The linker region of the ABC-transporter Ste6 mediates ubiquitination and fast turnover of the protein

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Upon block of endocytosis, the a-factor transporter Ste6 accumulates in a ubiquitinated form at the plasma membrane. Here we show that the linker region, which connects the two homologous halves of Ste6, contains a signal which mediates ubiquitination and fast turnover of Ste6. This signal was also functional in the context of another plasma membrane protein. Deletion of an acidic stretch in the linker region ('A-box') strongly stabilized Ste6. The A-box contains a sequence motif ('DAKTI') which resembles the putative endocytosis signal of the α -factor receptor Ste2 ('DAKSS'). Deletion of the DAKTI sequence also stabilized Ste6 but, however, not as strongly as the A-box deletion. There was a correlation between the half-life of the mutants and the degree of ubiquitination: while ubiquitination of the Δ DAKTI mutant was reduced compared with wild-type Ste6, no ubiquitination could be detected for the more stable Δ A-box variant. Loss of ubiquitination seemed to affect Ste6 trafficking. In contrast to wild-type Ste6, which was associated mainly with internal membranes, the ubiquitination-deficient mutants accumulated at the plasma membrane, as demonstrated by immunofluorescence and cell fractionation experiments. These findings suggest that ubiquitination is required for efficient endocytosis of Ste6 from the plasma membrane.

Keywords: ABC-transporter/a-factor/endocytosis/Ste6/ubiquitination

Introduction

The yeast Ste6 protein is a typical member of the ABC-transporter family consisting of 12 putative membrane-spanning segments and two ATP-binding domains (Kuchler *et al.*, 1989; McGrath and Varshavsky, 1989). By sequence comparison, it appears to be most closely related to the mammalian Mdr proteins. This is underscored by the finding that mouse *mdr3* is able to complement a *ste6* defect in yeast (Raymond *et al.*, 1992). Ste6 mutants are defective in the secretion of the mating pheromone a-factor. Because of its similarity to other proteins of the ABC-transporter family, which are known to transport substrates across membranes, it has been assumed that Ste6 acts as a transporter for a-factor at the plasma membrane. We found, however, that most of Ste6 is associated with internal membranes (Kölling and Hollenberg, 1994). However, there is also evidence that

at least a certain fraction of Ste6 travels to the plasma membrane. This evidence is derived from experiments with endocytosis mutants showing an accumulation of Ste6 at the plasma membrane upon block of endocytosis. Following endocytosis, the Ste6 protein seems to be degraded in the vacuole (Berkower *et al.*, 1994; Kölling and Hollenberg, 1994).

Curiously, the Ste6 protein which accumulates at the plasma membrane in endocytosis mutants is ubiquitinated. What is the role of this ubiquitination? Most thoroughly characterized is the role of ubiquitination in protein turnover. Ubiquitin, a 76 amino acid polypeptide, is covalently attached to protein substrates, thereby marking the proteins for degradation by the 26S proteasome, an ATP-dependent multicatalytic proteinase complex (Ciechanover, 1994; Hochstrasser, 1995; Jentsch and Schlenker, 1995). Despite extensive knowledge about the enzymology of ubiquitin-dependent proteolysis, only a limited number of natural substrates are known to date. In yeast, the ubiquitin system has been implicated in the degradation of several soluble proteins, including the transcriptional regulators Mat α 2 (Chen *et al.*, 1993) and Gcn4 (Kornitzer *et al.*, 1994), several yeast cyclins (Deshaies *et al.*, 1995; Seufert *et al.*, 1995; Yaglom *et al.*, 1995), fructose 1,6-bisphosphatase (Schork *et al.*, 1995), the G α subunit Gpa1 (Madura and Varshavsky, 1994) and the GTP exchange factor Cdc25 (Kaplon and Jacquet, 1995). Degradation via the ubiquitin–proteasome pathway, however, does not seem to be restricted to soluble proteins. There are also a few examples of integral membrane proteins that seem to be degraded via the proteasome. One example that is of special interest in this context is the human CFTR protein, because it is itself a member of the ABC-transporter family. The CFTR protein seems to have problems in attaining a properly folded conformation. A large fraction of the wild-type precursor protein and virtually all of a mutant form of CFTR (Δ F508), found in the majority of cystic fibrosis patients, is retained in the endoplasmic reticulum (ER) by the quality control system and is apparently degraded by the ubiquitin–proteasome pathway (Jensen *et al.*, 1995; Ward *et al.*, 1995). Similarly, a mutant form of the multispanning membrane protein Sec61, a yeast protein involved in protein translocation, seems to be degraded at the level of the ER by the ubiquitin–proteasome pathway (Biederer *et al.*, 1996).

However, the consequence of ubiquitination is not always the immediate degradation of the target proteins. The immunoglobulin E receptor is ubiquitinated in response to antigen binding and is deubiquitinated rapidly upon receptor disengagement (Paolini and Kinet, 1993). There is no evidence for a selective degradation of the ubiquitinated receptors. The cycle of ubiquitination–deubiquitination, therefore, seems to serve a purely regu-

Fig. 1. Predicted membrane topology of Ste6, structure of the linker region.

latory function. A role for ubiquitination other than targeting proteins for proteasomal degradation is further proposed by Hicke and Riezman (1996), based on experiments with the yeast α -factor receptor Ste2. Their data indicate that ubiquitination triggers endocytosis of the receptor–ligand complex leading to subsequent degradation in the vacuole. Other recent studies suggest that the same may be true for other plasma membrane proteins like uracil permease Fur4 (Volland *et al.*, 1994), the general amino acid permease Gap1 (Hein *et al.*, 1995), the multidrug permease Pdr5 (Egner *et al.*, 1995; Egner and Kuchler, 1996) and of course Ste6 (Berkower *et al.*, 1994; Kölling and Hollenberg, 1994).

A Ste6 mutant, which is no longer ubiquitinated, would be an important tool to analyze the function of Ste6 ubiquitination. We were interested, therefore, in identifying the signal(s) in Ste6 required for ubiquitination. Here, we show that ubiquitination and fast turnover of Ste6 are mediated by a signal in the linker region connecting the two ABC-transporter repeats. Our data indicate that ubiquitination is important for efficient endocytosis of Ste6.

Results

Mutations in the linker region affect the half-life of Ste6

Ste6 is an extremely unstable protein with a half-life of ~14 min (Kölling and Hollenberg, 1994). Experiments unrelated to the present study, where we generated chimeras between Ste6 and other ABC-transporters, indicated that the 100 amino acid long linker region (amino acids 609–716) (Figure 1), which connects the two homologous halves of Ste6, is important for the fast turnover. The linker region was therefore named ‘D-box’ for ‘destabiliza-

Fig. 2. Stability of Ste6 variants. Cells of strain JPY201, transformed with different *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 various time intervals, as indicated. The precipitated proteins were analyzed by SDS–PAGE and autoradiography. (A) JPY201/pRK257, (B) JPY201/pRK264, (C) signal intensities of experiment A (○) and B (●) plotted against the chase time, (D) JPY201/pRK265, (E) JPY201/pRK281, (F) signal intensities of experiment D (□) and E (■) plotted against the chase time.

tion box’. Upon closer inspection, we noticed an unusual distribution of charged amino acids in the D-box region. The ‘upstream’ half (amino acids 609–661) contains mostly acidic amino acids (D,E = 11; K = 3) and was therefore named ‘A-box’ for acidic box. The ‘downstream’ half (amino acids 662–716) contains two blocks of basic amino acids (K,R = 14; E = 4) and was named ‘B-box’ for ‘basic box’. Furthermore, we found that the A-box contains a sequence motif ‘DAKTI’, which closely resembles the putative endocytosis signal of Ste2 ‘DAKSS’ (Rohrer *et al.*, 1993).

A-box, B-box and DAKTI deletion mutants were constructed in order to assess the influence of these regions on the stability of Ste6. The half-lives of the Ste6 deletion mutants were determined by pulse–chase experiments in the *STE6* deletion strain JPY201, transformed with Ste6-encoding plasmids. Essentially the same results were obtained with single-copy and multi-copy *STE6* plasmids. As can be seen from Figure 2, deletion of the A-box strongly stabilized Ste6 [calculated half-life (τ) = 68 min, compared with 14 min for wild-type Ste6]. Deletion of the B-box and the DAKTI sequence led to an ~2- to 3-fold stabilization of Ste6 (τ = 43 and 36 min).

Recent data by Hicke and Riezman (1996) suggested that the lysine residue in the DAKSS sequence of Ste2 acts as an acceptor for ubiquitin. Ubiquitination seems to trigger endocytosis and subsequent degradation of Ste2 in the vacuole. To test whether the lysine residue is crucial for the destabilizing effect of the DAKTI sequence in Ste6, we generated a lysine to arginine mutation (‘K612R’). This exchange, however, had only a modest effect on the half-life of Ste6 (τ = 22 min, not shown). The K612R mutation was constructed by site-directed mutagenesis of *STE6*,

Fig. 3. Halo-assay for *a*-factor activity. Serial dilutions (1:1, 1:2, 1:4 and 1:8) of culture supernatants of JPY201, transformed with different single copy *STE6* plasmids, were spotted onto a lawn of the *a*-factor supersensitive *ssr2* strain XMW1-10D: (1) YEp420, (2) pRK109, (3) pRK182, (4) pRK256, (5) pRK266, (6) pRK282, (7) pRK308, (8) pRK309. *a*-factor activity is seen as a zone of growth inhibition.

while the other deletion mutants were constructed by the insertion of PCR-generated cassettes into a modified *STE6* gene that contained additional restriction sites flanking the D-box-encoding sequences. The altered Ste6 protein (Ste6*), encoded by this modified *STE6* gene, behaved like wild-type Ste6, with respect to function, localization and turnover. To rule out that the additional restriction sites introduced into *STE6* contributed to the stabilizing effect of the DAKTI deletion, a new deletion was constructed by site-directed mutagenesis which removes only the DAKTI sequence. This 'DAKTI*' deletion was indistinguishable from the original DAKTI deletion ($\tau = 32$ min, not shown).

The A-box and DAKTI deletion mutants are fully functional

To rule out that the observed stabilization is due to misfolding of the mutant proteins, we measured the *a*-factor transport activity in the *STE6* deletion strain JPY201, transformed with single-copy plasmids encoding the different Ste6 variants. The amount of *a*-factor in the culture supernatants was determined by a halo-assay. Serial dilutions of the supernatants were spotted onto a lawn of an *a*-factor supersensitive *ssr2* strain (Figure 3). This strain has problems in recovering from pheromone-induced cell cycle arrest due to a defect in the adaptation response (Dietzel and Kurjan, 1987). A growth inhibitory effect of culture supernatants comparable with wild-type Ste6 was observed with the A-box and DAKTI deletion mutants and the K612R mutant, i.e. the supernatants of these mutants contained *a*-factor activity in amounts comparable with wild-type Ste6. No growth inhibition was seen with the vector control and the B-box deletion.

In a second assay, we measured the mating activity

of strain JPY201, transformed with the different *STE6* plasmids, by determining the frequency of zygote formation with a *MAT α* tester strain. Again, the mating activity was the same for wild-type Ste6 and the A-box and DAKTI deletion variants and the K612R mutant (not shown). No zygotes were observed for the vector control and the B-box deletion. These results show that the A-box and DAKTI deletion mutants are fully functional, in contrast to the B-box deletion which is completely inactive. The lack of activity and an aberrant intracellular localization of the Ste6 Δ B-box protein (see below) suggest that the stabilizing effect of the B-box deletion is indeed due to misfolding of the protein and degradation of the protein by a proteolytic pathway 'slower' than the normal Ste6 degradative pathway. The A-box and DAKTI deletions, however, appear specifically to affect the turnover of Ste6.

Ste6 turnover is unaffected in proteasome mutants

The finding that Ste6 is stabilized ~3-fold in a *ubc4 ubc5* mutant (Seufert and Jentsch, 1990) suggested that the ubiquitin system is involved in the degradation of Ste6 (Kölling and Hollenberg, 1994). Ubiquitination could directly target Ste6 for degradation by the proteasome or could have an indirect effect on the turnover of the protein by affecting Ste6 trafficking. To test whether the proteasome is involved directly in the degradation of Ste6, we determined the half-life of Ste6 in proteasome mutants by pulse-chase experiments. A congenic set of strains, consisting of the wild-type strain WCG4a, the *pre1-1* strain WCG4-11a and the *pre1-1 pre2-2* strain WCG4-11/22a (Heinemeyer *et al.*, 1991, 1993), was transformed with a multi-copy *STE6* plasmid. Ste6 had about the same half-life in all three strains (wild-type, 31 min; *pre1-1*, 27 min; *pre1-1 pre2-2*, 27 min; not shown). These data argue against a role for the proteasome in Ste6 degradation. The dramatic stabilization of Ste6 in a vacuolar *pep4* mutant (Berkower *et al.*, 1994; Kölling and Hollenberg, 1994) also suggests that the proteasome is not directly involved in the degradation of Ste6.

The Ste6 Δ A-box mutant is no longer ubiquitinated

Wild-type Ste6 accumulates in a highly ubiquitinated form upon block of endocytosis (Kölling and Hollenberg, 1994). We were interested to see whether the stabilization observed with the Δ A-box and Δ DAKTI mutants was correlated with a change in the ubiquitination pattern. To facilitate detection of Ste6 ubiquitination, we made use of a hemagglutinin (HA)-tagged ubiquitin variant. The *end4 Δ ste6* strain RKY592 was transformed with the multi-copy plasmid YEp112 (Hochstrasser *et al.*, 1991), encoding the HA-tagged ubiquitin under the control of the *CUP1* promoter, and with multi-copy plasmids encoding different Ste6 variants marked with a c-myc epitope tag. The strains were grown for several generations at 25°C in the presence of copper to induce expression of HA-ubiquitin. After the cells had been shifted to restrictive temperature (37°C) for 30 min to block endocytosis, cell extracts were prepared. Proteins immunoprecipitated from these extracts by Ste6 antibodies were analyzed by Western blotting with anti-myc antibodies to detect Ste6 and with anti-HA antibodies to detect HA-ubiquitin covalently

Fig. 4. Ubiquitination of Ste6 variants. The *end4 Δste6* strain RKY592 was transformed with YEp112, expressing an HA-tagged ubiquitin variant (Hochstrasser *et al.*, 1991), and with a plasmid expressing a c-myc-tagged Ste6 variant. The *STE6* plasmids were: (1) no plasmid, (2) pRK257, (3) pRK264, (4) pRK281, (5) pRK310 and (6) pRK311. The proteins immunoprecipitated from cell extracts with anti-Ste6 antibodies were analyzed by Western blotting with the anti-myc antibody 9E10 (A). The blot was 'stripped' and reprobed with the anti-HA antibody 12CA5 (B).

attached to Ste6. As can be seen from Figure 4A, all Ste6 variants were detectable in the anti-Ste6 immunoprecipitates. Ubiquitination of Ste6 is detected as a high molecular weight 'smear' on the anti-HA Western blot. A ubiquitin smear was detected for all variants examined, except for the Δ A-box mutant (Figure 4B). Thus, in contrast to wild-type Ste6, the Δ A-box mutant does not seem to be ubiquitinated. Also, we consistently observed that ubiquitination was reduced compared with wild-type in the two DAKTI deletion variants. The K612R mutant behaved more or less like wild-type. An exact quantification of the results is difficult, since the expression level of HA-ubiquitin was somewhat variable between different experiments due to fluctuations in copy number of the 2μ vectors. However, in all experiments performed so far, we never observed ubiquitination of the Δ A-box mutant and we consistently saw less ubiquitination of the Δ DAKTI mutants compared with wild-type Ste6.

Intracellular distribution of ubiquitination-deficient Ste6 mutants

Ubiquitination seems to act as an endocytosis signal for the α -factor receptor Ste2 (Hicke and Riezman, 1996). To see whether ubiquitination similarly affects the localization of Ste6, we compared the intracellular distribution of the ubiquitination-deficient Ste6 mutants with the distribution of wild-type Ste6. The intracellular localization of myc-tagged Ste6 variants, expressed from multi-copy plasmids, was examined by immunofluorescence microscopy. Ste6 was detected with anti-myc antibodies and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies. We noticed that the observed Ste6 immunofluorescence staining was sensitive to the fixation conditions. Fixation according to the standard protocol (Pringle *et al.*, 1989) for 2 h at 30°C gave rise to the patchy, Golgi-like staining pattern, as reported previously (Kölling and Hollenberg, 1994); longer fixation (4 h, 30°C), however, revealed a vacuolar staining (Figure 5A). The Ste6 staining surrounded the vacuoles, which can be identified as white spots in the phase contrast image. In some cells, a few dots close to the vacuole were observed. These dots were somewhat more obvious in the K612R mutant (Figure 5E). In the Δ A-box (Figure 5B) and Δ DAKTI mutants (Figure 5C and D), we observed a pronounced cell surface staining, which was more intense in the bud than in the

Fig. 5. Localization of Ste6 variants by immunofluorescence. The c-myc-tagged Ste6 variants, expressed in strain JPY201, were detected with anti-myc primary antibodies and FITC-conjugated anti-mouse secondary antibodies. *STE6* plasmids: (A) pRK257, (B) pRK264, (C) pRK281, (D) pRK310, (E) pRK311, (F) YEp420. Left panels: FITC staining, right panels: phase contrast image.

mother cell. The surface staining was brighter in the Δ A-box mutant than in the Δ DAKTI mutants. These data show that the amount of Ste6 in the plasma membrane is higher in the Δ A-box and Δ DAKTI deletion mutants compared with wild-type.

To acquire more quantitative information about the intracellular distribution of the Ste6 variants, we performed cell fractionation experiments. Cell extracts of JPY201 Δ ste6, transformed with single-copy plasmids encoding the different Ste6 variants, were fractionated on sucrose density gradients. A thorough characterization of these types of gradients has been presented in a previous report (Kölling and Hollenberg, 1994). The plasma membrane marker Pma1 (Serrano *et al.*, 1986), which is very well separated from internal membranes on these gradients, was found mainly at high sucrose densities (fractions 15–18, Figure 6A). The main peak of Ste6 was found in the middle of the gradient around fraction 11 (Figure 6C). Only a small amount of wild-type Ste6 could be detected in the plasma membrane fraction. Essentially the same pattern was seen with the Δ A-box and Δ DAKTI variants, i.e. the main peak in fraction 11 and some Ste6 in the plasma membrane fraction. However, it is clear that the

Fig. 6. Fractionation of Ste6 variants on sucrose gradients. Whole cell extracts of strain JPY201, transformed with different *STE6* plasmids, were fractionated by density gradient centrifugation (20–50% sucrose, w/w, lowest density in fraction 1). Aliquots of the gradient fractions were analyzed by SDS–PAGE and Western blotting. The *STE6* plasmids were: (A, B and C) pRK109, (D) pRK256, (E) pRK282, (F) pRK266. (A) Anti-Pma1 blot, (B) anti-Vph1 blot, (C–F) anti-Ste6 blots.

fraction of Ste6 ΔA-box (Figure 6D) and Ste6 ΔDAKTI (Figure 6E) in the plasma membrane is significantly higher than with wild-type Ste6. (A densitometric quantification of these results is presented in Figure 7.) The Ste6 ΔB-box mutant fractionated very differently from the other variants (peak in fraction 6, Figure 6F), supporting the view that it represents a misfolded protein which is probably retained in the ER. The Ste6 distribution overlaps but does not exactly coincide with the distribution of the vacuolar marker Vph1 (Manolson *et al.*, 1992) (peak in fraction 10, Figure 6B). This result is compatible with the view that part of Ste6, as suggested by the immunofluorescence experiments, is located in the vacuolar membrane.

Our experiments show that the ubiquitination-deficient Ste6 mutants accumulate at the plasma membrane. The two methods used to assess the cellular distribution of Ste6, immunofluorescence and sucrose gradient fractionation, however, give a different picture regarding the magnitude of this effect. While the surface staining of the ΔA-

Fig. 7. Fractionation of Ste6 variants on sucrose gradients. Densitometric quantification of the Ste6 signals in Figure 6.

Fig. 8. Fractionation of Ste6 by differential centrifugation. Cell extracts were prepared from ConA-coated spheroplasts and centrifuged at 3000 g for 15 min to pellet the plasma membranes (P3). The supernatant was spun again at 100 000 g for 1 h to pellet the internal membranes (P100). S100 = supernatant from the 100 000 g spin. Equal portions of the fractions were assayed for the presence of Ste6 (lanes 1–3) and Pma1 (lanes 4–6) by Western blotting. Extracts were prepared from (A) JPY201 Δste6 + pRK109 (Ste6*) and (B) JPY201 Δste6 + pRK256 (ΔA-box).

box and ΔDAKTI variants in the immunofluorescence experiments looks impressive, the sucrose gradients show only a comparatively small accumulation of the proteins in the plasma membrane fraction. To obtain additional information about the intracellular distribution of Ste6, we prepared plasma membranes by a ‘ConA precipitation’ experiment (Patton and Lester, 1991). Spheroplasts were coated with the lectin concanavalin A (ConA) which binds to mannoproteins at the cell surface. ConA-stabilized plasma membrane sheets can be recovered in the pellet fraction after a low speed spin. The low speed supernatant was spun again at 100 000 g for 1 h to pellet the remaining cellular membranes. As can be seen from Figure 8A and B, most of the plasma membrane marker Pma1 (76–90%) was found in the low speed pellet (P3) while only a small amount of Ste6* (13%) could be recovered in this fraction (Figure 8A). The amount of Ste6 ΔA-box in the P3 pellet, on the other hand, was substantially higher (Figure 8B). More than half of the protein (57%) was found in this fraction.

Taken together, our localization experiments show that

there is a substantial accumulation of the ubiquitin-deficient mutants at the plasma membrane, indicating that ubiquitination is important for efficient endocytosis of Ste6. However, the fractionation experiments also show that not all of the Δ A-box protein is present at the cell surface. A substantial fraction of the protein still appears to be associated with internal membranes.

The ubiquitination-deficient Ste6 mutants accumulate at the plasma membrane upon block of endocytosis

One reason why the ubiquitination-deficient mutants do not accumulate in large amounts at the plasma membrane could be that they are impaired in their transport to the plasma membrane. To test whether the mutants are able to reach the plasma membrane, the *end4* Δ ste6 strain RKY592 was transformed with single-copy plasmids encoding the Ste6 variants. The cells were grown at 25°C for several generations and were then shifted for 1 h to the restrictive temperature (37°C) to block endocytosis (Raths *et al.*, 1993). Cell extracts were prepared and fractionated on sucrose gradients. Both, Ste6 Δ A-box (Figure 9B) and Ste6 Δ DAKTI (Figure 9D) showed a pronounced accumulation in the plasma membrane fraction (fractions 15–18). The typical ‘ubiquitin-smear’ was seen for wild-type Ste6 (Figure 9A). This ‘smear’ was reduced in the Δ DAKTI mutant and was absent in the Δ A-box mutant, in agreement with the results of the ubiquitination experiment. The Δ B-box mutant did not accumulate in the plasma membrane (Figure 9C). These results show that the ubiquitination-deficient mutants are not impaired in their transport to the plasma membrane. This is a further indication that the deletion of the A-box and the DAKTI sequence does not disturb the overall structure or function of the protein.

In addition to the normal Ste6 band ≥ 140 kDa, a degradation band of ~ 55 kDa was observed. This band was virtually absent in the Δ A-box mutant and was not present at all in the Δ B-box mutant. It increased in intensity upon addition of trypsin (not shown). Thus, no specific proteolytic activity seems to be required to generate this Ste6 fragment. There is, however, a correlation between the ubiquitination status of Ste6 and the appearance of this band. One interpretation of this finding is that ubiquitination leads to a conformational change in Ste6 which exposes the proteinase-sensitive site. The proteolytic fragment must be derived from the C-terminus of Ste6, since the antibodies used to detect it were raised against a C-terminal Ste6 fragment. A C-terminal fragment with a calculated mol. wt of 55 kDa could be obtained by cleavage in the third intracellular loop of Ste6. A similar C-terminal proteolytic fragment has been described for human Mdr1 (Yoshimura *et al.*, 1989).

The D-box affects stability and localization of Pma1

The A-box and DAKTI deletions could affect the turnover of Ste6 either by removing a distinct degradation or transport signal or by changing the overall structure of the protein. If the D-box contains a distinct signal, it should in principle be transferable to another membrane protein. To test this prediction, we generated fusions between the plasma membrane protein Pma1 and the

Fig. 9. Distribution of Ste6 variants in the endocytosis mutant *end4*. Cell extracts of the *end4* Δ ste6 strain RKY592, transformed with different *STE6* plasmids, were fractionated on sucrose gradients (20–50% sucrose, w/w, lowest density in fraction 1). The cells were grown at 25°C and shifted to 37°C for 1 h prior to extract preparation. Aliquots of the gradient fractions were analyzed by SDS-PAGE and Western blotting with anti-Ste6 antibodies. The *STE6* plasmids were: (A) pRK109, (B) pRK256, (C) pRK266, (D) pRK282.

A-box, B-box and D-box sequences. Pma1 is an extremely stable protein with a reported half-life of 11 h (Benito *et al.*, 1991). The Ste6 sequences were fused to the C-terminus of an HA-tagged variant of Pma1. The stability of the fusions was analyzed by a gal-depletion experiment (Figure 10). Strain JD52, expressing the different fusions under the control of the *GAL1* promoter, was pre-grown on galactose medium, where the *GAL1* promoter is active. Then the cells were transferred to glucose medium to turn off transcription from the *GAL1* promoter. Samples were taken at various time intervals and analyzed by Western blotting with anti-HA antibodies. During the time course of the experiment, the amount of HA-Pma1 (Figure 10A) stayed more or less constant, in contrast to the amount of the HA-Pma1–D-box fusion protein which was rapidly diminished (Figure 10B). The estimated half-life of the HA-Pma1–D-box fusion is ~ 30 min. The half-life of Pma1 was not significantly affected by the A-box (Figure 10C) or the B-box (Figure 10D). This experiment shows that the D-box not only destabilizes Ste6 but also the foreign Pma1 protein. The A-box and B-box sequences alone,

Fig. 10. Stability of HA-Pma1 fusions. Strain JD52, transformed with different *PMA1* plasmids, was grown on galactose medium to induce expression of the Pma1 fusion proteins from the *GALI* promoter. At time $t = 0$ the cells were transferred to glucose medium. Cell extracts were prepared at the time intervals indicated and analyzed by SDS-PAGE and Western blotting with anti-HA antibodies. *PMA1* plasmids: (A) pRK315, (B) pRK318, (C) pRK319, (D) pRK320.

however, were not sufficient to cause a destabilization of Pma1.

Next, we were interested to see whether the localization of Pma1 was affected by the Ste6 sequences. The intracellular distribution of the fusion proteins was analyzed by cell fractionation. Cell extracts from fusion protein-expressing strains, grown on galactose medium, were fractionated on sucrose gradients. Again, the main portion of HA-Pma1 was found in the densest fractions of the gradient (fractions 15–18, Figure 11A). However, a substantial amount of HA-Pma1 was also observed in the middle of the gradient. This differs from what we have seen with wild-type Pma1 in glucose-grown cells (Figure 6A), where the internal pool of Pma1 represented only a minor fraction compared with the plasma membrane pool (fractions 15–18). The distribution pattern of Pma1 may vary with growth conditions (galactose versus glucose) or may be affected by the insertion of the HA-tag. The important point, however, is that the distribution of HA-Pma1–D-box (Figure 11B) is different from the distribution of HA-Pma1 (Figure 11A). Most of HA-Pma1–D-box is found in the internal pool and only a small amount is found in the plasma membrane fraction. Again, the complete D-box was required to see the effect, since neither A-box (Figure 11C) nor B-box (Figure 11D) alone affected the distribution of Pma1. The mostly intracellular localization of the Pma1–D-box suggests that the fusion protein is internalized efficiently by endocytosis. To exclude other possibilities, such as intracellular retention due to misfolding of the protein, it is important to

Fig. 11. Fractionation of Pma1 fusion proteins on sucrose gradients. Whole-cell extracts of strain JD52, transformed with different *PMA1* plasmids, were fractionated by density-gradient centrifugation (20–50% sucrose, w/w, lowest density in fraction 1). The gradient fractions were analyzed by SDS-PAGE and Western blotting with anti-HA antibodies. The *PMA1* plasmids were: (A) pRK315, (B) pRK318, (C) pRK319, (D) pRK320.

show that the protein still travels to the plasma membrane. Fractionation experiments with an *end4* mutant showed that the fusion protein accumulates at the plasma membrane upon block of endocytosis (not shown). The D-box, therefore, seems to specifically affect the half-life of Pma1 by stimulating endocytosis.

The diffuse ‘smear’ on top of the HA-Pma1–D-box fusion protein bands suggests that the fusion protein is ubiquitinated. To test this prediction, an experiment similar to the one described in Figure 4 was performed (Figure 12). Strain JD52 was transformed with two plasmids, one plasmid expressing either normal ubiquitin or c-myc-tagged ubiquitin and another plasmid expressing either HA-Pma1 or HA-Pma1–D-box. Proteins immunoprecipitated from cell extracts with anti-HA antibodies were assayed by Western blotting for the presence of HA-Pma1 with anti-HA antibodies (Figure 12A) and for the presence of c-myc-tagged ubiquitin, covalently attached to Pma1, with anti-c-myc antibodies (Figure 12B). On the c-myc blot a strong signal was detected for the HA-Pma1–D-box protein (Figure 12B, lane 4), demonstrating that this protein is ubiquitinated. A faint signal was also detected for the HA-Pma1 protein (Figure 12B, lane 2). HA-Pma1 alone, therefore, seems to be already ubiquitinated to a certain degree. This ubiquitination, however, is drastically enhanced by the addition of the D-box. No signals were obtained for the controls expressing only normal ubiquitin (Figure 12B, lanes 1 and 3).

Fig. 12. Ubiquitination of the HA-Pma1–D-box fusion. Strain JD52 was transformed with a plasmid expressing either normal ubiquitin (YEp96) or c-myc-tagged ubiquitin (YEp101⁺) (Hochstrasser *et al.*, 1991) and with a plasmid expressing either HA-tagged Pma1 (pRK315) or the HA-tagged Pma1–D-box fusion (pRK318). The plasmid combinations were: (1) YEp96, pRK315; (2) YEp105, pRK315; (3) YEp96, pRK318; (4) YEp105, pRK318. Proteins immunoprecipitated from cell extracts with anti-HA antibodies were analyzed by Western blotting with anti-HA antibodies (A). The blot was 'stripped' and reprobed with anti-c-myc antibodies (B).

Discussion

The Ste6 turnover signal

In this study, we show that the Ste6 linker region contains a signal which mediates ubiquitination and fast turnover of Ste6. Which part of the linker region forms the signal? The linker region is composed of an acidic half (A-box) and a basic half (B-box). Deletion of the acidic half has a strong impact on the stability of Ste6 and completely prevents ubiquitination of the protein. The A-box, therefore, either contains the turnover signal or is part of a larger signal which is inactivated upon deletion of the A-box. A potential candidate for a turnover signal is the sequence motif 'DAKTI' found in the A-box region. This sequence resembles the 'DAKSS' signal shown to be required for the endocytosis of a truncated Ste2 receptor (Rohrer *et al.*, 1993). Deletion of the DAKTI sequence indeed affects the turnover of Ste6. The stabilizing effect, however, is not as strong as the effect of the A-box deletion, indicating that the DAKTI sequence is only part of the signal. Alternatively, additional redundant signals may exist in the A-box region.

The lysine residue in the Ste2 DAKSS sequence has been shown to be essential for the internalization of a C-terminally truncated receptor (Rohrer *et al.*, 1993; Hicke and Riezman, 1996). An exchange of this lysine residue for arginine eliminates ubiquitination of the truncated receptor, supporting the view that ubiquitination triggers endocytosis of Ste2. A lysine to arginine mutation in the Ste6 DAKTI sequence, however, had only a minor effect on the Ste6 turnover ($\tau = 14 \text{ min} \rightarrow \tau = 22 \text{ min}$). Also, ubiquitination of Ste6 did not seem to be affected. However, we still cannot exclude that this lysine functions as an acceptor for ubiquitin. Apparently, the ubiquitination machinery seems to be able to use a number of alternative lysine residues as acceptors in the vicinity of a degradation signal (Kornitzer *et al.*, 1994). This seems to be true even for the Ste2 receptor, since the effect of the lysine mutation in the DAKSS sequence has only been demonstrated for a truncated version of Ste2 where the C-terminus, which contains several lysines, has been deleted.

That the B-box also contributes to ubiquitination and turnover of Ste6 is suggested by the analysis of the Pma1–Ste6 fusions. In contrast to the D-box, which caused a marked destabilization and ubiquitination of Pma1, the

Table 1. STE6 plasmids

Plasmid	Ste6 variant	Mutant type	Vector ^a
pRK109	Ste6*	PCR cassette	pRK182
pRK256	Δ A-box	PCR cassette	pRK182
pRK266	Δ B-box	PCR cassette	pRK182
pRK282	Δ DAKTI	PCR cassette	pRK278
pRK308	Δ DAKTI*	mutagenesis	pRK278
pRK309	K612R	mutagenesis	pRK278
pRK257	Ste6*	PCR cassette	pYKS1
pRK264	Δ A-box	PCR cassette	pYKS1
pRK265	Δ B-box	PCR cassette	pYKS1
pRK281	Δ DAKTI	PCR cassette	pYKS1
pRK310	Δ DAKTI*	mutagenesis	pYKS1
pRK311	K612R	mutagenesis	pYKS1

^apRK182 and pRK278 = single-copy plasmids, pYKS1 = multi-copy plasmid.

A-box alone was not sufficient to destabilize Pma1. The importance of the B-box for Ste6 turnover could not be evaluated by deletion analysis, since deletion of the B-box gave rise to a non-functional and probably misfolded protein. This is not totally unexpected, since deletion of the B-box places the A-box, which contains many negatively charged amino acids, just upstream of the first transmembrane span of the second half of Ste6. It has been shown that a net positive charge just upstream of a membrane-spanning segment is required for a N_{in}–C_{out} orientation (Gafvelin and von Heijne, 1994, a net negative charge in this position may, therefore, inverse the membrane orientation of the membrane span, which would change the membrane topology of the protein.

The role of Ste6 ubiquitination

Analysis of the different Ste6 variants established a correlation between the Ste6 half-life and the extent of ubiquitination. This correlation could be explained by postulating a role for ubiquitination in targeting Ste6 for degradation by the proteasome. The proteasome has indeed been implicated in the degradation of another ABC-transporter, the CFTR protein (Jensen *et al.*, 1995; Ward *et al.*, 1995). However, the finding that the half-life of Ste6 was unaffected by mutations in the proteasome subunits Pre1 and Pre2 (Heinemeyer *et al.*, 1991, 1993) argues against a role for the proteasome in Ste6 degradation. Moreover, previous experiments, which point to the vacuole as the major site of Ste6 degradation (Berkower *et al.*, 1994; Kölling and Hollenberg, 1994), provide a strong argument against a direct involvement of the proteasome in Ste6 turnover.

As described for Ste2, ubiquitination may also indirectly affect the turnover of Ste6 by inducing endocytosis and subsequent degradation in the vacuole (Hicke and Riezman, 1996). Our localization experiments show that the ubiquitination-deficient Ste6 mutants accumulate at the plasma membrane, suggesting that ubiquitination indeed stimulates endocytosis of Ste6. The different methods used to assess the cellular distribution of the Ste6 mutants give somewhat different results regarding the magnitude of this effect. The immunofluorescence experiment and the ConA experiment suggest that the loss of ubiquitination has a strong impact on the internalization of Ste6. The sucrose gradients, on the other hand, show only a compar-

Table II. Yeast strains

Strain	Genotype	Source
DBY2058	<i>MATα ura3-52 his4-619</i>	D.Botstein, Stanford
JD52	<i>MATα ura3-52 his3-Δ200 leu2-3,112 trp1-Δ63 lys2-801</i>	J.Dohmen, Düsseldorf
JPY201	<i>MATα ste6-Δ1::HIS3 gal2 his3-Δ200 leu2-3,112 lys2-801 trp1-1 ura3-52</i>	J.McGrath (McGrath and Varshavsky, 1989)
RKY592	<i>ura3 trp1ste6-Δ1::HIS3 end4 his3 leu2 gal</i>	backcrossed from strain RH 268-1C (Raths <i>et al.</i> , 1993)
WCG4a	<i>MATα ura3 leu2-3,112 his3-11,15 CANs</i>	W.Heinemeyer, Stuttgart
WCG4-11a	<i>MATα pre1-1 ura3 leu2-3,112 his3-11,15 CANs</i>	W.Heinemeyer, Stuttgart
WCG4-11/22a	<i>MATα pre1-1 pre2-2 ura3 leu2-3,112 his3-11,15 CANs</i>	W.Heinemeyer, Stuttgart
XMW1-10D	<i>MATα cry1-11 thr4 ssl2-1</i>	W.Duntze, Bochum

atively small accumulation of the ubiquitination-deficient proteins in the plasma membrane fraction. The plasma membrane fraction on the sucrose gradients, however, is only operationally defined as the fraction where Pma1, the plasma membrane ATPase, is located. It is conceivable that the plasma membrane consists of subdomains which do not fractionate together. It could well be possible, therefore, that a fraction of Ste6 is localized to plasma membrane subdomains, e.g. domains actively involved in endocytosis, which do not contain Pma1. Separation of plasma membrane subdomains should not occur in the ConA precipitation experiment. Therefore, the ConA precipitation experiment would be expected to give a more accurate picture of the amount of Ste6 at the cell surface.

Although all localization experiments show that the ubiquitination-deficient Ste6 Δ A-box mutant accumulates at the cell surface, it is also clear that a substantial fraction of the protein is still associated with internal membranes. If endocytosis of the mutant protein from the plasma membrane were the only rate-limiting step in the degradation pathway, most of Ste6 Δ A-box should be found in the plasma membrane. There are several possible explanations why not all of the ubiquitination-deficient Ste6 protein is found in the plasma membrane. One explanation is that endocytosis still occurs in the absence of ubiquitination, albeit at a slower rate. Another possibility is that not all of the Ste6 protein reaches the vacuole via the plasma membrane. A certain fraction of the protein could travel directly from the Golgi to the vacuole via a pre-vacuolar compartment. The finding that Ste6 is stabilized only 3-fold in late secretory mutants is in line with this interpretation. A third possibility is that Ste6 ubiquitination stimulates another internal transport step which becomes rate-limiting for the Ste6 Δ A-box protein due to the absence of ubiquitination.

Materials and methods

Plasmids and yeast strains

To facilitate the construction of *STE6* linker mutants, additional *Bam*HI sites were introduced by PCR at the end of repeat 1 of *STE6*, 5'-...AATGAC (position 2289) GGATCC-3' and at the beginning of repeat 2 of *STE6*, 5'-GGATCC (position 2617) TAATC...-3' (for sequence positions see Kuchler *et al.*, 1989; the original *STE6* sequences are in bold print). The following PCR-generated cassettes with one *Bam*HI and one *Bgl*II end were inserted between the two *Bam*HI sites. Ste6*, 5'-GGATCCATAATGT (position 2293) CTGAT... CAAAAA (position 2612) GATCT-3'; Δ A-box, 5'-GGATCC (position 2452) AAAAA... CAAAAA (position 2612) GATCT-3'; Δ B-box, 5'-GGATCCATAATGT (position 2293) CTGAT... GCAATC (position 2448) CAGATCT-3'; Δ DAKTI, 5'-GGATCCG (position 2311) TAGAT... CAAAAA (position 2612) GATCT-3'.

Site-directed mutagenesis was performed with the Bio-Rad Muta-Gene™ kit based on the method of Kunkel *et al.* (1987). A 1.2 kb internal *Pst*I *STE6* fragment (position 1620–2830) was mutagenized with the following mutagenic primers (exchanges marked in bold print): Δ DAKTI*, 5'-C (position 2278) TACAGAATGACTACTCTGTGCG-ACACAGAGACTGAAGAAAAATC; K612R, 5'-G (position 2283) AATGACTACTCTGTGCGAGAACGATCGTAGATACAGAGACTG-AAG. The mutations were confirmed by sequencing.

The 1.2 kb internal *Pst*I fragments, modified by mutagenesis or by insertion of the PCR cassettes, were cloned into pRK182, pRK278 and pYKS1 (Kuchler *et al.*, 1993), as summarized in Table I. The plasmids pRK182 and pRK278 are based on the single-copy vectors YCp50 (Rose *et al.*, 1987) and YCplac33 (Gietz and Sugino, 1988) and contain a 6.2 kb *Bgl*III–*Sall* *STE6* fragment. pYKS1 is a multi-copy vector, based on YEpl352, containing a c-myc-tagged *STE6* gene (Kuchler *et al.*, 1993).

Plasmid pRK315, based on the single copy vector YCp50, encodes a modified *PMA1* gene under the control of the *GAL1* promoter. The N-terminal part of *PMA1*, containing an HA tag, was derived from pXZ28 (kindly provided by Jim Haber). Two modifications were introduced into the C-terminal part, which was generated by assembly PCR. First, the internal *Bam*HI site (position 2535; Serrano *et al.*, 1986) was removed, without changing the amino acid sequence and, second, a new *Bam*HI site was placed behind the last codon [5'...GAAACC (position 3690) GGATCC-3']. Furthermore, stop codons in all three reading frames were placed downstream from the *Bam*HI site. In-frame fusions with the *STE6* D-box (pRK318), A-box (pRK319) and B-box (pRK320) were generated by inserting the PCR fragments described above into the C-terminal *Bam*HI site of the modified *PMA1* gene.

The yeast strains used are listed in Table II.

Pulse-chase experiments and immunoprecipitation

The procedure for the pulse-chase experiments and immunoprecipitation has been described in detail previously (Kölling and Hollenberg, 1994). Aliquots corresponding to ≈ 1 OD₆₀₀ unit cells were removed at 20 min intervals and lysed by vortexing with glass beads for 3 min. The relative amount of Ste6 at each time point was quantified by scanning of autoradiograms with a Howtek Scanmaster 3 scanner and analysis with the Imagemaster 1D software.

Quantitative mating assay and halo-assay

To determine the mating activity, equal numbers (1×10^7) of the exponentially growing *MAT α* strain JPY201, transformed with different Ste6 plasmids, and the *MAT α* tester strain DBY2058 were mixed. The cells were pelleted, overlaid with 5 ml of YPD medium and incubated for 4 h at 30°C. Aliquots of serial dilutions were plated on rich medium, to determine the total cell number and on selective plates allowing only for growth of the zygotes. The fraction of cells which were able to grow on the selective plates was determined. The frequency of zygote formation for a wild-type *MAT α* mating partner was usually $\sim 10\%$. For the *Δste6* *MAT α* strain JPY201 no zygotes were detected among 5×10^6 cells (frequency $< 2 \times 10^{-7}$).

For the halo-assay, overnight cultures of JPY201, transformed with different Ste6 plasmids, were diluted to an OD₆₀₀ = 0.2 in SD + casamino acids medium and were grown for 4 h at 30°C. The culture supernatants were spotted onto a lawn of *ssl2* (= *ssl2*) cells (2×10^6 cells of strain XMW1-10D in 3 ml of YPD top agar poured onto a YPD plate) using a 32-point inoculator ('frogger').

Immunofluorescence experiments

The immunofluorescence experiments were performed essentially as described in Pringle *et al.* (1989). Cells were grown to exponential

phase ($A_{600} = 0.5\text{--}0.8$, $3\text{--}4 \times 10^7/\text{ml}$) and fixed directly for 4 h with formaldehyde (final 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% polylysine (Sigma). The cells were first incubated with the anti-c-myc mouse monoclonal primary antibody (9E10, Berkeley Antibody Co, Inc.) (1:200 dilution in PBS + 1 mg/ml BSA) for 90 min and then another 90 min with FITC-conjugated anti-mouse secondary antibodies (Dianova, 1:300 dilution in PBS/BSA). Finally, the cells were incubated for 5 min with 4,6 -diamidino-2-phenylindole (DAPI) (1 mg/ml). The cells were examined with a Zeiss Axioskop and photographed with Ilford FP4 black and white film.

Fractionation experiments

The sucrose density gradient fractionation was performed, according to a protocol obtained from R.Serrano, as described previously (Kölling and Hollenberg, 1994).

The ConA precipitation experiment is based on a protocol by Patton and Lester (1991). Forty A_{600} units of cells from an exponentially growing culture ($A_{600} = 0.4$, $2 \times 10^7/\text{ml}$) were harvested, washed in H_2O and incubated in pre-treatment buffer (100 mM EDTA, 0.5% β -mercaptoethanol, 10 mM Tris-HCl, pH 7.5) at 4 ml/g cells (wet weight) for 20 min at 30°C. Then 10 mM NaN_3 was added to the culture and subsequently to the buffers to stop ATP-dependent transport processes. The cells were then washed and resuspended in S buffer (1.2 M sorbitol, 0.5 mM MgCl_2 , 40 mM HEPES, pH 7.5) to 4 ml/g cells and spheroplasted by the addition of 50 μg of oxalyticase (Enzogenetics) for 45–60 min at 30°C. Spheroplasts were centrifuged through a 5 ml cushion of $1.7 \times$ S buffer for 5 min at 500 g. The spheroplasts were washed in 5 ml of S buffer and in 5 ml of ConA buffer (1.2 M sorbitol, 1 mM Mg acetate, 1 mM CaCl_2 , 1 mM MnCl_2 , 50 mM Tris-HCl, pH 7.5) and were carefully resuspended in ConA buffer at 20 ml/g cells. One volume of ConA buffer containing ConA (3.5 mg/g cells) was added dropwise while gently stirring at room temperature. After addition of ConA, the stirring was continued for another 10 min. Then the spheroplasts were harvested by a 5 min centrifugation at 500 g, washed twice in ConA buffer and resuspended in cold lysis buffer [2 mM EDTA, 1 mM dithiothreitol (DTT), 5 mM HEPES, pH 7.5] at 50 ml/g cells. The lysis buffer contained the following protease inhibitors: 0.5 $\mu\text{g}/\text{ml}$ each of aprotinin, antipain, chymostatin, leupeptin, pepstatin A, 1.6 $\mu\text{g}/\text{ml}$ benzamidin, 1 $\mu\text{g}/\text{ml}$ phenanthroline, 170 $\mu\text{g}/\text{ml}$ phenylmethylsulfonyl fluoride. The following steps were performed at 4°C. The spheroplasts were lysed with 20 strokes in a Dounce homogenizer using a tight fitted pestle. One ml of the homogenate was transferred to a microcentrifuge tube and centrifuged twice for 5 min at 100 g. The supernatant was centrifuged for 15 min at 3000 g. The plasma membrane-containing pellet (P3) was then resuspended in 500 μl of storage buffer (20% glycerol, 1 mM DTT, 0.1 mM EDTA, 10 mM Tris-HCl, pH 7.5). The supernatant was again centrifuged for 1 h at 100 000 g (45 000 r.p.m. in a Beckman TLA45 rotor). The pellet (P100) was again resuspended in storage buffer.

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