immunoprecipitated from the cell extracts and analyzed by SDS–PAGE and autoradiography as described previously [8]. X-ray films (Kodak) were exposed for about 5 h for 2μ-*STE6* and 3 days for single-copy *STE6*. Pulse-chase experiments were performed as described previously [8].

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2.4. Immunofluorescence experiments

Immunofluorescence experiments were performed as described in [17].

3. Results

3.1. A-box dependent phosphorylation of Ste6

To test for Ste6 phosphorylation, cells of the *STE6* deletion strain RKY959, transformed with a multicopy *STE6* plasmid or with a vector control, were labeled with [^{32}P]orthophosphate. As can be seen in Fig. 1B (lane 3), a strong phosphorylation signal could be detected for wild-type Ste6 while no signal was detected with the vector control (Fig. 1B, lane 1). Phosphorylation of Ste6 has also been demonstrated previously [10]. We then examined the phosphorylation status of the Ste6 ΔA-box variant which is defective in ubiquitination and strongly stabilized but fully functional in **a**-factor secretion and mating [9]. With this A-box deletion variant no phosphorylation signal could be detected (Fig. 1B, lane 2). The Western blot control in Fig. 1A shows that the ΔA-box protein is expressed under these conditions. The concurrent loss of phosphorylation and ubiquitination in the A-box mutant points to a role of phosphorylation in Ste6 turnover.

3.2. Potential targets for serine/threonine phosphorylation in the A-box region

In our search for potential phosphorylation sites, we focused on serine/threonine residues contained in two short sequence motifs resembling the 'DAKSS' motif, found in the α-factor receptor Ste2, which acts as a phosphorylation and ubiquitination signal [7] and is required for efficient Ste2 endocytosis [5]. To assess the contribution of these residues to phosphorylation and turnover of Ste6, mutants were constructed by site-directed mutagenesis. Threonine 613 in the DAKTI sequence was replaced by alanine (T613A), serine 623 was replaced by leucine (S623L). Furthermore, a double mutant was constructed (T613A S623L). The stability of these mutants was determined by pulse-chase experiments. As can be seen in Fig. 2, both the T613A mutation (Fig. 2B, $\tau = 45$ min) and the S623L mutation (Fig. 2C, $\tau = 80$ min) stabilize Ste6 about two- to three-fold. The stabilizing effects were additive. When the two mutations were combined, a five-fold stabilization comparable to that of the A-box deletion was observed (Fig. 2D, $\tau = 110$ min). The half-life of wild-type Ste6 in this strain background was about 25 min (Fig. 2A). A quantification of the results is presented in Fig. 2E.

To exclude that the observed stabilization is caused by misfolding of the proteins and degradation by a slower alterna-

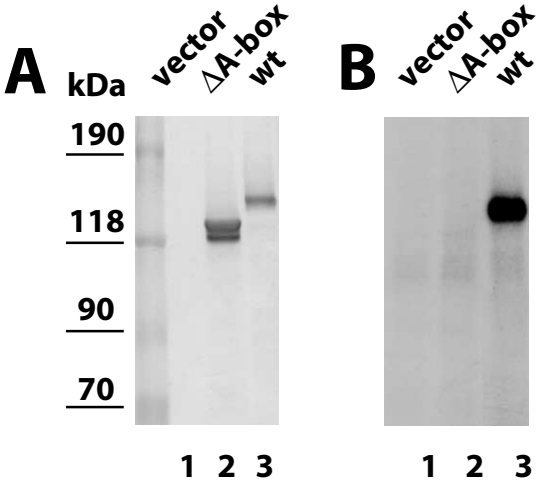
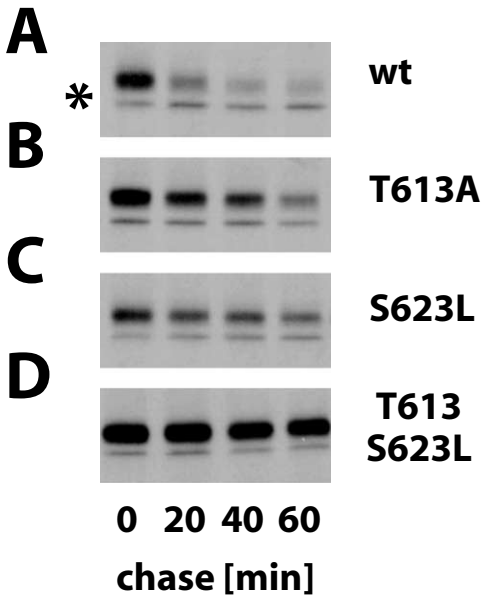


Fig. 1. A-box dependent phosphorylation of Ste6. Cells of the *STE6* deletion strain RKY959, transformed with different *STE6* plasmids, were labeled with [^{32}P]orthophosphate: (1) YEplac195 (vector), (2) pRK264 (ΔA-box), (3) pYKS2 (wild-type *STE6*). A: Western blot of the cell extracts with anti-Ste6 antibodies. B: Autoradiogram of the proteins immunoprecipitated with anti-Ste6 antibodies.



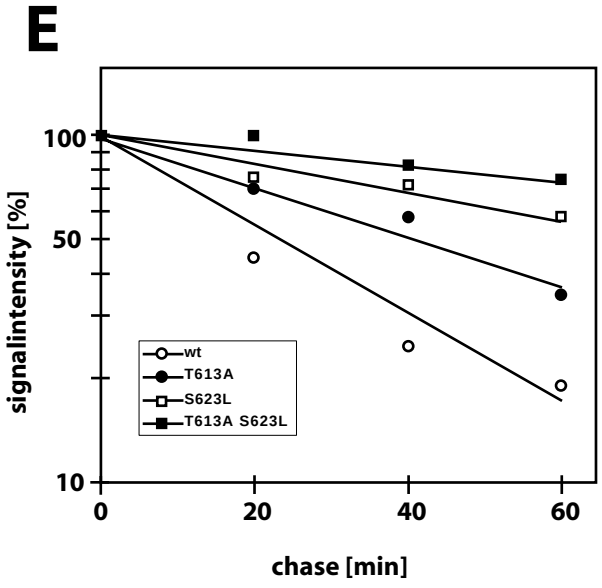


Fig. 2. Pulse-chase experiment: stability of Ste6 variants. Cells of the *STE6* deletion strain RKY959, transformed with different single copy *STE6* plasmids, were labeled with [^{35}S]methionine for 15 min and were then chased with an excess of cold methionine. Ste6 was immunoprecipitated from cell extracts prepared at time intervals, as indicated. The precipitated proteins were analyzed by SDS–PAGE and autoradiography. Plasmids used were: A: pRK182 (wild-type *STE6*); B: pRK321 (T613A); C: pRK402 (S623L); D: pRK337 (T613A S623L); E: Quantification of Ste6 signals. A background band unrelated to Ste6 is marked by an asterisk.

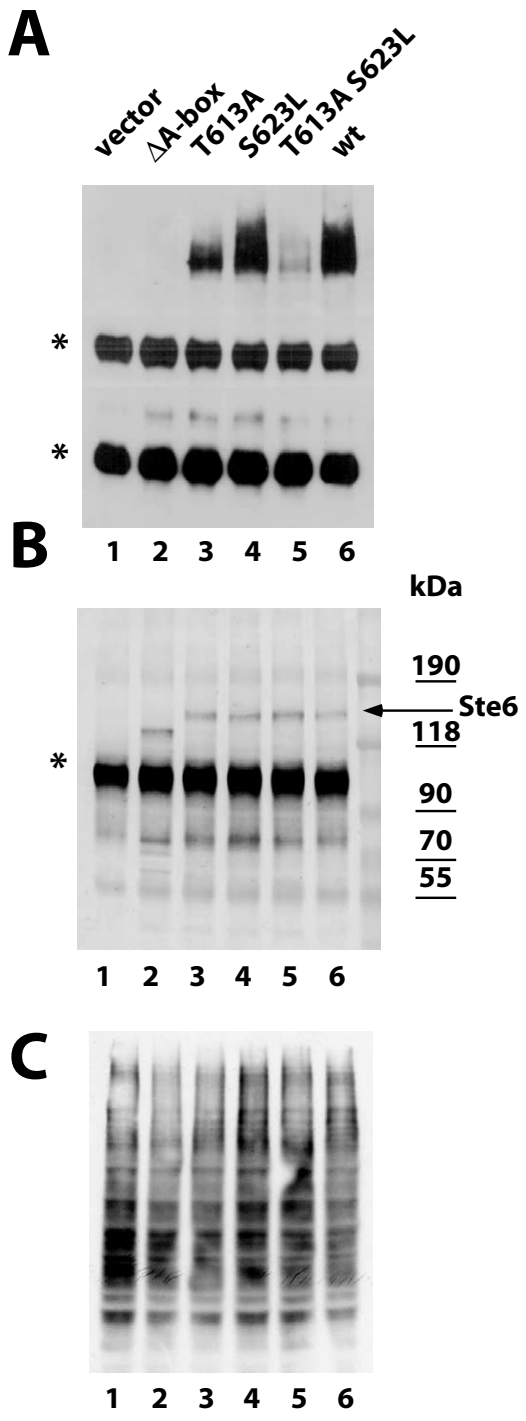


Fig. 3. Ubiquitination of Ste6 variants. RKY959 was transformed with YEp112, expressing HA-tagged ubiquitin, and with 2μ-plasmids, expressing different Ste6 variants. The plasmids were: (1) YEplac195 (vector), (2) pRK264 (ΔA-box), (3) pRK322 (T613A), (4) pRK427 (S623L), (5) pRK339 (T613A S623L), (6) pYKS2 (wt *STE6*). Proteins immunoprecipitated with Ste6 antibodies were analyzed by Western blotting with the anti-HA antibody, to detect ubiquitin (A) and with anti-Ste6 antibodies to detect Ste6 (B). Background bands from immunoprecipitation (IgG, protein A) are marked by asterisks. The position of Ste6 is indicated by an arrow. C: Detection of HA-ubiquitin in cell extracts by Western blotting with HA-antibodies.

tive pathway, the mating activity mediated by the Ste6 variants was determined by a patch mating assay. The Ste6 mutants displayed a mating activity indistinguishable from wild type (data not shown). Therefore, the mutations appear to specifically affect the normal degradation pathway of Ste6.

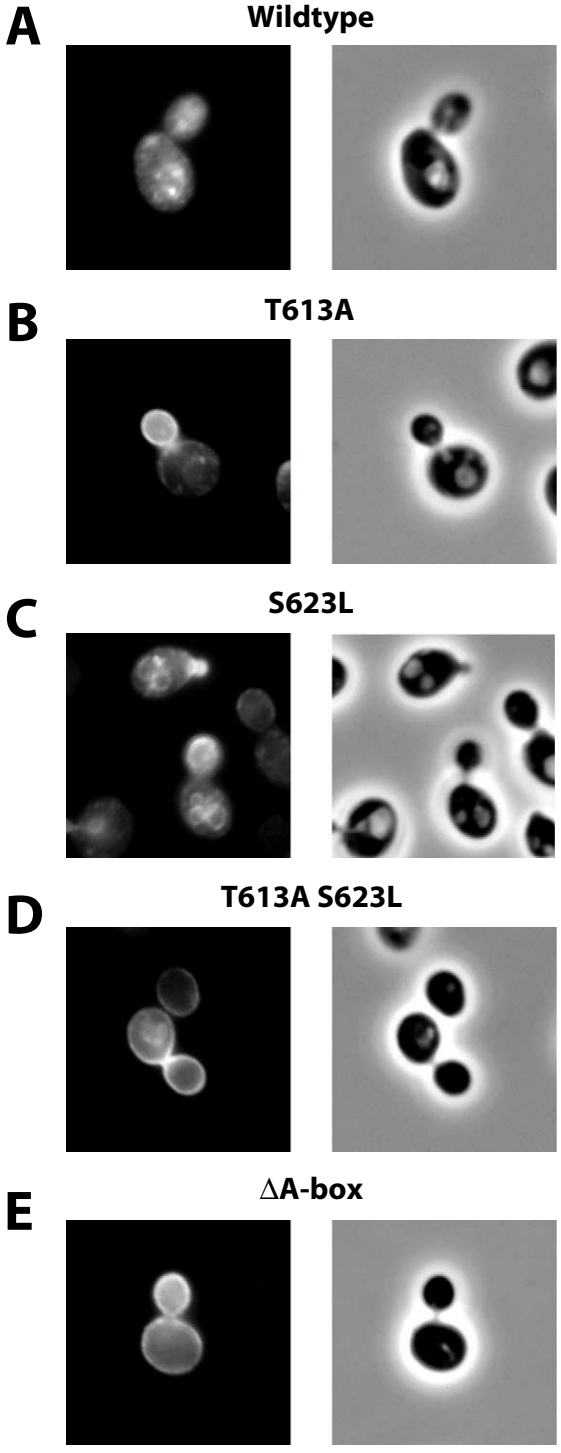


Fig. 4. Localization of Ste6 variants by immunofluorescence. Detection of c-myc-tagged Ste6 variants, expressed in strain RKY959, with anti-myc primary antibodies and DTAF-conjugated anti-mouse secondary antibodies. A: pYKS2 (wt *STE6*); B: pRK322 (T613A); C: pRK427 (S623L); D: pRK339 (T613A S623L); E: pRK264 (ΔA-box). Left panels: DTAF-staining; right panels: phase contrast image.

3.3. Modification and localization of the Ste6 variants

To determine the degree of ubiquitination of the different Ste6 variants, Ste6 protein immunoprecipitated from cell extracts was assayed for the presence of covalently attached HA-tagged ubiquitin. Ubiquitination of Ste6 was detected as a 'ubiquitin-smear' on the upper part of the gel. A strong ubiquitination signal was observed for wild-type Ste6 (Fig. 3A, lane 6) while no signal was detectable with the ΔA-box variant (Fig. 3A, lane 2), as reported previously [9]. The T613A mutant showed a reduced level of ubiquitination (Fig. 3A, lane 3) which was further reduced by the introduction of the S623L mutation (Fig. 3A, lane 5). But in contrast to the ΔA-box variant there was still some residual ubiquitination visible. Surprisingly, however, the S623L mutant alone did not show a detectable ubiquitination defect. As a control, the immunoprecipitates were probed with anti-Ste6 antibodies by Western blotting (Fig. 3B), demonstrating that comparable amounts of Ste6 protein had been precipitated. Furthermore, care had been taken to achieve comparable levels of expression of HA-tagged ubiquitin (Fig. 3C).

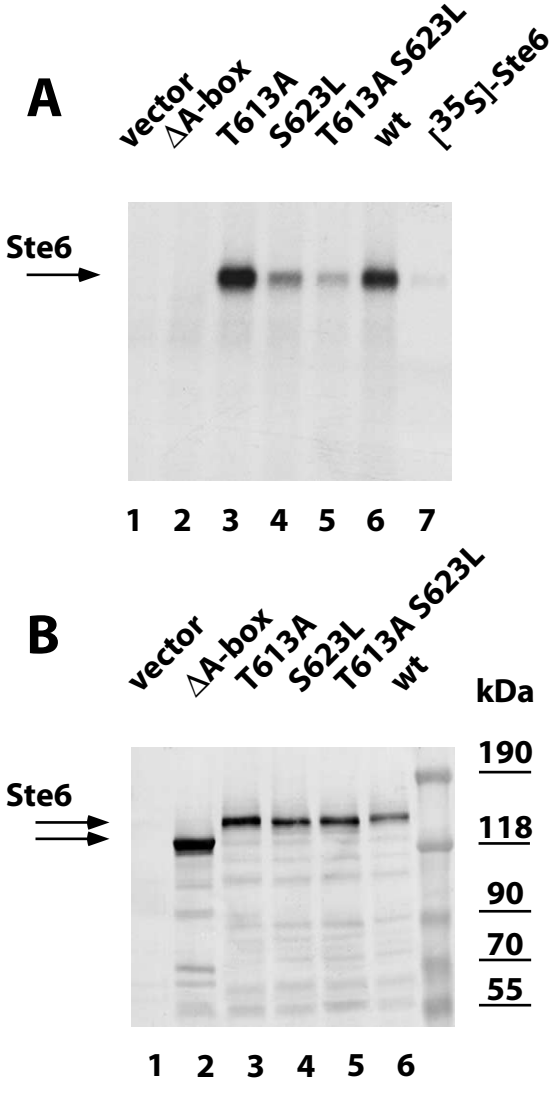


Fig. 5. Phosphorylation of Ste6 variants. RKY959, transformed with different 2μ *STE6* plasmids, was labeled with [^{32}P]orthophosphate. A: Autoradiogram of the proteins immunoprecipitated with anti-Ste6 antibodies. Ste6 is marked by an arrow. B: Western blot of corresponding cell extracts with anti-Ste6 antibodies. (1) YEplac195 (vector), (2) pRK264 (ΔA-box), (3) pRK322 (T613A), (4) pRK427 (S623L), (5) pRK339 (T613A S623L), (6) pYKS2 (wt *STE6*); lane 7 in (A) [^{35}S]methionine-labeled Ste6.

The intracellular localization of the Ste6 variants was determined by immunofluorescence experiments (Fig. 4). Wild-type Ste6 exhibited a mostly intracellular, patchy staining (Fig. 4A). Patches were often seen associated with the vacuole which can be seen as white spots in the phase contrast image. With the T613A mutant, which also showed reduced ubiquitination, a bright cell surface staining was observed in addition to the intracellular signal (Fig. 4B). This indicates, as suggested previously [9], that ubiquitination may be involved in internalization of the Ste6 protein. The S623L mutant, however, showed a very different staining pattern (Fig. 4C). With this mutant, a perivacuolar staining was observed in many cells in addition to some cell surface staining, which was not as pronounced as in the T613A mutant. The ring-like staining around the vacuole points to a defect in sorting to the multi-vesicular-bodies (MVB) pathway [18,19]. The double mutant T613A S623L had a staining similar to the T613A mutant (Fig. 4D), i.e. the T613A mutation appears to be 'dominant' over the S623L mutation. This could indicate that the DAKTI signal acts before the EEKSI signal in a sequential pathway. The ΔA-box mutant was included for comparison (Fig. 4E).

We then examined whether the observed effects on ubiquitination and turnover are correlated with a reduction in Ste6 phosphorylation. Again, no phosphorylation signal could be detected for the ΔA-box deletion (Fig. 5A, lane 2). However, with the other mutants no clear-cut correlation between the effects on ubiquitination and turnover and phosphorylation could be established. Phosphorylation was not affected by the T613A mutation despite its marked effect on ubiquitination and turnover (Fig. 5A, lane 3). In contrast, phosphorylation was reduced for the S623L variant (Fig. 5A, lane 4), which displays a wild-type ubiquitination level. The T613A S623L double mutant had a somewhat lower phosphorylation level than the S623L single mutant (Fig. 5A, lane 5).

4. Discussion

In this report, we demonstrate that Ste6 phosphorylation is dependent on the presence of a 50 amino acid stretch in the linker region ('A-box') connecting the two homologous halves of Ste6. In our attempt to localize the phosphorylation site(s) within the A-box region, we focused on two short sequence stretches ('DAKTI' and 'EEKSI') resembling a sequence motif ('DAKSS') shown to be required for endocytosis of the α-factor receptor Ste2 [5]. Mutation of the serine/threonine residues in the two DAKSS-like sequences resulted in a stabilization of Ste6. The stabilizing effects of the single exchanges were additive. When the two mutations were combined (T613A S623L) the observed stabilization was about as strong as that of the whole A-box deletion. This shows that the turnover signal, present in the A-box region, can be inactivated by two single point mutations. Although both single point mutations stabilized Ste6 to a similar extent, the phenotype of these mutations was different. The T613A mutation in the DAKTI sequence resulted in reduced ubiquitination and in accumulation of Ste6 at the plasma membrane. This is in line with the proposed role of ubiquitination in endocytosis of yeast plasma

membrane proteins [20]. From this result, it can be concluded that the DAKTI motif is required for the ubiquitination and internalization of Ste6, as suggested previously [9]. In the EEKSI motif mutant, however, ubiquitination was normal and the mutant protein was mainly found internal around the vacuole. The most likely explanation for this finding is that the EEKSI motif affects another, presumably internal, trafficking step, which could be sorting of Ste6 to the MVB pathway [18,19].

The T613A S623L double mutant showed a Ste6 immunofluorescence staining pattern very similar to the T613A single mutant. The T613A mutation, therefore, appears to be 'dominant' over the S623L mutation, at least with respect to immunofluorescence staining. This can be taken as an indication that the trafficking step affected by T613A lies before the S623L-dependent step. The ubiquitination defect of the double mutant, however, was much more severe than in the T613A single mutant. The reason for that could be that the S623L mutation not only inactivates the EEKSI motif but also further impairs recognition of the mutated DAKTI motif by the ubiquitination machinery through a change in the secondary structure around the DAKTI sequence.

Although the examined mutations had profound effects on Ste6 turnover, there was no simple correlation between the phosphorylation state of Ste6 and its ubiquitination. In the case of T613A, ubiquitination was reduced while phosphorylation was normal. The opposite effect was seen with the S623L mutant. Here, phosphorylation was reduced and ubiquitination was normal. The most likely explanation for these findings is that the serine/threonine mutations do not act through the inactivation of specific phosphorylation sites. Where then are the phosphorylation site(s) and what is their function? Phosphorylation may occur at other serine/threonine residues in the A-box region. These residues may not directly be part of the turnover signal(s) but could regulate their availability to the ubiquitination machinery. In any case, the data presented for Ste6 cannot be easily reconciled with the idea that phosphorylation is a prerequisite for ubiquitination, as proposed for other yeast plasma membrane proteins [7,21].

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