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Ubiquitin Lys63 is involved in ubiquitination of a yeast plasma membrane protein

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We have recently reported that the yeast plasma membrane uracil permease undergoes cell-surface ubiquitination, which is dependent on the Npi1/Rsp5 ubiquitin–protein ligase. Ubiquitination of this permease, like that of some other transporters and receptors, signals endocytosis of the protein, leading to its subsequent vacuolar degradation. This process does not involve the proteasome, which binds and degrades ubiquitin–protein conjugates carrying Lys48-linked ubiquitin chains. The data presented here show that ubiquitination and endocytosis of uracil permease are impaired in yeast cells lacking the Doa4p ubiquitin–isopeptidase. Both processes were rescued by overexpression of wild-type ubiquitin. Mutant ubiquitins carrying Lys→Arg mutations at Lys29 and Lys48 restored normal permease ubiquitination. In contrast, a ubiquitin mutated at Lys63 did not restore permease polyubiquitination. Ubiquitin–permease conjugates are therefore extended through the Lys63 of ubiquitin. When polyubiquitination through Lys63 is blocked, the permease still undergoes endocytosis, but at a reduced rate. We have thus identified a natural target of Lys63-linked ubiquitin chains. We have also shown that monoubiquitination is sufficient to induce permease endocytosis, but that Lys63-linked ubiquitin chains appear to stimulate this process.

Keywords: endocytosis/Lys63 chains/transporter/ubiquitin/yeast

Introduction

The covalent linkage of ubiquitin (Ub) to substrate proteins is a common means used by eukaryotic cells to signal their degradation by the 26S proteasome (Ciechanover, 1994). Ub molecules are transferred to lysine residues of target proteins via an E1–E2–E3 enzyme thioester cascade (Ub-activating enzyme/Ub-conjugating enzyme/Ub–protein ligase). The acceptor protein usually undergoes polyubiquitination, resulting in chains in which the carboxy-terminus of each Ub is linked to Lys48 of the preceding Ub (Hershko and Ciechanover, 1992). This type of chain binds to the human proteasome subunit 5a (Deveraux *et al.*, 1994; Baboshina and Haas, 1996). Ub-conjugates are then degraded to small peptides, while Ub is released in a free, functional form, by proteasome-associated Ub–isopeptidases (Hershko and Ciechanover, 1992). Biochemical and genetic evidence indicate that the Ub/proteasome pathway is involved in the degradation of abnormal proteins, and regulatory proteins with short half-lives (Hochstrasser, 1996b).

It has long been known that ubiquitination does more than simply signal degradation by the proteasome. Histone H2A is a stable protein, despite being conjugated to Ub (Rechsteiner, 1988). A number of cell surface mammalian receptors are also modified by Ub but, until recently, the function of the modification has remained obscure (for review, see Ciechanover, 1994). It was reported that the yeast ABC transporter, Ste6p, accumulates in a ubiquitinated form in an endocytosis mutant, suggesting that ubiquitination is involved in Ste6p endocytosis (Kölling and Hollenberg, 1994). More recent work on certain receptors and transporters in yeast and mammals indicates that ubiquitination might indeed function as a signal triggering endocytosis of these proteins (Egner and Kuchler, 1996; Galan *et al.*, 1996; Hicke and Riezman, 1996; Roth and Davis, 1996; Strous *et al.*, 1996; Kölling and Losko, 1997). The receptors of the pheromones **a** and α in *Saccharomyces cerevisiae* undergo ligand-induced ubiquitination. Deficient ubiquitination due to either mutations within the receptor, or the depletion of Ub greatly impairs the endocytosis of these receptors (Hicke and Riezman, 1996; Roth and Davis, 1996). Chinese hamster ovary cells, which have a thermolabile E1, are defective for both ubiquitination and endocytosis of the growth hormone receptor (Strous *et al.*, 1996). These proteins are degraded in the lysosome/vacuole, without the involvement of the proteasome. This raises the question of how the ubiquitination of these proteins triggers their internalization, rather than their recognition by the proteasome. It is not simply because they are membrane-bound proteins. The CFTR is a transmembrane protein. Its mutant form commonly found in cystic fibrosis patients, is multiubiquitinated when in the endoplasmic reticulum, and then degraded by the proteasome (Ward *et al.*, 1995). Certain cell-surface tyrosine-kinase receptors would also be degraded by the proteasome (Mori *et al.*, 1995; Jeffers *et al.*, 1997). It has been suggested that the fate of proteins undergoing Ub-dependent endocytosis could be determined by monoubiquitination, as opposed to the multiubiquitination required for recognition by the proteasome (Hicke and Riezman, 1996), or by polyubiquitination involving a specific type of Ub chains (Hochstrasser, 1996a). There is now considerable evidence supporting the existence of multiubiquitin chains bearing linkage specificities distinct from Lys48. Multiubiquitin chains linked through Lys6 and Lys11 of Ub have been observed *in vitro* (Baboshina and Haas, 1996), as well as multi-Ub chain assembly that utilize non-lysine linkages (Hodgins *et al.*, 1996). In yeast, Ub molecules appear to be linked together through Lys29 and Lys63 in addition to Lys48 (Arnason and Ellison, 1994). Ubiquitination at Ub Lys29

has been shown to play a role in the Ub fusion degradation pathway (UFD), for which physiological substrates have not yet been identified (Johnson *et al.*, 1995). Genetic evidence indicates the involvement of Lys63-linked Ub chains in stress response (Arnason and Ellison, 1994) and DNA repair (Spence *et al.*, 1995). Ub–protein conjugates carrying Lys63-linked polyubiquitin chains have been reported, but the corresponding protein moieties have not yet been identified (Spence *et al.*, 1995).

We have used the plasma membrane uracil permease of *S.cerevisiae* to characterize the type of ubiquitination required for endocytosis. This transporter is targeted, via the secretory pathway, to the plasma membrane (Jund *et al.*, 1988), where it undergoes phosphorylation on multiple serines (Volland *et al.*, 1992). It has a constitutive turnover which is accelerated under several stress conditions, such as nutrient starvation, heat stress or the inhibition of protein synthesis (Volland *et al.*, 1994). Both basal and stress-stimulated degradation of the permease are triggered by its ubiquitination depending on the Npi1p/Rsp5p ubiquitin–protein ligase (Galan *et al.*, 1996). This protein belongs to a class of E3s defined by a domain homologous to the human E6AP carboxy-terminus (hect domain) (Hein *et al.*, 1995; Huibregtse *et al.*, 1995). Five members of this class have been identified in yeast (Hochstrasser, 1996b). We show in this report that permease ubiquitination and endocytosis are defective in mutant cells lacking the Doa4p Ub-isopeptidase. This phenotype could be complemented with an overproduction of Ub. We therefore systematically expressed in Δ*doa4* cells mutant Ubs carrying Lys→Arg mutations preventing the formation of various kinds of Ub chain. This allowed us to show that Ub–permease conjugates are extended through the Lys63 of Ub. When formation of this type of chain is blocked, permease endocytosis is reduced, suggesting that Lys63-linked Ub chains stimulate endocytosis.

Results

Impaired permease ubiquitination and endocytosis in mutant cells lacking the Doa4p deubiquitinating enzyme

S.cerevisiae contains numerous Ub-isopeptidases. No significant phenotype has been reported in cells lacking a number of these enzymes (Ciechanover, 1994; Hochstrasser, 1996b). In contrast, the Doa4p Ub-isopeptidase, which is homologous to the human tre2 oncogene, has been shown to have a key role in Ub-dependent degradation *in vivo* (Papa and Hochstrasser, 1993). Cells in which the *DOA4* gene is disrupted are viable, but display strong inhibition of the turnover of several soluble substrates of the Ub pathway. They accumulate low-molecular mass ubiquitinated species, with a concomitant loss of larger conjugates (Papa and Hochstrasser, 1993; Hochstrasser, 1996b). We therefore set out to determine whether disruption of the *DOA4* gene would affect the Ub-dependent endocytosis of uracil permease. We compared the rates of uracil permease turnover in cells lacking the *DOA4* gene and in isogenic wild-type cells after inhibition of protein synthesis. The permease activity, corresponding to plasma membrane-located protein, decreased rapidly when cycloheximide was added to control wild-type cells ($t_{1/2}$ = 23 min; Figure 1A). There

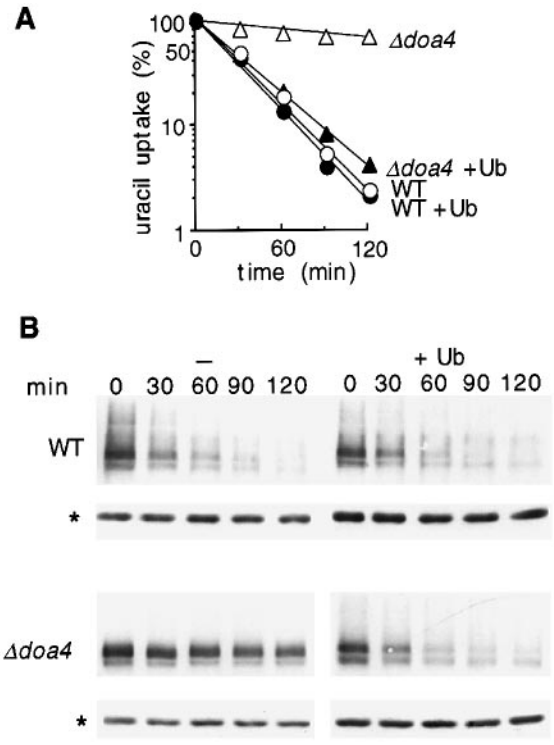

Fig. 1. Stabilization of uracil permease in Δ*doa4* cells is reversed by overexpression of Ub. MHY501 (WT), and MHY623 (Δ*doa4)* cells transformed with YEp352fF (2 μ *URA3 FUR4*) or transformed by both YEp352fF and YEp96 (2 μ *TRP1* Ub) plasmids, were grown as described in Materials and methods. During logarithmic growth, $CuSO_4$ (0.1 mM) was added to the medium to induce Ub synthesis from the *CUP1* promoter. Cycloheximide (100 μg/ml) was added 30 min later. (**A**) Uracil uptake was measured at the times indicated. Results are percentages of initial activities. The values are averages of at least three independent experiments, with standard deviations <5%. (**B**) Protein extracts were prepared at the times indicated. Proteins from 0.2 ml culture were analysed for uracil permease by Western immunoblotting. Permease appeared in the total extracts as several bands corresponding to the various phosphorylated states of uracil permease (Volland *et al.*, 1992). Detection of an unspecific band (✷) which remained stable during the experiment shows that all the lanes were equally loaded.

was a parallel drop in immunodetected permease (Figure 1B). In contrast, the amounts of immunodetected protein in Δ*doa4* cells hardly changed for 2 h after incubation with cycloheximide. Moreover, mutant cells exhibited protection against stress-induced loss of uracil uptake ($t_{1/2}$ = 150 min compared with 23 min in wild-type cells), indicating that the permease was stabilized at the plasma membrane. Disruption of the *DOA4* gene therefore strongly impaired permease internalization. The protection (~6-fold) is reminiscent of the protection provided against Ub-dependent degradation of soluble substrates of the Ub pathway (Papa and Hochstrasser, 1993).

The proteolysis deficiency in some mutants lacking Ub-isopeptidases can be rescued by overexpression of Ub (Baker *et al.*, 1995). Wild-type and Δ*doa4* cells were transformed with a multicopy plasmid encoding a synthetic Ub gene under the control of the *CUP1* inducible promoter. Overexpression of Ub did not affect permease turnover in wild-type cells ($t_{1/2}$ = 22 min), but overexpression of Ub for only 30 min produced a wild-type permease turnover ($t_{1/2}$ = 26 min) in Δ*doa4* cells. These data strongly suggest that the permease is stabilized in Δ*doa4* cells because

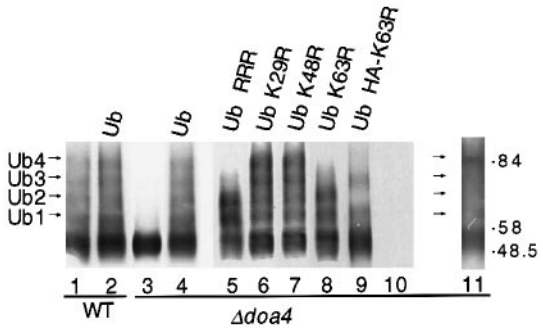

Fig. 2. Effect of the overexpression of wild-type and mutant Ubs on the ubiquitination of uracil permease in wild-type and Δ*doa4* cells. Lanes 1–10: MHY623 (Δ*doa4*) and MHY501 (WT) cells transformed with YEp352fF (2 μ *URA3 FUR4*) alone (lanes 1 and 3), or transformed with YEp352fF plus YEp96 (2 μ *TRP1* Ub*)* (lanes 2 and 4), YEp352fF plus plasmids encoding the various mutant Ubs (lanes 5–9), or YEp96 alone (lane 10) were grown as described in Figure 1. Cells were collected 30 min after adding $CuSO_4$ and used to prepare plasma membrane-enriched fractions. Aliquots were analysed for uracil permease by Western immunoblotting. The molecular masses of the markers are given in kDa. Arrows indicate mono- (Ub1), di- (Ub2), tri- (Ub3) and tetra- (Ub4) ubiquitinated permease. Chromosomal-encoded permease was strictly undetectable in membrane fractions prepared from MHY501 cells (lane 10). Lane 11: MHY623 cells (Δ*doa4*) transformed with YEp352fF and YEp96 in exponential growth were supplemented with $CuSO_4$ (0.1 mM) for 30 min. Cycloheximide (100 μg/ml) was then added. An aliquot withdrawn 30 min later was used to prepare a plasma membrane-enriched fraction, which was analysed for uracil permease by Western immunoblotting.

these cells lack free Ub after inhibition of protein synthesis. The above experiment was performed in cells overexpressing uracil permease from a multicopy plasmid (see Materials and methods) in order to allow immunological detection of the transporter. In Δ*doa4* cells synthesizing only chromosomal-encoded permease, a strong stabilization of uracil uptake activity reversed by overproduction of Ub was also observed after inhibition of protein synthesis (data not shown), showing that the impairment of permease turnover in Δ*doa4* cells was not a consequence of its overexpression.

We monitored the ubiquitination of uracil permease in Δ*doa4* and wild-type cells. In plasma membrane-enriched fractions prepared from exponentially growing cells, there are small amounts of Ub–permease conjugates, and their relative amounts are enriched in mutants that are deficient for the internalization step of endocytosis (Galan *et al.*, 1996). Western blot analysis of a plasma membrane-enriched fraction from wild-type cells with or without Ub overexpression (Figure 2, lanes 2 and 1, respectively) showed a ladder of four minor bands with molecular weights of ~65, 72, 78 and 85 kDa, in addition to the main permease band. These bands are believed to be mono-, di-, tri- and tetra-ubiquitinated permease, depending on their size. Membranes prepared from Δ*doa4* cells had no Ub–permease conjugates (Figure 2, lane 3). Overproduction of Ub for only 30 min induced the formation of Ub–permease conjugates in Δ*doa4* cells, with the same pattern as those in wild-type cells (Figure 2, lane 4). Thus, the endocytosis of uracil permease is strongly impaired in Δ*doa4* cells because they are deficient in the ubiquitination of the protein. This, in turn, appears to be due to the small amount of Ub available in these cells. The possibility to trigger the formation of Ub–permease conjugates by copper induction of Ub in Δ*doa4* cells enabled us to check possible modifications in the pattern of permease ubiquitination under conditions accelerating permease degradation. Cycloheximide was added 30 min after the addition of copper to Δ*doa4* cells. After 30 min of treatment, cells were collected and used to prepare plasma membrane-enriched fraction. Under these conditions, which trigger ~50% permease degradation (see Figure 1), we observed an enrichment of tetra-ubiquitinated permease species (Figure 2, lane 11). Transient enrichment of these particular Ub–permease species was also occasionally observed in membranes of wild-type cells submitted to various stress conditions that induce sudden acceleration of permease turnover (data not shown). Therefore, uracil permease seems to undergo progressive polyubiquitination (at least up to tetra-ubiquitination) before endocytosis.

Ub–permease conjugates are extended through Ub Lys63

The ability to complement Δ*doa4* cells with plasmid-encoded Ub has provided a powerful tool to check the incorporation of different Ub moieties into target proteins. We used these cells to test the effect of various mutated Ubs on uracil permease ubiquitination. A variant Ub in which Lys29, Lys48 and Lys63 are replaced by Arg (UbRRR) does not promote multiubiquitin chains *in vivo* (Arnason and Ellison, 1994). Overexpression of UbRRR in Δ*doa4* cells caused the accumulation of mono- and di-ubiquitinated permease, but not that of higher molecular weight Ub–permease conjugates (Figure 2A, lane 5). This suggests that uracil permease is ubiquitinated on at least two lysine residues. The presence of tri- and tetra-Ub–permease conjugates in wild-type cells (Figure 2A, lanes 1 and 2) indicates that permease normally undergoes polyubiquitination.

Overproduction of UbK29R and UbK48R in Δ*doa4* cells restored the normal ubiquitination pattern of the permease (Figure 2A, lanes 6 and 7). In contrast, overexpression of UbK63R triggered the formation of only mono- and di-ubiquitinated permease conjugates (lane 8). Both bands appeared to have a higher molecular weight when mutant UbK63R was extended by the addition of a tag derived from haemagglutinin (HA) (Figure 2A, lane 9), confirming that they indeed result from incorporation of plasmid-encoded Ub. Δ*doa4* cells overproducing wild-type Ub, mutant UbK63R, or UbRRR all had similar levels of free Ub after Cu induction (not shown), indicating that the under-ubiquitination of uracil permease in cells producing mutant Ubs was not caused by a reduction in their pool of free Ub. The above data therefore strongly suggest that the inability of UbK63R to induce tri- and tetra-ubiquitinated permease is due to its incapacity to form polyubiquitinated chains on uracil permease.

We determined the fate of another substrate of the Ub system in our experimental conditions in order to check whether the K63R mutation affects the availability of Ub for conjugation in Δ*doa4* cells. The fusion protein Ub–Proline–β galactosidase (Ub–Pro–βgal), a substrate of the UFD pathway (Johnson *et al.*, 1995), is only slowly cleaved at the X–βgal junction, unlike other fusion Ub–X–βgal proteins (Bachmair *et al.*, 1986). It undergoes polyubiquitination on its Ub moiety (Johnson *et al.*, 1992),

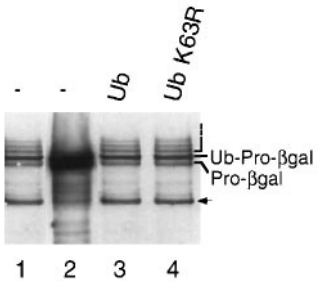

Fig. 3. Effect of the overexpression of wild-type Ub or UbK63R on the steady-state concentration and ubiquitination pattern of Ub–Pro–βgal in Δ*doa4* cells. MHY501 (WT) transformed with the plasmid encoding Ub–Pro–βgal (lane 1), MHY623 (Δ*doa4*) cells transformed with the plasmid encoding Ub–Pro–βgal (lane 2), or transformed with this plasmid plus plasmid encoding wild-type Ub (lane 3), or UbK63R (lane 4) were grown as indicated in Materials and methods. Cells were collected 3 h after adding $CuSO_4$, and used to prepare total protein extracts (Galan *et al.*, 1996). Aliquots containing the same amount of proteins were analysed using an antibody against β-galactosidase (Galan *et al.*, 1996). The position of Pro–βgal which results from slow deubiquitination of Ub–Pro–βgal (Bachmair *et al.*, 1986) is indicated. An arrow indicates the stable 90 kDa derivative of βgal. Brackets denote multiubiquitinated Ub–Pro–βgal species.

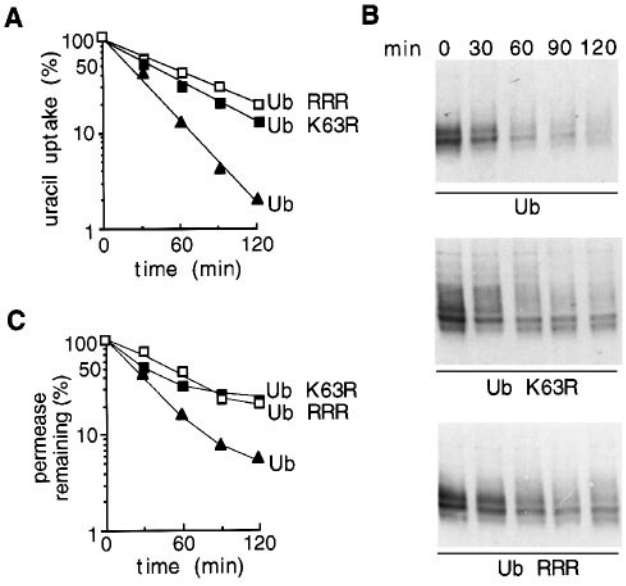

Fig. 4. Permease turnover in Δ*doa4* cells overexpressing wild-type Ub, UbK63R or UbRRR. MHY623 (Δ*doa4*) cells transformed with both YEp352fF and plasmids encoding wild-type Ub, UbK63R or UbRRR, were grown and induced for Ub synthesis for 30 min as described in Figure 1. Cycloheximide (100 μg/ml) was then added. (**A**) Uracil uptake was measured at the times indicated. The values are averages of at least three independent experiments with standard deviations <5%. (**B**) Protein extracts were prepared at the times indicated. Proteins from 0.2 ml culture were analysed for uracil permease by Western immunoblotting. (**C**) Bands corresponding to the main permease signal were quantified in each lane along with a control band that was present at constant level over the entire experiment (as shown in Figure 1). Average values were determined for several film exposures of at least two independent experiments. The relative amounts of permease are plotted versus time.

followed by rapid degradation by the proteasome ($t_{1/2}$ ~5 min) (Richter-Ruoff *et al.*, 1992; Ghislain *et al.*, 1993). Ub–Pro–βgal is a very sensitive indicator of general defects in proteolysis (van Nocker *et al.*, 1996). In cells in which the *DOA4* gene is disrupted, Ub–Pro–βgal is strongly protected against degradation (Papa and Hochstrasser, 1993). In agreement with these findings, we observed that steady-state amounts of Ub–Pro–βgal accumulated in Δ*doa4* cells as compared with wild-type cells (Figure 3, compare lanes 1 and 2). Overexpression of wild-type Ub in Δ*doa4* cells caused a decrease in the steady-state amount of Ub–Pro–βgal (Figure 3, lane 3). We also observed the appearance of multi-ubiquitinated Pro–βgal conjugates, as well as an increase in the relative amount of a characteristic 90 kDa cleavage product that is specific for βgal-containing proteins with short half-lives (Bachmair *et al.*, 1986). Overproduction of UbK63R triggered the degradation of Ub–Pro–βgal, and an identical ubiquitination pattern (Figure 3, lane 4) as for wild-type Ub was observed, indicating that this mutant Ub can form multi-Ub chains in Δ*doa4* mutant cells.

Taken together, the above results indicate that overproduction of UbK63R in Δ*doa4* cells does not rescue ubiquitination of uracil permease, in contrast to that of a known proteasome substrate. This strongly suggests that permease carries Lys63-linked Ub chains.

Roles of monoubiquitination and Lys63-linked Ub chains in permease endocytosis

In order to determine whether or not Lys63-linked Ub chains play a role in the internalization of uracil permease, we compared the fate of uracil permease in Δ*doa4* cells overexpressing wild-type Ub, mutant UbK63R and UbRRR that impair the formation of multi-Ub permease conjugates. Overexpression of UbK63R and UbRRR in Δ*doa4* cells led to a loss of uracil uptake with a $t_{1/2}$ of 40 min for UbK63R and of 50 min for UbRRR (Figure 4A), instead of 150 min in control Δ*doa4* cells which did not overexpress Ub (Figure 1). Thus, the mutant Ubs were clearly able to allow permease internalization. However, they were not as efficient as wild-type Ub for that function. In their presence, the rate of permease internalization was ~2-fold lower than that observed in the presence of wild-type Ub ($t_{1/2}$ 26 min; Figure 4). In agreement with these observations, some permease degradation was observed after inhibition of protein synthesis in cells overproducing the mutant Ubs, but the extent of degradation was reduced as compared with cells overproducing wild-type Ub (Figure 4B). In addition to the main permease signals (non-ubiquitinated protein), traces of mono/diubiquitinated species remained detectable throughout experiments in cells expressing the mutant Ubs (more specifically UbK63R). Quantification of the immunoblots allowed us to estimate an ~2-fold reduction in the rate of permease degradation in cells expressing the mutant Ubs, versus wild-type Ub (Figure 4C).

Although Δ*doa4* cells clearly depend on plasmid-encoded Ub for permease endocytosis, they are not entirely devoid of Ub. In order to ascertain that the partial restoration of permease endocytosis upon overexpression of UbRRR and UbK63R in these cells did not result from an increased pool of chromosomal-encoded Ub, we investigated the role of Lys63-linked chains in permease endocytosis using another experimental system. The physiological roles of various kinds of Ub chains has been determined using yeast strains in which the four natural Ub genes have been disrupted, and which express plasmid-encoded wild-type Ub (Finley *et al.*, 1994) or any of several mutant Ubs. Such mutant cells, expressing UbK63R as

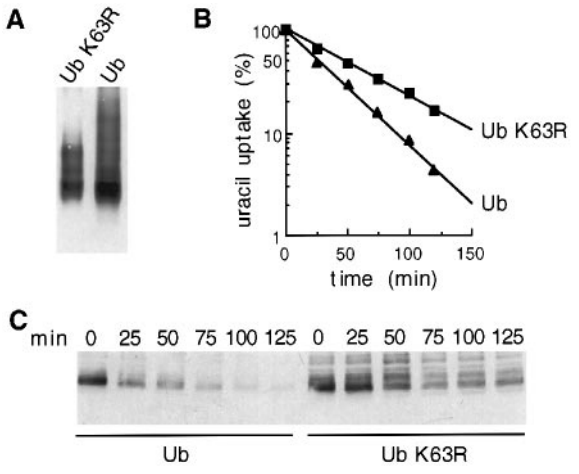

Fig. 5. Permease ubiquitination and endocytosis in cells having UbK63R as their sole source of Ub. SUB280 and SUB413, expressing wild-type Ub and UbK63R, respectively, were transformed with Yep352fF. Cells were grown as described in Materials and methods, and induced for Ub overexpression for 2 h. (**A**) Cells were used to prepare plasma membrane-enriched fractions. Aliquots were analysed for uracil permease by Western immunoblotting. (**B**) Uracil uptake was measured after addition of cycloheximide (100 μg/ml). (**C**) Protein extracts were prepared at the times indicated after addition of cycloheximide and analysed for uracil permease by Western immunoblotting.

their sole source of Ub, were demonstrated to be fully viable (Spence *et al.*, 1995). We compared the fate of uracil permease in cells expressing either wild-type Ub, or UbK63R. The pattern of Ub–permease conjugates in these two types of cell was clearly comparable with that observed in Δ*doa4* cells overexpressing the same Ubs. Four bands of mono to tetra-Ub–permease conjugates were detected in the presence of wild-type Ub, and only two species of mono/diubiquitinated permease in cells having UbK63R as their sole source of Ub (Figure 5A). Permease endocytosis was followed in these two types of cell after inhibition of protein synthesis. Cycloheximide induced in both strains a loss of uracil uptake, with a $t_{1/2}$ of 25 and 42 min in the presence of wild-type and mutant Ub, respectively (Figure 5B). Accordingly, permease was degraded in both type of cells, but at lower rate in cells expressing only UbK63R (Figure 5C). These results are quite similar, if not identical, to those observed in Δ*doa4* cells. The overall data indicate that monoubiquitination is sufficient to induce permease endocytosis, and suggest that the occurrence of polyubiquitin chains with Lys63 Ub–Ub linkages speeds up the internalization of uracil permease, and its subsequent degradation.

It seems unlikely that the slow rate of permease endocytosis in cells lacking Lys63-linked Ub chains should correspond to an indirect general effect of UbK63R. Δ*doa4* cells overexpressing UbK63R, or cells having UbK63R as their sole source of Ub exhibit a normal growth rate. General perturbation of protein trafficking was not observed either, since the rate of maturation of the vacuolar carboxypeptidase Y is normal in both types of cell (data not shown).

Discussion

We have used a yeast strain lacking the Doa4p ubiquitin–isopeptidase in order to examine how ubiquitination acts as a signal for the endocytosis of plasma membrane uracil permease. Proteolysis of several substrates of the ubiquitin/proteasome pathway is strongly inhibited in Δ*doa4* cells (Papa and Hochstrasser, 1993). Disruption of the *DOA4* gene severely impaired the ubiquitination and endocytosis of uracil permease, and both processes were rescued by the overexpression of Ub. These data show that the amount of intracellular Ub available for ubiquitination is limiting in Δ*doa4* cells, at least in our experimental conditions, in agreement with the observation that free Ub drops dramatically in these cells under certain physiological conditions (Chen and Hochstrasser, 1995). Even though the physiological role of this deubiquitinating enzyme is not yet clear, Δ*doa4* cells provide a simple experimental system for testing the effects of various Ub mutations upon Ub-dependent processes. Δ*doa4* cells, that appear to lack sufficient Ub to fulfil a number of Ub-dependent processes, represent an alternative tool to the more refined mutant cells, which are disrupted for the four natural Ub genes and express only plasmid-encoded Ub (Finley *et al.*, 1994; Spence *et al.*, 1995).

We overproduced in Δ*doa4* cells mutant Ubs carrying Lys→Arg mutations in one to three of the lysine residues that can be coupled *in vivo* to additional Ub. Wild-type Ub, and mutant Ubs carrying Lys→Arg mutations at Lys29 and/or Lys48 restored normal permease ubiquitination, with the formation of mono- to tetra-Ub–permease conjugates. Tetra-Ub-conjugates were transiently enriched under several conditions, leading to the acceleration of permease turnover rate, indicating that their formation normally preceded permease degradation. Overproduction of the triple mutant UbRRR and of the mutant UbK63R in Δ*doa4* cells resulted only in permease diubiquitination. Similarly, only diubiquitinated permease conjugates were observed in cells deleted for all Ub genes, and producing UbK63R as their sole source of Ub. This strongly suggests that two lysine residues in permease serve as Ub-acceptor sites, and that permease normally carries Ub chains extended through Lys63 of Ub.

While Ub-conjugates carrying Lys63-linked chains have been reported (Spence *et al.*, 1995), to our knowledge none has been identified. Uracil permease, a natural target for Lys63 chain assembly, clearly differs from substrates of the Ub–proteasome pathway, which carry Lys48-linked multiubiquitinated chains (Hochstrasser *et al.*, 1991; Schork *et al.*, 1995). The efficient binding of these chains to the proteasome subunit 5 requires at least four Ubs (Deveraux *et al.*, 1994; Baboshina and Haas, 1996). The observation that uracil permease carries short Lys63-linked chains provides various reasons for understanding why these Ub-conjugates are not recognized by the proteasome. The structural configuration of Lys63 chains is entirely different from that of Lys48-linked chains (Arnason and Ellison, 1994). Crystal structure studies of Ub have shown that Lys63 lies close to the N-terminus of the protein (Vijay-Kumar *et al.*, 1987), which seems to be important for recognition by the proteasome (Ellison and Hochstrasser, 1991). Lys63 Ub–Ub linkages would thus prevent recognition by the multiprotease complex.

The formation of selective Ub–Ub linkages is thought to be a property of Ub–protein ligases, together with associated Ub-conjugating enzymes (Jentsch, 1992; Johnson *et al.*, 1995; Baboshina and Haas, 1996). E3

Ubr1p interacts physically with E2/Rad6p (Dohmen *et al.*, 1991) and the resulting complex is involved in the formation of Lys48-linked chains on substrates of the N-end rule (Varshavsky, 1996). Ufd4p, a member of the E3 hect family, would probably act with Ubc4p and Ubc5p for ubiquitination at Lys29 of substrates of the UFD pathway (Johnson *et al.*, 1995). Hence, extension of Ub chains by Lys63 could be a property of the E2/E3 pair involved in the ubiquitination of uracil permease. We have not yet identified the E2 enzyme(s) involved in permease ubiquitination. Ubc4p and Ubc5p (or their close homologue Ubc1p) were likely candidates, since they are reported to be involved in the formation of Ub–protein conjugates carrying Lys63 chains (Arnason and Ellison, 1994; Spence *et al.*, 1995), and to be essential for ubiquitination and/or endocytosis of several yeast plasma membrane proteins (Kölling and Hollenberg, 1994; Hicke and Riezman, 1996; Roth and Davis, 1996). However, experiments performed on Δ*ubc4*Δ*ubc5*, and on Δ*ubc1* cells did not reveal impairment in permease ubiquitination and endocytosis, neither after inhibition of protein synthesis, nor after heat stress (data not shown) known to induce Ubc4p and Ubc5p (Seufert and Jentsch, 1990. Current investigations are in progress in order to test the potential involvement of the other 10 Ubcp recognized in the yeast genome. Although the ubiquitination and degradation of several proteins have been shown to depend on particular E2 enzymes, the possible overlap of function of these enzymes (Hochstrasser, 1996b), and the participation of multiple E2 to ubiquitination of a given substrate (Chen *et al.*, 1993) might complicate identification of the Ubcp possibly involved in Ub Lys63-dependent ubiquitination of uracil permease. Even though the E2(s) responsible for permease ubiquitination are not yet identified, we already know that permease ubiquitination depends on the Ub–protein ligase Npi1p/Rsp5p *in vivo* (Galan *et al.*, 1996). The *Npi1* strain was identified in a screen for mutant cells impaired in catabolite inactivation of several amino acid permeases (Grenson, 1992), a process that corresponds to ammonium-induced internalization and degradation in the case of the general amino acid permease, Gap1p (Hein *et al.*, 1995). It was recently shown that Npi1p is also required for glucose-induced endocytosis and degradation of the maltose transporter when protein synthesis is impaired (Lucero and Lagunas, 1997). Recent reports suggest that rNedd4, the rat homologue of Npi1p is involved in the ubiquitination of some plasma membrane epithelial Na channel subunits, since it interacts with domains within these subunits (Staub *et al.*, 1996) that are critical for their turnover (Schild *et al.*, 1996). In addition to its role in the turnover of several plasma membrane proteins, Npi1/Rsp5p displays other functions. Mutations in *NPI1/RSP5* also known as *MDP1*, impair mitochondrial/cytoplasmic distribution of a tRNA-modifying enzyme (Zoladek *et al.*, 1997). Npi1p/Rsp5p is involved in the *in vitro* ubiquitination, and *in vivo* turnover, of the soluble large subunit of RNA polymerase II (Huibregste *et al.*, 1997). In that case, the type of Ub chains linkage was not reported, but it was observed that *in vitro* ubiquitination could be supported by *Arabidopsis thaliana* Ubc8p, belonging to the same E2 subfamily as the *S.cerevisiae* Ubc4p/Ubc5p. Latter enzymes can catalyse different Ub–Ub linkages under different circumstances (Dohmen *et al.*, 1991; Arnason and Ellison, 1994). It will be critical to understand whether Lys63 chain extension is a property of proteins of the E3/ Npi1p family, or whether these proteins, in association with different Ubcps, or other regulatory proteins, have different Ub chain specificities regarding different classes of substrates. In this respect, it is notable that Npi1p/Rsp5p was found in large complexes (Yashiroda *et al.*, 1996), like E3 components for mitotic cyclin ubiquitination (King *et al.*, 1995; Sudakin *et al.*, 1995).

The role played by Ub, and more specifically by Ub63 chains, in endocytosis remains to be determined. Permease endocytosis in Δ*doa4* cells is partially rescued by the overproduction of UbK63R and UbRRR. In agreement with these observations, stress-induced permease endocytosis is observed in cells having UbK63R as their sole source of Ub. Therefore, monoubiquitination, i.e. the attachment of a single Ub to two permease target lysines, is sufficient to signal permease endocytosis. However, the rate of permease endocytosis is significantly lower when Ub–permease conjugates cannot be extended through Ub Lys63. Optimization of permease endocytosis when Ub chains are extended by Lys63 might result either from the specific configuration of these chains, or merely from the increase in the number of ubiquitin residues carried by uracil permease. Defining the mechanisms underlying the helper function of Lys63-linked Ub chains in permease endocytosis largely depends on understanding the role of ubiquitination as a signal for endocytosis. Single Ub residues, or more efficiently Ub chains, may provide a binding site for a component of the endocytic machinery. Alternatively, they may play the role of molecular chaperones that help to uncover cryptic endocytic signals in the targeted proteins. It is also possible that they play a role in clustering their targets to regions of active endocytosis. There is clearly much left to discover before we can decipher Ub-dependent endocytosis.

Materials and methods

Yeast strains and growth conditions

MHY501 (*MAT*α *his3-*Δ*200 leu2-3, 112 ura3-52 lys2-801 trp1-1*) and its isogenic Δ*doa4* derivative MHY623 are described by Papa and Hochstrasser (1993). In strain SUB280 (*MAT***a** *lys2-801 leu2-3,112 ura3-52 his3-*Δ*200 trp1-1 [am] ubi1-*Δ*1::TRP1 ubi2-*Δ*2::ura3 ubi3-*Δ*ub-2 ubi4-*Δ*2::LEU2* [pUB39] [pUB100]) (Finley *et al*., 1994), the unique source of Ub is the multicopy plasmid pUB39, which carries a synthetic Ub gene under the control of the *CUP1* promoter. SUB413 (Spence *et al*., 1995) is identical to SUB280 except that it expresses a mutant UbK63R derivative instead of wild-type Ub. Yeast strains were transformed according to Gietz *et al*. (1992). Strains were grown at 30°C in minimal medium (YNB) containing 0.67% yeast nitrogen base without amino acids (Difco) supplemented with appropriate nutrients, and 2% glucose as a carbon source for experiments on uracil permease, and with 4% galactose as a carbon source for experiments on Ub–Pro–βgal. Overexpression of the *CUP1* promoter was induced with 0.1 mM $CuSO_4$.

Plasmids

The plasmid YEp96 contains a synthetic yeast Ub gene under the control of the copper-inducible *CUP1* promoter (Ecker *et al*., 1987). Plasmids encoding mutant Ubs, in which Lys29 (UbK29R), Lys48 (UbK48R), Lys63 (UbK63R), or all three lysines (Lys29, -48- and -63; UbRRR) are replaced by arginine, are also derivatives of YEp96 (Arnason and Ellison, 1994). HA-UbK63R is identical to UbR63K, except that it bears a haemagglutinin (HA) tag on the N-terminus of Ub. Plasmids encoding UbK48R, UbK63R and HA-UbK63R were constructed using the Stratagene chameleon double-stranded site-directed mutagenesis kit. The

resulting genes encoding mutant Ubs were sequenced using double-strand DNA and the Sequenase 2.0 kit (USB). The 2 μ plasmid YEp352fF carries the *FUR4* gene (encoding uracil permease) under the control of its endogenous promoter (Galan *et al.*, 1996). The 2 μ plasmid encoding Ub–Pro–βgal under the control of the *GAL10* promoter is derived from pUb23 (Bachmair *et al.*, 1986). The permease activity of cells transformed with YEp352fF is amplified 25-fold, which enables uracil permease to be immunodetected. A 100-fold overexpression of active uracil permease did not induce any associated phenotypes (Silve *et al.*, 1991).

Measurement of uracil uptake

Uracil uptake was measured in exponentially growing cells as previously described (Volland *et al.*, 1992). Yeast cultures (1 ml) were incubated with 5 μM [^{14}C]uracil (Amersham) for 20 s at 30°C, then quickly filtered through Whatman GF/C filters. These were washed twice with ice-cold water and then counted for radioactivity.

Yeast cell extracts and Western immunoblotting

Cell extracts were prepared and proteins analysed by immunoblots as previously described using an antiserum against either the last 10 residues of uracil permease (Volland *et al.*, 1994), or β-galactosidase (Sigma) (Galan *et al.*, 1996). Primary antibodies were detected with a horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody followed by chemiluminescence (ECL, Amersham). When indicated, band intensity was quantified by scanning densitometry using the NIH 1.59 software. Peak areas in each lane were quantified in pixels. Quantification was performed in the range where signal intensity was observed to be proportional to protein concentration.

Membrane preparation

Yeast cells (A_{600} = 80) in exponential growth phase were harvested by centrifugation in the presence of 10 mM sodium azide, and used to prepare plasma membrane-enriched fractions (Galan *et al.*, 1996). Membrane-bound proteins were analysed by Western blotting as described above.

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