Molecular Biology of the Cell
Vol. 11, 3365–3380, October 2000

The Doa4 Deubiquitinating Enzyme Is Functionally Linked to the Vacuolar Protein-sorting and Endocytic Pathways

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Submitted June 6, 2000; Revised July 12, 2000; Accepted July 27, 2000
Monitoring Editor: Juan Bonifacino

The *Saccharomyces cerevisiae DOA4* gene encodes a deubiquitinating enzyme that is required for rapid degradation of ubiquitin–proteasome pathway substrates. Both genetic and biochemical data suggest that Doa4 acts in this pathway by facilitating ubiquitin recycling from ubiquitinated intermediates targeted to the proteasome. Here we describe the isolation of 12 spontaneous extragenic suppressors of the *doa4-1* mutation; these involve seven different genes, six of which were cloned. Surprisingly, all of the cloned *DID* (Doa4-independent degradation) genes encode components of the vacuolar protein-sorting (Vps) pathway. In particular, all are class E Vps factors, which function in the maturation of a late endosome/prevacuolar compartment into multivesicular bodies that then fuse with the vacuole. Four of the six Did proteins are structurally related, suggesting an overlap in function. In wild-type and several *vps* strains, Doa4–green fluorescent protein displays a cytoplasmic/nuclear distribution. However, in cells lacking the Vps4/Did6 ATPase, a large fraction of Doa4–green fluorescent protein, like several other Vps factors, concentrates at the late endosome–like class E compartment adjacent to the vacuole. These results suggest an unanticipated connection between protein deubiquitination and endomembrane protein trafficking in which Doa4 acts at the late endosome/prevacuolar compartment to recover ubiquitin from ubiquitinated membrane proteins en route to the vacuole.

INTRODUCTION

Protein degradation plays an important part in numerous cellular processes (Gottesman and Maurizi, 1992). In eukaryotes, proteins that must be rapidly destroyed are generally recognized and degraded by the ubiquitin system (Hochstrasser, 1996; Pickart, 1997; Varshavsky, 1997; Ciechanover, 1998). Attachment of ubiquitin to substrate proteins has distinct mechanistic roles in two different intracellular proteolytic pathways. For many short-lived cellular proteins, attachment to a polyubiquitin chain(s) facilitates their binding to a large protease called the 26S proteasome (Coux *et al.*, 1996; Pickart, 1997). The ubiquitin molecules in these chains are linked by amide bonds between Lys-48 of one ubiquitin and the C-terminal carboxyl group of the next ubiquitin. After binding of the ubiquitin conjugate, the proteasome degrades the substrate to small peptides. Many membrane proteins are degraded by a different ubiquitin-dependent mechanism (Hicke, 1997; Bryant and Stevens, 1998). Their attachment to either a single ubiquitin or short Lys-63–linked ubiquitin oligomers appears to trigger their endocytosis and/or transport through a series of endosomal compartments to the vacuole/lysosome, where the proteins are destroyed by vacuolar hydrolases (Roth and Davis, 1996; Galan and Haguenauer-Tsapis, 1997; Kölling and Losko, 1997; Levkowitz *et al.*, 1998; Loayza and Michaelis, 1998; Terrell *et al.*, 1998).

Ubiquitin is a long-lived protein in the yeast *Saccharomyces cerevisiae*, so it must be removed from ubiquitin–substrate conjugates before or during substrate degradation (Swaminathan *et al.*, 1999). In yeast, 17 deubiquitinating enzymes (Dubs) are predicted from the completed genome sequence. Several have been studied to a limited degree, but relatively little is known about their physiological functions or natural substrates (Wilkinson and Hochstrasser, 1998). Among the most extensively characterized Dubs is the yeast Doa4 enzyme, which has been shown to play crucial roles in both ubiquitin-dependent proteolysis and ubiquitin homeostasis (Papa and Hochstrasser, 1993; Singer *et al.*, 1996; Papa *et al.*, 1999; Swaminathan *et al.*, 1999).

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Table 1. Yeast strains

Strain	Genotype
MHY500	*MAT***a** *his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1*
MHY501	*MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1*
MHY606	MHY500 × MHY501
MHY623	*MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 doa4-Δ1::LEU2*
MHY1080	*MATα leu2-3,112::LEU2-Deg1-lacZ ura3-52 lys2-801*
MHY1096	*MATa leu2-3,112::LEU2-Deg1-lacZ ura3-52 lys2-801 doa4-1*
MHY1230	*MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 did1-Δ1::HIS3*
MHY1232	*MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 did3-Δ1::HIS3*
MHY1234	*MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 did2-Δ1::HIS3*
MHY1250	*MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 doa4-Δ1::LEU2 did1-Δ1::HIS3*
MHY1251	*MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 doa4-Δ1::LEU2 did3-Δ1::HIS3*
MHY1253	*MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 doa4-Δ1::LEU2 did2-Δ1::HIS3*
MHY1269	*MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 vps27Δ::LEU2*
MHY1275	*MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 doa4-Δ1::LEU2 vps27Δ::LEU2*
MHY1307	*MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 doa4-Δ1::LEU2 vps45Δ::HIS3*
MHY1309	*MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 vps45Δ::HIS3*
MHY1334	*MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 did4-Δ1::LEU2*
MHY1370	*MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 doa4-Δ1::LEU2 did4-Δ1::LEU2*
MHY1387	*MATa leu2-3,112::LEU2-Deg1-lacZ ura3-52 lys2-801 doa4-1 did6-1*
MHY1388	*MATa leu2-3,112::LEU2-Deg1-lacZ ura3-52 lys2-801 doa4-1 did7-1*
MHY1389	*MATα leu2-3,112::LEU2-Deg1-lacZ ura3-52 lys2-801 did6-1*
MHY1391	*MATα leu2-3,112::LEU2-Deg1-lacZ ura3-52 lys2-801 did7-1*
MHY1556	*MATα leu2-3,112 ura3-52 lys2-801 trp1-1 doa4-Δ1::LEU2 ypt1-A136D*
MHY1558	*MATa his leu2-3,112 ura3-52 doa4-Δ1::LEU2 vps33-Δ1::kanR*
MHY1640	*MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-Δ901 suc2-Δ9 DOA4-GFP*
MHY1641	*MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-Δ901 suc2-Δ9 vps4-Δ1::TRP1 DOA4-GFP*
MH11D5-8a	*MATa leu2-3,112::LEU2-Deg1-lacZ ura3-52 lys2-801 doa4-1*
MBY3	*MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-Δ901 suc2-Δ9 vps4-Δ1::TRP1*
SEY6210	*MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-Δ901 suc2-Δ9*
SGY18	*MATα his4-519 leu2-3,112 ura3-52 ade6 gal2 vps33-Δ1::kanR*

Doa4 appears to function late in the ubiquitin–proteasome pathway by recycling ubiquitin from proteasome-targeted substrates. A significant fraction of the enzyme is associated with 26S proteasomes, and *doa4* mutations interact genetically with mutations in components of the proteasome. In exponentially growing cultures of *doa4* mutants, small ubiquitinated species accumulate; these species were suggested to be the proteolytic remnants of ubiquitinated proteins (Papa and Hochstrasser, 1993; Papa *et al.*, 1999). In addition, intracellular ubiquitin pools in the mutant become depleted, particularly in stationary-phase cultures, as a result of the proteolysis of ubiquitin itself. Partial suppression of ubiquitin depletion by mutations in components of the 26S proteasome suggests that this protease is at least partly responsible for the degradation of ubiquitin (Swaminathan *et al.*, 1999). Conceivably, the inability to release ubiquitin chains from proteasome-targeted substrates leads to degradation of the entire ubiquitin–protein conjugate. Such degradation may be inefficient, and the resulting ubiquitin–peptide intermediates could accumulate on proteasomes, thereby inhibiting overall rates of proteasomal degradation.

Recent studies of ubiquitin homeostasis in yeast led to the surprising finding that inactivation of genes important for vacuolar proteolysis and endocytosis significantly reduced ubiquitin depletion in *doa4* cells (Swaminathan *et al.*, 1999). These data suggested that a substantial flux of cellular ubiquitin is involved in endocytosis and vacuolar targeting of yeast membrane proteins and that Doa4 has a direct or indirect role in this pathway as well. Here we provide evidence that the participation of Doa4 in this pathway is very likely direct and appears to be at the late endosome/prevacuolar compartment (PVC) stage. We have identified suppressor mutations that largely bypass the requirement for Doa4 in yeast cells; the mutants were named Doa4-independent degradation or *did* mutants. Unexpectedly, all of the *did* mutations are in genes important for the step in which the Golgi-to-vacuole and endosome-to-vacuole protein trafficking pathways converge (Bryant and Stevens, 1998). On their own, the *did* mutations have little or no effect on the proteolysis of several tested proteasome substrates, but several of the *did* mutations lead to substantial accumulation of cellular ubiquitin–protein conjugates. All suppress the *doa4* defects in proteasome substrate degradation by a mechanism other than (or in addition to) restoring cellular ubiquitin levels. These data suggest that the two major intracellular proteolytic pathways—vacuolar and proteasomal—have in common a requirement for protein deubiquitination by the Doa4 enzyme.

MATERIALS AND METHODS

Strains and Materials

Yeast strains used in this study are listed in Table 1. The *Escherichia coli* strains used were JM101 and WM1100. Yeast and bacterial media were prepared as described, and standard yeast genetic and recombinant DNA methods were used (Ausubel *et al.*, 1989). Monoclonal mouse antibodies against the T7 and hemagglutinin (HA) epitopes were purchased from Novagen (Madison, WI) and BAbCO

(Richmond, CA), respectively, and a mAb to ubiquitin was from D. Gottschling (Hutchinson Cancer Research Center, Seattle, WA). Polyclonal rabbit antibodies used were against ubiquitin (Swaminathan *et al.*, 1999), Ste3 (Roth and Davis, 1996) (from N. Davis, Wayne State University, Detroit, MI), carboxypeptidase Y (CPY) (from A. Cooper, University of Missouri, Kansas City, MO), and green fluorescent protein (GFP; from P. Silver, Dana-Farber Cancer Center, Boston, MA).

Isolation of did Mutants

The *did1* to *did5* mutants were originally isolated during a screen for *Schizosaccharomyces pombe* orthologues of the *S. cerevisiae DOA4* gene (A.Y. Amerik. and M. Hochstrasser, unpublished data) that was based on an X-gal plate assay for enhanced *Deg1-β*gal degradation in the *doa4-1* mutant MHY11D5-8a (Papa and Hochstrasser, 1993). Seven plasmid-independent revertants were identified from ~70,000 colonies. Backcrossing revealed that the suppressor mutations were unlinked to *doa4-1*, and suppression in all cases was recessive. The mutants were sorted into five complementation groups based on matings between different *did doa4-1* double mutants. When separated from the *doa4-1* allele, all of the mutants except *did5-1* were found to have readily scored recessive defects as well. During several unsuccessful attempts to clone *DID5* by suppression of *Deg1-β*gal degradation in *did5-1 doa4-1* cells, we also performed control transformations of the *doa4-1* strain MHY1096 with an empty vector. Five additional *did* suppressors unlinked to *doa4-1* were isolated, and suppression was also recessive. Three of the suppressors were new *did3* alleles based on complementation by the cloned *DID3* gene, and two, *did6-1* and *did7-1*, were in genes that did not correspond to *DID1–DID4*. None of the cloned *DID* genes on low-copy plasmids was able to reverse the *did5-1 doa4-1* suppressor phenotype.

The *DID* genes were cloned from either of two *CEN/URA3*-based yeast genomic libraries, one made in YCp50 (Rose *et al.*, 1987) and the other in YCplac33 (A.Y. Amerik. and M. Hochstrasser, unpublished data). By means of a plate-based selection, putative *DID* gene–containing clones were identified by their ability to suppress the sensitivity of the corresponding *did* single mutants to 0.8 μg/ml canavanine sulfate. To eliminate plasmid-independent revertants, canavanine-resistant clones were streaked onto plates containing 5-FOA, which is toxic to cells expressing the *URA3* gene, to identify cells that had lost the library plasmid. Plasmids from transformants that were no longer canavanine-resistant after 5-FOA treatment were recovered in *E. coli* and then retested in mutant yeast cells. DNA subcloning was used to trace the complementing activity from the original plasmid inserts to single ORFs except in the case of *DID2* (see below). Linkage of the six cloned genes with the respective chromosomal *did* mutations was verified by subcloning *DID* gene–containing DNA fragments into YIp352 (Hill *et al.*, 1986), directed integration of the resulting plasmids into the yeast genome, and linkage analysis of the YIp352-borne *URA3* marker and the canavanine hypersensitivity caused by the *did* mutations.

To determine whether the *DID2* ORF (*YKR035w-A*), which was originally not annotated in the *Saccharomyces* Genome Database (SGD), was expressed as protein, the ORF was fused at its 5′ end with a sequence encoding the T7-Tag epitope (Novagen). A two-step PCR procedure was used for epitope tagging (Papa *et al.*, 1999). PCR products were cloned in pGEM-T/Easy (Promega, Madison, WI), excised with *Not*I, and subcloned into the *CEN/URA3* yeast–*E. coli* shuttle vector pRS316 (Sikorski and Hieter, 1989). The pRS316-T7-DID2 plasmid was transformed into MHY1234 cells (*did2Δ::HIS3*). Because the putative *DID2/YKR035w-A* ORF was completely bracketed by the *YKR035c* ORF on the opposite strand, selective inactivation of *DID2* was achieved by mutating the presumptive *DID2* start codon to an ATA codon. The mutation, *did2-3*, did not alter the predicted protein sequence of *YKR035c*. The next ATG in the *DID2* ORF is codon 89, which if used to initiate translation would result in a truncated protein. The *did2-3* allele was cloned into pRS316 as described for the T7-Tag addition, and the allele was verified by DNA sequencing.

By DNA sequence analysis, we noticed that a sequence upstream of the predicted *DID4/YKL002w* ORF bore significant similarity to the 5′ region of the *DID3* ORF. Perfect matches to consensus 5′ and 3′ splice site and branch point sequences were found just upstream of the SGD-annotated *YKL002w* ORF, suggesting the presence of an intron. We verified this by PCR amplification from a cDNA library with the use of primers predicted to flank the intron position. Sequencing of the PCR fragment confirmed the absence of a 68-base pair (bp) DNA element that is in the genomic sequence and that corresponds to the predicted intron. The intron-encoding sequence spans nucleotides 437,476 to 437,543 in chromosome XI (SGD).

Yeast Strain and Plasmid Construction

To make deletion alleles of *DID1*, *DID2*, and *DID3*, the yeast *HIS3* gene was amplified by PCR with the use of primers with 5′ sequences that corresponded to the regions just upstream of the start codons and just downstream of the termination codons of the respective *DID* genes. The amplified fragments were used for transformation of MHY606 cells. The resulting heterozygous diploids were sporulated, and tetrads were dissected. His^+ haploid segregants were checked by colony PCR. A two-step procedure was used to make a null allele of *DID4*. Fragments of 400 bp bearing the immediate 5′ or 3′ sequence flanking the *DID4* ORF were amplified, as was a 1500-bp DNA fragment containing *LEU2*. The 5′ sequences within the primers used to amplify *LEU2* also corresponded to sequences immediately adjacent to the start and termination codons of *DID4*. In a second round of amplification, the overlapping *LEU2* and *DID4* flanking DNA fragments were annealed, extended by *Taq* polymerase, and then amplified with the use of the outermost *DID4* primers. The resulting DNA fragment contained the *LEU2* gene flanked by 400 bp of *DID4* upstream and downstream sequences. After transformation of MHY606 with this PCR fragment, the diploid was sporulated and tetrads were dissected as described above.

Plasmids carrying *vps27Δ::LEU2* and *vps45Δ::URA3* were obtained from Robert Piper, University of Iowa, Iowa City, IA (Piper *et al.*, 1994, 1995). The inserts were transformed into yeast. Integration of the mutant alleles was verified by colony PCR, and mutant segregants were identified by tetrad analysis. Yeast strains with multiple gene deletions were made by the appropriate genetic crosses. For high-copy expression of *DID1–DID4*, the four genes were subcloned separately into the 2-μm vector YEplac195 (Gietz and Sugino, 1988). The resulting plasmids were transformed into the various *didΔ* mutants.

Constructs for the expression of HA-tagged versions of Did1 and Did3 were generated by PCR amplification of the corresponding genes with the use of primers matching the 5′ and 3′ ends of each ORF. *Sac*I and *Xho*I restriction sites were built into the 5′ and 3′ primers, respectively, and PCR products were digested with *Sac*I and *Xho*I and subcloned into the expression vectors YATAG200 (CEN/ARS) and YRTAG310 (2 μm), which resulted in the placement of the genes behind the *CUP1* promoter and fused at their 3′ ends to a sequence encoding an in-frame HA epitope tag (see Li and Hochstrasser, 1999).

A *DOA4*–GFP gene fusion (S65T GFP variant) was made previously for expression from a low-copy centromeric plasmid (F.R. Papa and M. Hochstrasser, unpublished data). This fusion construct, which uses the *DOA4* promoter, completely complemented the canavanine sensitivity of the *doa4Δ* mutant. The insert DNA encoding the Doa4–GFP protein was excised with *Hin*dIII and *Kpn*I and subcloned into YIp352. The resulting plasmid was cleaved with *Bgl*II, which cuts at a unique site in *DOA4*, to direct integration of the plasmid into the chromosomal *DOA4* locus in yeast strains SEY6210 and MBY11. Ura^+ transformants were selected, and the site of integration was verified by linkage analysis.

Table 2. The *did* mutants

Mutant	Alleles	Other names	References	ORF
did1	1	*snf7*, *vps32*	Tu *et al.*, 1993; Babst *et al.*, 1998	YLR025w
did2	2	–	–	YKR035w-A
did3	5	*vps24*	Babst *et al.*, 1998	YKL041w
did4	1	–	–	YKL002w
did5	1	–	–	?
did6	1	*vps4*	Babst *et al.*, 1997	YPR173c
did7	1	*vps27*	Piper *et al.*, 1995	YNR006w

Anti-Ubiquitin Immunoblot Analysis

Anti-ubiquitin immunoblot analysis was done essentially as described previously (Amerik *et al.*, 1997). Cell were grown at 30°C to midlogarithmic phase, collected by centrifugation, and resuspended in Laemmli gel-loading buffer. After heating to 100°C for 10 min and spinning down cell debris, the supernatants were loaded onto 16% Tricine-SDS-polyacrylamide gels (Schägger and von Jagow, 1987). Proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA), and the blots were boiled for 30 min in water before antibody incubations. Antibody binding was detected with the use of ECL reagents (Amersham, Arlington Heights, IL). Under the conditions used, the reactivity of free ubiquitin was significantly weaker than that of ubiquitin–protein conjugates, particularly with the mouse mAb.

Degradation Assays

Pulse-chase and pulse-labeling analyses were conducted as described previously (Chen *et al.*, 1993). Cells were labeled for 5–10 min with ^{35}S-TransLabel (ICN Pharmaceuticals, Costa Mesa, CA). Aliquots of yeast cells were disrupted by mixing with an equal volume of 2% SDS, 90 mM HEPES, pH 7.5, and 30 mM DTT and heating at 100°C for 10 min. Cleared and diluted cell extracts were precipitated with antibodies against α2 (Hochstrasser and Varshavsky, 1990), β-galactosidase (Organon Teknika, Malvern, PA), or T7-Tag (Novagen). To measure the degradation of Ste3, 10 ml of cells was grown in minimal medium to $OD_{600} \sim 0.8$, pelleted, and resuspended in 1 ml of minimal medium. Cycloheximide was added to a final concentration 0.5 mg/ml. At the appropriate times, equal aliquots of cells were removed and heated for 10 min at 100°C, and debris was removed by centrifugation at 14,000 × *g*. Proteins were resolved on 10% SDS-polyacrylamide gels and analyzed by anti-Ste3 immunoblot analysis with ECL detection.

Fluorescence Microscopy

Staining of yeast cell membranes with the FM 4-64 lipophilic dye was performed as described previously with minor modifications (Vida and Emr, 1995). All strains were grown at 30°C in 10 ml of YPD to $OD_{600} \sim 1$. Cells were harvested and resuspended in 166 μl of YPD. FM 4-64 (0.4 μl of a 16 mM solution in DMSO) was added to each tube and incubated at 30°C for 20 min. Cells were harvested by centrifugation, resuspended in 0.2 ml of fresh YPD, and incubated for 1 h at 30°C. Cells were collected by centrifugation and resuspended in YPD, and a drop of the cell suspension was placed on a slide and viewed by fluorescence microscopy. Similar conditions were used to view GFP fusion proteins by intrinsic GFP fluorescence.

Subcellular distributions of Did1, Did2, Did3, and Doa4 were examined in fixed yeast strains by indirect immunofluorescence as described (Li and Hochstrasser, 1999). Formaldehyde was added to a final concentration of 3.7% to exponentially growing cultures (10 ml). After 2 h, cells were centrifuged and washed with 10 ml of buffer B (0.1 M potassium phosphate, pH 6.8, 0.5 mM $MgCl_2$). Cells were collected by centrifugation, washed with 10 ml of buffer C (0.1 M potassium phosphate, pH 6.8, 0.5 mM $MgCl_2$, 1.2 M sorbitol), centrifuged, and resuspended in 1 ml of buffer C. After addition of 5 μl of β-mercaptoethanol and 10 μl of zymolase 100T (Seikagaku America, Rockville, MD; 5 mg/ml in buffer C), cells were incubated at 30°C for 1 h, harvested, washed with 5 ml of buffer C, and resuspended in 1 ml of buffer C. Aliquots of cells (15 μl) were placed on polylysine-coated multiwell slides, incubated for 10 min, and washed with 15 μl of PBS, pH 7. Cells were treated with 15 μl of 0.2% Triton X-100 in PBS for 10 min, washed three times with PBS, and incubated in PBS containing 0.5% BSA for 10 min. Primary antibodies were then added. After overnight incubation, cells were washed four times with PBS and twice with PBS containing 0.5% BSA and then were incubated for 1 h with secondary antibodies (Oregon Green goat anti-mouse and Texas Red anti-rabbit immunoglobulin G conjugates; Molecular Probes, Eugene, OR). After several washings with PBS and air drying, mounting medium was added to each well, and slides were covered with coverslips. Samples were viewed on a Zeiss (Thornwood, NY) LSM 510 confocal fluorescence microscope.

Analysis of CPY Sorting

Yeast cells were grown in 10 ml of minimal medium to $OD_{600} \sim 1$, harvested, and resuspended in 200 μl of zymolase buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 1 M sorbitol) supplemented with yeast nitrogen base [YNB plus $(NH_4)_2SO_4$], glucose, and amino acids. Zymolase 100T (10 μg/2 × 10^7 cells) was added, and the culture was incubated at 30°C for 1 h. Cells were washed twice in 1 ml of wash buffer (1 M sorbitol, 0.67% YNB, 2% glucose) and resuspended in 200 μl of labeling buffer (50 mM potassium phosphate, pH 7.4, 0.5% glucose, 1 M sorbitol). Twenty microliters of ^{35}S-TransLabel was added, and cells were labeled at 30°C for 30 min, collected, and resuspended in chase buffer (0.67% YNB, 2% glucose, 10 mM methionine, 10 mM cysteine, 1 M sorbitol). After a 45-min chase period, cultures were centrifuged at 14,000 × *g* for 1 min to generate intracellular (pellet) and extracellular (supernatant) fractions. Levels of CPY in each fraction were determined by anti-CPY immunoprecipitation and quantified from data collected on a Storm 860 Phosphorimager (Molecular Dynamics) with the use of ImageQuant software.

RESULTS

Identification of doa4-1 Suppressors

Spontaneous suppressors of the *doa4-1* mutation (Papa and Hochstrasser, 1993) were identified with the use of plate-based screens for reversion of the defect in degradation of a normally short-lived reporter protein, *Deg1*-βgal (see MATERIALS AND METHODS). *Deg1*-βgal contains a degradation signal from the Matα2 transcriptional repressor but accumulates to abnormally high levels in *doa4* cells (Hoch-

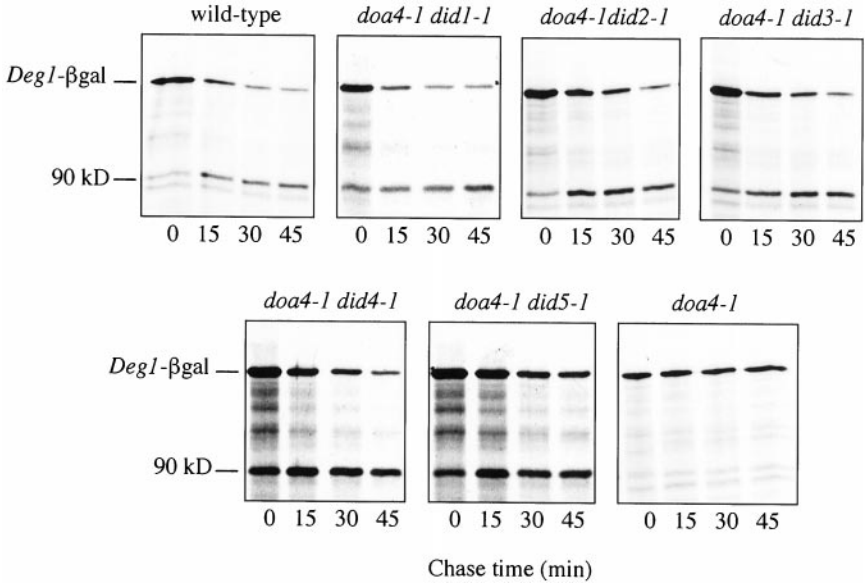

Figure 1. Suppression of the *doa4* degradation defect by *did* mutations. *Deg1-*βgal pulse-chase analysis in *doa4-1* and *did doa4-1* mutants. Average *Deg1-*βgal half-lives from one to five independent measurements were as follows: wild type, 11 min; *did1-1 doa4-1*, 13 min; *did2-1 doa4-1*, 12 min; *did4-1 doa4-1*, 14 min; and *did5-1 doa4-1*, 22 min. Half-lives were calculated from quantitative Phosphorimager data from time points up to 30 min. During this time, degradation is close to first order; as described originally by Hochstrasser and Varshavsky (1990), *Deg1-*βgal degradation rates slow gradually, particularly with long chase times. The reporter protein was expressed from a chromosomally integrated copy of *Deg1-lacZ*. 90 kD indicates a proteolytic fragment of *Deg1-*βgal generated in cells in which the reporter protein is short-lived.

strasser and Varshavsky, 1990). Twelve recessive suppressors unlinked to the original *doa4-1* mutation were identified in two separate screens. The mutants fell into seven different complementation groups (Table 2). Subsequently, we found that a *doa4*Δ null allele was also efficiently suppressed by the new mutations, indicating that they are bypass suppressors. Therefore, the new mutations in these pseudorevertants were named Doa4-independent degradation or *did* mutations.

To confirm that the reduced steady-state levels of *Deg1-*βgal in the *did doa4-1* double mutants resulted from enhanced *Deg1-*βgal degradation, pulse-chase analyses were performed (Figure 1). In all of the mutants except *doa4-1 did5-1*, the degradation defect was nearly completely suppressed. In *doa4-1 did5-1* cells, suppression was incomplete but still significant. As with many mutants in the ubiquitin–proteasome pathway, the *doa4-1* mutant is extremely sensitive to the arginine analogue canavanine and grows poorly at high temperatures (Papa and Hochstrasser, 1993). Both of these defects were also partially suppressed in *did doa4-1* cells with the exception of *did5-1 doa4-1* (Figure 2, A and C). Although the *did* mutations could partially suppress the *doa4-1* canavanine and temperature sensitivities, the *did* single mutants were themselves sensitive to these treatments if higher concentrations of canavanine or higher growth temperatures were used (Figure 2, B and D).

Isolation of the DID Genes

The *DID1*, *DID2*, *DID3*, *DID4*, *DID6*, and *DID7* genes were cloned from yeast genomic DNA libraries by functional complementation of the canavanine hypersensitivity of the corresponding yeast mutants (see MATERIALS AND METHODS). DNA subcloning from the original genomic inserts allowed identification of the genes responsible for the complementing activity in each case except for *DID2* (see below) (Table 2). Four of the *DID* genes were identified previously from genetic screens unrelated to the present one. *DID1* is the same as *SNF7*/*VPS32*, which encodes a protein involved in overcoming glucose repression of transcription (Tu *et al.*, 1993) and in the trafficking of proteins to the vacuole (Babst *et al.*, 1998). *DID3* is identical to *VPS24*, which was also recently implicated in vacuolar protein sorting (Babst *et al.*, 1998). Finally, *DID6* and *DID7* were found to be the same as *VPS4* (Babst *et al.*, 1997) and *VPS27* (Piper *et al.*, 1995), respectively. Both of these genes also encode proteins that participate in endosomal transport. *DID2* and *DID4* encoded previously uncharacterized proteins.

For *DID2*, the smallest complementing subclone included two potential ORFs, *YKR035c* and *YKR035w-A*. The latter sequence has similarity to other *DID* products (see below) but is on the opposite strand from and completely bracketed by the initially annotated *YKR035c* ORF. Because of this unusual arrangement, we first wished to determine whether *YKR035w-A* was in fact translated into protein. The *YKR035w-A* ORF was fused at its 5′ end with a sequence encoding a T7 epitope tag, and a low-copy plasmid encoding the putative T7-tagged protein was transformed into a *did2* null mutant. Wild-type growth on canavanine and at high temperature was restored, and a protein of the predicted size was specifically immunoprecipitated (Figure 3A). To selectively inactivate *YKR035w-A* without affecting the predicted protein sequence of *YKR035c*, the initiation codon of the former ORF was mutated to yield the *did2-3* allele. The predicted protein, if expressed, would be missing the first 88 residues of the wild-type YKR035w-A protein. The *did2-3* construct failed to complement the canavanine hypersensitivity of a *did2*Δ strain (Figure 3B) or to prevent the suppression of *Deg1-*βgal degradation in a *did2-1 doa4-1* double mutant. We conclude that Did2 is encoded by *YKR035w-A*, a conclusion reinforced by the sequence similarities discussed below.

Four of the DID Genes Encode Related Proteins

Unexpectedly, when the predicted Did proteins were compared, Did1, Did2, Did3, and Did4 were found to be related in sequence (Figure 4A). All four are relatively small, highly

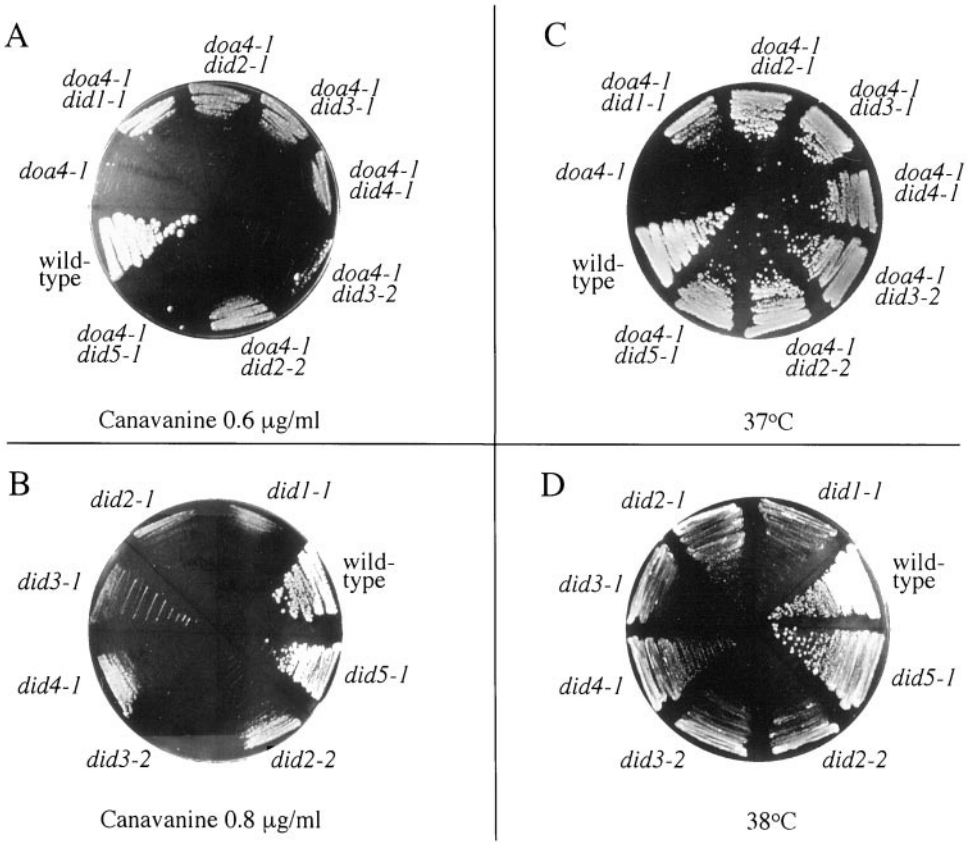

Figure 2. Mutations in *DID* genes suppress the canavanine- and temperature-sensitivity of the *doa4* mutant and cause phenotypic abnormalities associated with mutants with impaired intracellular proteolysis. Growth of *did* single and *doa4 did* double mutants on canavanine-containing media (A and B) and at increased temperatures (C and D) is shown.

charged proteins that are predicted to be largely α-helical and to have coiled-coil protein-interaction domains in their N-terminal regions (Lupas *et al.*, 1991). All have acidic isoelectric points, but they also all share a bias in charge distribution, with basic residues concentrated in their N-terminal halves and acidic residues in the C-terminal segments. These sequence and structural similarities suggest that the Did1–Did4 proteins are related by descent and may have comparable mechanisms of action.

Several genetic interactions between *DID* genes support this last inference. When performing complementation analysis of the original *did* mutants, we noticed that the *did2-2/+ did4-1/+* double heterozygote was still partially canavanine-sensitive, in contrast to the analogous double heterozygote involving *did2-1*, which was fully canavanine-resistant. This was true even though the *did2-1* single mutant was slightly more sensitive to canavanine than was *did2-2* (Figure 2B). Such allele-specific "unlinked noncomplementation" is often an indication that the two encoded proteins act in the same protein complex (Stearns and Botstein, 1988). We also placed each of the *DID1–DID4* genes in high-copy plasmids and determined whether any of them could suppress the defects associated with a null allele of the other *DID* genes. High-copy expression of *DID4* suppressed the temperature- and canavanine-sensitivity of *did3*Δ, but cross-suppression was not observed in any other case. Did3 and Did4 are the most closely related of the yeast proteins shown in Figure 4A, sharing 30% identity and 57% similarity in a 161-residue overlap.

Although the yeast Did1–Did4 proteins were clearly related to one another, much stronger similarities between individual yeast Did proteins and proteins from other eukaryotes were evident (Figure 4). The functional specialization of the Did proteins, therefore, appears to have occurred early in eukaryotic evolution, and the high degree of conservation (~40–50% identity) supports the importance of these factors for normal cell function. We note several intriguing sequence similarities between Did2 and Did4 and proteins from other organisms. Did2, which is expressed from an ORF completely embedded in another ORF, showed 47% identity with a previously overlooked predicted human polypeptide (Figure 4B) that is also expressed from an ORF embedded in another gene, in this case from an alternative reading frame on the same strand as *PRSM1* (Scott *et al.*, 1996). *PRSM1* is predicted to encode a secreted metalloprotease, and a single mRNA is detected in a variety of cell types. The *PRSM1* locus, which would include the *DID2*-like sequence, is a candidate for a recently mapped breast cancer susceptibility gene (Whitmore *et al.*, 1998) and for a lymphedema-distichiasis gene (Mangion *et al.*, 1999), which both mapped to chromosome 16q24.3. Did2 also displayed strong similarity to DG1118, a *Dictyostelium* protein required for normal morphological development (GenBank accession number 3789911). Yeast Did4 was 45% identical (68% similar) to the human BC-2 protein, a putative breast adenocarcinoma marker (Figure 4C). Thus, two of the

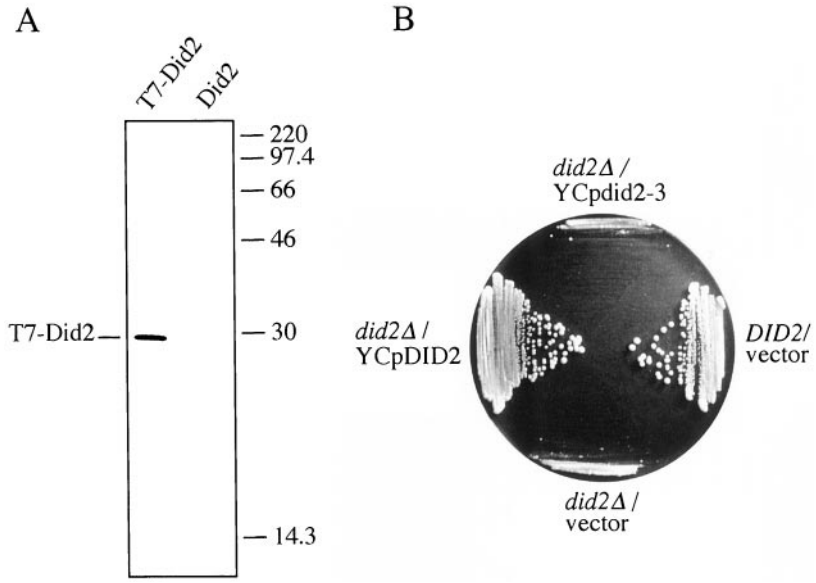

Figure 3. Did2 is encoded by *YKR035w-A*, an ORF embedded in another gene. (A) Protein expression from *YKR035w-A* assayed by epitope tagging and immunoprecipitation from radiolabeled yeast cells. Molecular mass standards are indicated (kDa). (B) A mutation that disrupts the predicted protein-coding sequence of *YKL035w-A* but not that of *YKR035c* on the opposite strand cannot complement a *did2*Δ mutation. Cells transformed with the indicated plasmids were plated on 1.0 μg/ml canavanine at 30°C.

yeast Did proteins have human orthologues that may be altered in breast tumors.

Defects in Other Ubiquitin Pathway Components Are Not Suppressed by did Mutations

We tested whether *did* alleles could suppress mutations in components of the ubiquitin–proteasome pathway other than Doa4. Double mutants involving *did1* and *did3* and mutations in several well-characterized components of the ubiquitin system were constructed through genetic crosses. The alleles used were *doa3-1*, *uba1-2*, and *ubp14*Δ. *DOA3* encodes the essential β5 catalytic subunit of the 20S proteasome (Chen and Hochstrasser, 1995). Uba1 is an essential enzyme responsible for the activation of ubiquitin (McGrath *et al.*, 1991), and *uba1-2* is a nonlethal hypomorphic allele (Swanson and Hochstrasser, 2000). Ubp14 is a Dub that disassembles unanchored polyubiquitin chains and, like Doa4, is required for normal rates of proteasomal degradation (Amerik *et al.*, 1997). No detectable suppression of the degradation defects associated with these mutations was found by pulse-chase analysis of several substrates. Moreover, growth of the double mutants was generally worse than for the single mutants, most strikingly for *uba1-2 did1*Δ and *ubp14*Δ *did3*Δ, which failed to form colonies at 35 and 37°C, respectively. Therefore, suppression by the *did* mutations was specific to *doa4*.

Suppression by did Mutations Is Substrate-Specific

To investigate the effects of *did* mutations in *doa4* cells on the proteolysis of substrates other than *Deg1-*βgal, we measured the degradation of α2, Leu-βgal and Ub-Proβgal by pulse-chase analysis (Figure 5). The latter two proteins are artificial test substrates that are ubiquitinated by distinct mechanisms in vivo (Varshavsky, 1997). We found that the α2 degradation defect was completely suppressed in all tested *doa4*Δ *did*Δ double mutants. Leu-βgal, an N-end rule substrate, was also degraded at close to wild-type rates in *doa4*Δ *did1*Δ, *doa4*Δ *did3*Δ, and *doa4*Δ *did4*Δ cells, but in the *doa4*Δ *did2*Δ strain, little if any suppression was seen. Ub-Proβgal remained long-lived in this latter mutant as well, whereas in *doa4*Δ *did1*Δ, *doa4*Δ *did3*Δ, and *doa4*Δ *did4*Δ cells, very weak suppression of the *doa4*Δ Ub-Proβgal degradation defect was observed. Ub-Proβgal degradation is known to be the most sensitive of the tested substrates to perturbations of the ubiquitin–proteasome pathway (Papa and Hochstrasser, 1993; van Nocker *et al.*, 1996), which would be consistent with a strong but partial bypass of the *doa4*Δ proteolytic defect caused by mutation of the *DID* genes. The inability of *did2*Δ but not other *did* deletions to suppress the *doa4*Δ defect in either Ub-Proβgal or Leu-βgal degradation supports the idea that the structurally related Did proteins make overlapping but distinct contributions to ubiquitin-dependent processes mediated by Doa4.

Ubiquitin and Ubiquitin Conjugates in did and doa4 did Mutants

For some ubiquitin–proteasome pathway substrates, such as *Deg1-*βgal and α2, the *doa4* degradation defect can be suppressed significantly by augmenting ubiquitin levels, which become depleted in *doa4* cells. In contrast, ubiquitin overexpression results in little if any suppression of Leu-βgal and Ub-Proβgal degradation defects (Swaminathan *et al.*, 1999). Hence, the turnover of some proteins in *doa4* cells is limited primarily by decreased ubiquitin availability, whereas for other substrates, reduced proteolysis is caused by a distinct *doa4* defect(s). This latter defect is thought to arise from impaired ubiquitin recycling from proteasome-targeted substrates (Papa and Hochstrasser, 1993; Papa *et al.*, 1999). Some of the substrate-specific suppression effects noted above, therefore, might reflect differences in the way the *did* mutations affect one or the other of these *doa4* molecular defects, e.g., they might primarily increase cellular ubiquitin pools.

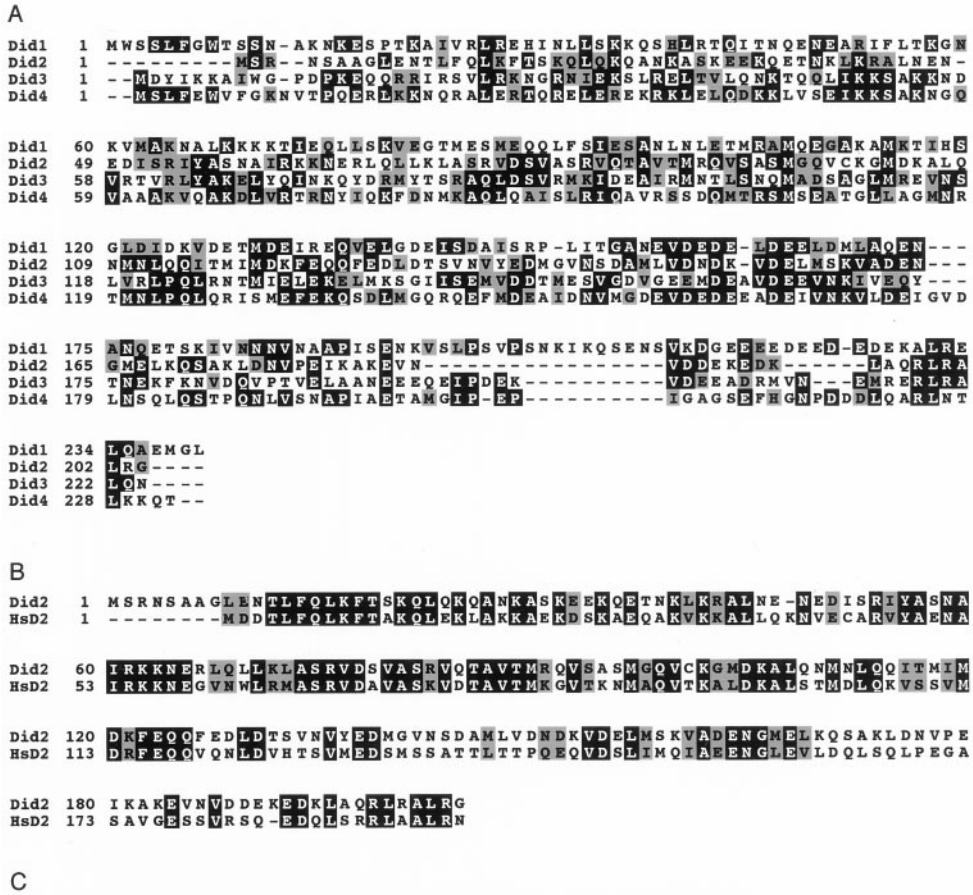

Figure 4. The Did1–Did4 proteins are related. (A) Sequence alignment of Did1 (YLR025w), Did2 (YKR035w-A), Did3 (YKL041w), and Did4 (YKL002w). (B) Alignment of Did2 with an alternative reading frame in the human *PRSM1* gene (Scott *et al.*, 1996). (C) Similarity between yeast Did4 and the human BC-2 breast adenocarcinoma marker protein (GenBank accession number 2828147).

To investigate this idea, we examined ubiquitin and ubiquitin conjugates in the various mutants by anti-ubiquitin immunoblotting (Figure 6). In all of the *doa4*Δ *did*Δ double mutants, levels of free ubiquitin were restored to wild-type levels or nearly so. Moreover, the intracellular concentration of the signature low-molecular-mass ubiquitinated species found in *doa4* cells was greatly reduced in the double mutants. For the strains that combined *doa4*Δ with *did1*Δ, *did3*Δ, or *did4*Δ, these ubiquitinated species were present at very low concentrations. In the *doa4*Δ *did2*Δ double mutant, the levels of the small conjugates were slightly but reproducibly higher, a finding congruent with the weaker proteolytic suppression by *did2*Δ (see above). Interestingly, both *did*Δ single and *doa4*Δ *did*Δ double mutants accumulated a broad array of higher-molecular-mass ubiquitin–protein conjugates, which were difficult to distinguish from the species that accumulated in proteasome mutants such as *doa3-1* (Figure 6) (see DeMarini *et al.*, 1995). This was clearest in the case of *did1*Δ and *doa4*Δ *did1*Δ cells.

The did Mutants Are All Class E Vacuolar Protein-sorting Mutants

We had previously found a link between Doa4-regulated ubiquitin homeostasis and the endocytic pathway in yeast (Swaminathan *et al.*, 1999). Moreover, four of the six mutants identified in the present study, *did1/vps32*, *did3/vps24*, *did6/vps4*, and *did7/vps27*, had been identified as class E vacuolar protein-sorting (*vps*) mutants (Piper *et al.*, 1995; Babst *et al.*, 1998). These findings suggested a close connection between suppression of the *doa4* proteolytic defect by inactivation of Did proteins and intracellular protein trafficking. Mutations in *VPS* genes result in the missorting of newly synthesized

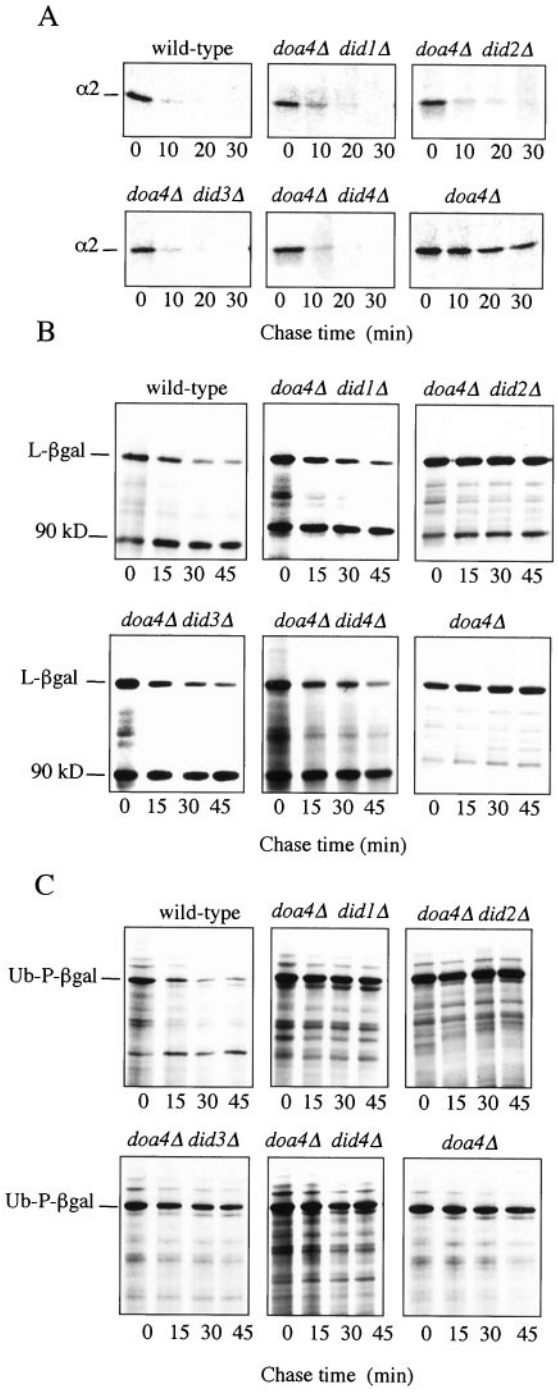

Figure 5. Substrate-specific suppression of *doa4* degradation defects by mutations in the *DID* genes. Pulse-chase analysis of α2 (A), Leu-βgal (B), and Ub-Proβgal (C) in congenic wild-type, *doa4*Δ, and *doa4*Δ *did*Δ strains. For analysis of α2 degradation, cells expressed the transcriptional repressor from the chromosomal *MAT* locus. For analysis of Leu-βgal and Ub-Proβgal degradation, expression of plasmid-derived fusion proteins was induced with galactose (Bachmair *et al.*, 1986).

vacuolar proteins such as CPY to the culture medium (Bryant and Stevens, 1998). The VPS pathway merges with the endocytic pathway at a late endosome compartment also called the PVC. Thus, proteins from both the *trans*-Golgi network and the plasma membrane are routed to the vacuole via this compartment. The distinguishing feature of the subset of *vps* mutants called class E mutants is the accumulation in the perivacuolar region of aberrant multilamellar structures known as the class E compartment, which is thought to be an exaggerated late endosome.

To determine whether Did2 and Did4 are also Vps proteins, CPY sorting in the *did2*Δ and *did4*Δ mutants was analyzed by pulse-chase experiments. Spheroplasts were radiolabeled with ^{35}S-TransLabel and chased for 30 min in the presence of excess unlabeled methionine and cysteine. CPY was immunoprecipitated from intracellular and extracellular fractions, and the immunoprecipitated proteins were separated by SDS-PAGE and visualized by fluorography (Figure 7A). After a 30-min chase in wild-type and *doa4*Δ cells, the endoplasmic reticulum precursor form of CPY (p1) had been transported to the Golgi, where the sugar chains were modified to yield the p2 precursor, and finally to the vacuole, where p2 was proteolytically processed to mature enzyme (mCPY). In contrast, in all of the *did*Δ mutants, a significant fraction of a p2-like CPY isoform was secreted into the culture medium. Therefore, *did2* and *did4* are *vps* mutants.

Vacuolar membranes and the class E compartment can be visualized in living cells by incubation with the lipophilic fluorescent dye FM 4-64 (Vida and Emr, 1995). In wild-type cells, the dye is taken up into endosomal membranes and transported to the vacuolar membrane. In class E *vps* mutants, the perivacuolar class E compartments are prominently stained. Our experiments demonstrated that similar structures were also present in *did2* and *did4* mutants (Figure 7B). As another measure of class E Vps function, we examined degradation of Ste3, the yeast α-factor receptor. This protein is ubiquitinated at the plasma membrane, endocytosed, and transported to the vacuole for degradation (Roth and Davis, 1996). Inactivation of vacuolar proteolysis by deletion of the *PEP4* gene strongly stabilizes the receptor, whereas deletion of the class E *VPS2/REN1* gene also inhibits Ste3 degradation, but in this case the effect is relatively moderate (Davis *et al.*, 1993). Degradation of Ste3 in the *did*Δ mutants was analyzed by following the disappearance of the receptor when protein translation was blocked by adding cycloheximide to the medium and monitoring protein by anti-Ste3 immunoblotting with chemiluminescence detection. Based on this semiquantitative assay, Ste3 proteolysis was modestly impaired in all of the mutants tested (Figure 8). Thus, a partial block to the degradation of endocytosed membrane proteins is likely a common property of class E mutants. A more severe Ste3 degradation defect was observed in *doa4*Δ cells, presumably as a result of ubiquitin limitation, inasmuch as *doa4*Δ has the same effect on the Ste2 receptor (Terrell *et al.*, 1998) and uracil permease (Galan and Haguenauer-Tsapis, 1997). Collectively, these observations establish Did2 and Did4 as novel class E Vps proteins.

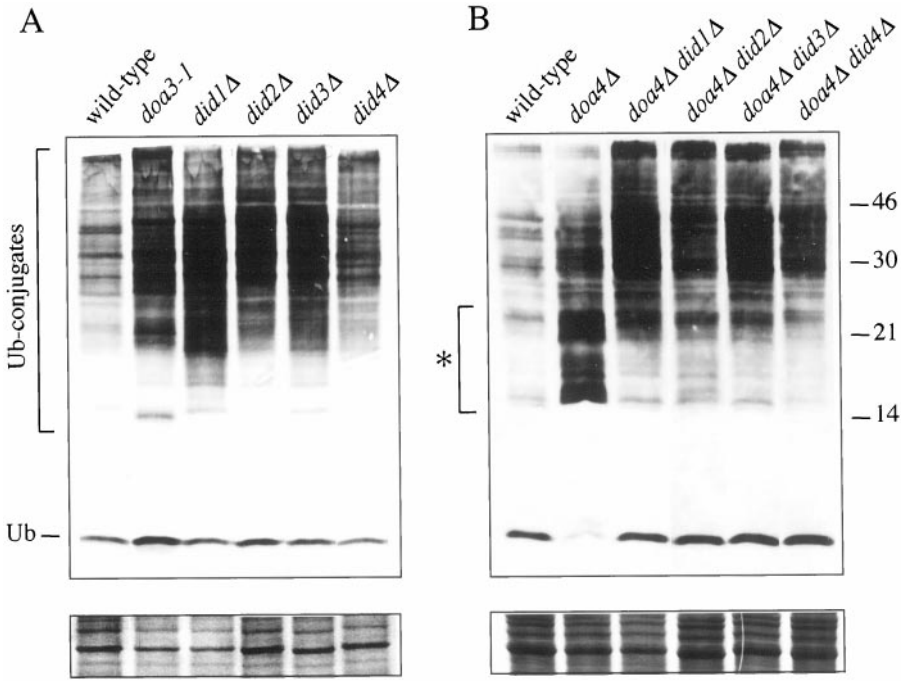

Figure 6. Suppression by *did* mutations of the small ubiquitinated species that accumulate in *doa4* cells and accumulation of ubiquitinated proteins in *did* single mutants. Extracts from a congenic set of strains were analyzed by anti-ubiquitin Western immunoblotting. Proteins were separated on a 16% Tricine gel; *doa4* cell-specific species are marked with an asterisk. Monoubiquitinated species are detected poorly with the anti-ubiquitin mAb. The bottom panels show a section of the Coomassie blue–stained filters used for immunoblotting to indicate the relative loading of protein in each lane.

Mutations in VPS Class C and Class D Genes Also Suppress doa4Δ

To assess the consequences of perturbing other steps in the secretory and VPS pathways on the *doa4*Δ degradation defect, we constructed *doa4*Δ *vps45*Δ, *doa4*Δ *vps33*Δ, and *doa4*Δ *ypt1-A136D* double mutants and measured *Deg*1-βgal, Leu-βgal, and/or α2 degradation in these cells. Vps45 belongs to the class D Vps proteins and is involved in protein transport between the *trans*-Golgi network and the PVC (Cowles *et al.*, 1994; Piper *et al.*, 1994), whereas Vps33 is a class C Vps protein important for the final step of vacuolar protein sorting, the delivery of transport intermediates to the vacuole (Banta *et al.*, 1990; Rieder and Emr, 1997). Ypt1 is required for vesicle trafficking both from the endoplasmic reticulum to the Golgi and within the Golgi complex (Jedd *et al.*, 1995). Deletion of *VPS33* or *VPS45* suppressed the *Deg1*-βgal and Leu-βgal degradation defects of *doa4*Δ cells (Figure 9A). In contrast, the *ypt1-A136D* mutation, when introduced into *doa4*Δ cells, did not change the rate of *Deg1*-βgal or α2 degradation (Figure 9B). We also attempted to test in *doa4*Δ cells the effect of introducing the *sec4-8* mutation, which inhibits protein transport from the *trans*-Golgi network to the plasma membrane (Salminen and Novick, 1987). However, *sec4-8* appears to be lethal in combination with *doa4*Δ, which might indicate a role for the ubiquitin system in this part of the secretory pathway.

In summary, loss of Doa4 function can be at least partially overcome by mutations that impair late steps of vacuolar protein sorting, i.e., transport of proteins to the late endosome or vacuole, but defects in the protein secretion pathway may actually exacerbate *doa4*Δ defects.

Did2 and Doa4 Relocalize to a Class E–like Compartment in vps4/did6 Mutants

A subset of class E Vps factors, which are normally soluble and found primarily in the cytoplasm, relocalize to the class E compartment membrane in cells defective for the Vps4/Did6 ATPase (Babst *et al.*, 1998). Specifically, Snf7/Vps32/Did1 and Vps24/Did3 relocalize, whereas Vps28 does not. Vps4 is thought to act as a dissociation factor for a complex of class E proteins bound to the cytoplasmic face of the late endosome. We confirmed that in *vps4*Δ cells, Vps24/Did3 concentrated at the class E compartment (Figure 10D). In addition, we examined the localization of a T7 epitope-tagged Did2 protein in a strain with a temperature-sensitive allele of *vps4* at a permissive temperature (24°C) or after a 45-min incubation at a nonpermissive temperature (37°C) (Figure 10A). At 24°C, Did2 localized to numerous cytoplasmic dots, suggestive of small membranous organelles, but after the shift to 37°C, Did2 was predominantly in one to three large, bright-staining perivacuolar spots, which also stained strongly with antibodies to CPY, a protein known to concentrate in class E structures in *vps4* cells (Babst *et al.*, 1998). Therefore, Did2, like the structurally related Did1 and Did3 proteins, appears to associate reversibly with the late endosome.

An analogous set of experiments was used to define the subcellular distribution of Doa4. We constructed derivatives of wild-type and *vps4*Δ strains that carried a chromosomally integrated DNA construct that expressed a functional fusion between Doa4 and the GFP of *Aequorea victoria* (Chalfie *et al.*, 1994) and examined the cells by scanning laser confocal microscopy (see MATERIALS AND METHODS). In wild-type cells, most of the Doa4–GFP fusion protein was diffusely localized to the nucleus/cytoplasm. In cells express-

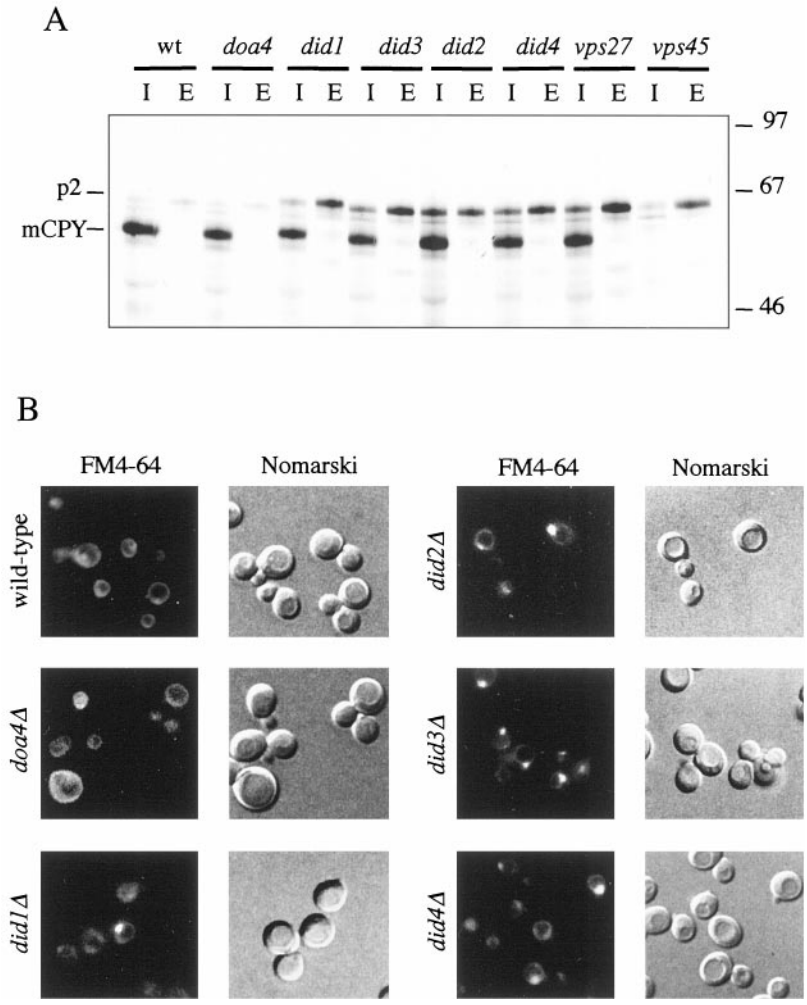

Figure 7. Vacuolar protein-sorting defect of *did* mutants. (A) Aberrant secretion of a vacuolar enzyme in the *did* mutants. Sorting of the vacuolar hydrolase CPY was determined by pulse-chase analysis. Cells spheroplasts were labeled for 10 min with ^{35}S-TransLabel, and after a 30-min chase period, CPY from intracellular (I) and extracellular (E) fractions was precipitated with anti-CPY antibodies and analyzed by SDS-PAGE. The intermediate secreted from *did1* cells had a slightly slower electrophoretic mobility than did the p2 form observed in wild-type cells and the other strains. This might be due to altered glycosylation of CPY in the *did1* mutant, although this has not been tested. (B) FM 4-64 staining of *did1*–*did4* mutants. Cells were labeled with FM 4-64 for 10 min and chased for 1 h at 30°C. The cells were then viewed with epifluorescence optics.

ing Doa4–GFP from a centromeric plasmid, which increases levels of the protein fusion, a similar distribution was observed but with a relatively higher concentration in the nucleus. Strikingly, in the *vps4*Δ mutant, a large fraction of the Doa4–GFP protein was found in one to three large spots located close to the vacuole (Figure 10C), even though Doa4–GFP expression was similar in the mutant and wild-type strains (Figure 10B). This effect was *vps4*-specific: no relocalization of Doa4–GFP was observed in *did1*Δ and *did3*Δ mutants. To confirm that the bright Doa4–GFP foci represent class E compartments, cells expressing both Doa4–GFP and Did3/Vps24-HA, a class E marker, were costained with antibodies to the respective tags and examined by immunofluorescence microscopy. The bright foci observed with the two antibodies colocalized (Figure 10D). Thus, Doa4 localization in the cell was controlled by Vps4/Did6 in a way that paralleled the regulation of multiple class E factors by the ATPase, suggesting that Doa4 could also function at the late endosome surface. It is important to note that the *doa4* mutant does not have an obvious *vps* phenotype (Figure 7A), and Doa4 is evidently present at levels below those of the Did/Vps class E proteins, based on the relative difficulty of detecting it by a variety of methods.

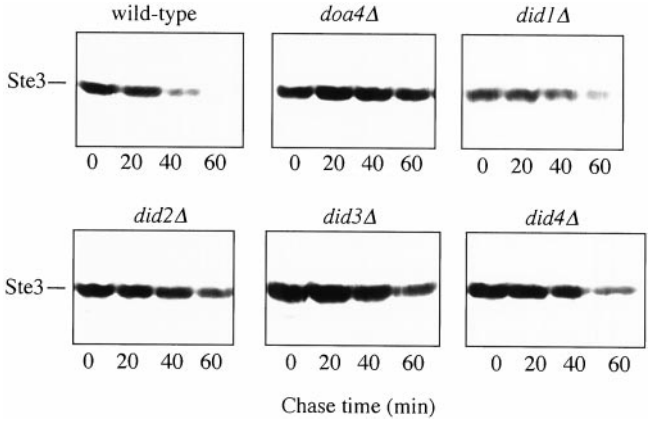

Figure 8. Ste3 receptor turnover in *doa4*Δ and *did*Δ mutants as measured by anti-Ste3 immunoblotting after the addition of the protein synthesis inhibitor cycloheximide. Logarithmically growing cells at 30°C were sampled (250 μl) at the indicated times.

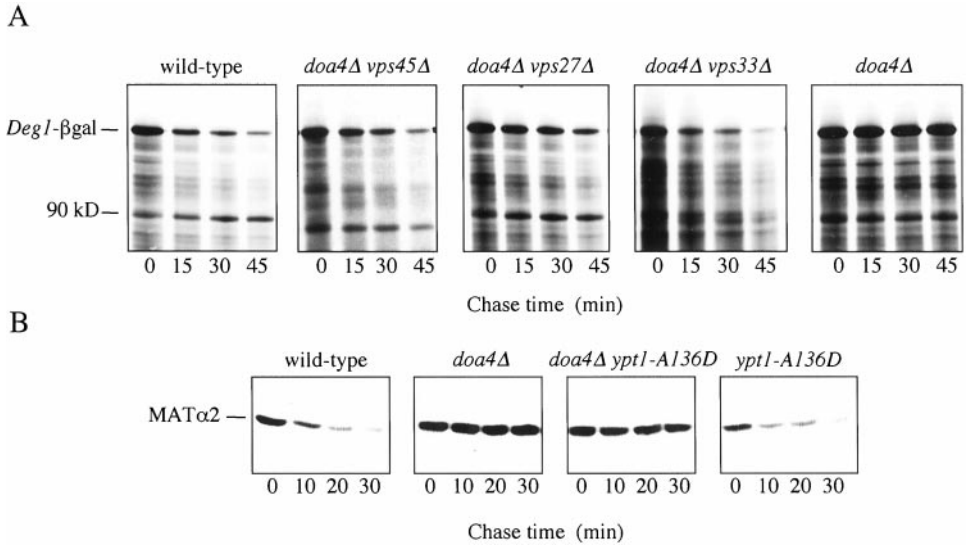

Figure 9. Mutations in genes involved in late stages of vacuolar protein sorting compensate for *doa4* deficiency in yeast. (A) Pulse-chase analysis of *Deg1*-βgal degradation in *doa4*Δ *vps45*Δ, *doa4*Δ *vps27*Δ, and *doa4*Δ *vps33*Δ double mutants. (B) Pulse-chase analysis of α2 degradation in *doa4*Δ *ypt1-A136D* double mutant.

Therefore, Doa4 is unlikely to function as a stoichiometric component of the late endosome coat complex.

The accumulation of Doa4 in the class E compartment in *vps4*Δ cells might require other class E factors. To test this, we examined Doa4–GFP localization in *did1*Δ *vps4*Δ and *did3*Δ *vps4*Δ (Figure 10C) double mutants. In both cases, the bright Doa4-GFP foci were no longer observed (but based on FM 4-64 and anti-Vph1 staining, class E compartments were present), suggesting that the protein complex formed on the late endosome surface by specific class E factors is necessary to recruit the Doa4 enzyme to these sites.

DISCUSSION

Here we have described a set of mutants in which the *doa4*Δ defects in ubiquitin homeostasis and proteasome-mediated proteolysis are efficiently overcome. All of the corresponding cloned genes encode factors important for a specific step in membrane trafficking to the vacuole. Most interestingly, the Doa4 deubiquitinating enzyme appears to localize reversibly with the late endosome/PVC, along with a group of proteins essential for targeting of membrane proteins to the vacuole. Doa4, therefore, may partition dynamically between a soluble, at least partly proteasome-associated pool (Papa *et al.*, 1999) and an endosomal membrane pool (Figure 10). These results point to a previously unsuspected function for the ubiquitin system in intracellular membrane protein trafficking and suggest that ubiquitinated membrane proteins can be deubiquitinated by Doa4 at the late endosome to recover ubiquitin and, possibly, to control the fate of the tagged protein.

The Did Proteins

Strikingly, all six of the identified *doa4* suppressor mutations inactivate class E Vps factors. Inactivation of these proteins results in the accumulation of perivacuolar structures consisting of stacked membrane cisternae (Rieder *et al.*, 1996). This "class E compartment" is thought to represent an exaggerated late endosome/PVC that accumulates because of the failure of the late endosome to mature into a multivesicular body (MVB), which would normally then fuse with the vacuole (Futter *et al.*, 1996; Odorizzi *et al.*, 1998). The internal vesicles in MVBs are enriched for specific lipids (Kobayashi *et al.*, 1999) and for cell surface receptors destined for vacuolar/lysosomal degradation (Futter *et al.*, 1996). Therefore, important lipid- and protein-sorting steps must occur during the formation of MVBs.

Several results argue for confluent mechanistic roles for the Did proteins in MVB maturation. First, four of the six Did proteins are related in primary sequence. Second, genetic interactions are observed among several of these factors, including nonallelic noncomplementation and high-copy suppression. Third, four of the six Did proteins are observed to concentrate in the class E compartment when another Did protein, the Did6/Vps4 ATPase, is inactive, and ATPase-defective Vps4 proteins behave similarly (Babst *et al.*, 1998). These hydrophilic and generally soluble factors are thought to form a supramolecular complex on the surface of the late endosome/PVC that drives or facilitates membrane invagination (Odorizzi *et al.*, 1998). Interestingly, Did1–Did4, Did6, and Did7 all have putative coiled-coil domains, and the coiled-coil region in Did6/Vps4 is known to be important for its localization to the PVC (Babst *et al.*, 1998). A potential coiled coil is also predicted (Lupas *et al.*, 1991) in Doa4 (residues 683–704), which may be necessary for its reversible association with Did/Vps coiled-coil proteins.

Strong sequence similarity is shared between the two novel class E Vps factors identified in this study (Did2 and Did4) and two mammalian gene products implicated in breast cancer (see RESULTS). TSG101, the likely mammalian orthologue of another yeast class E Vps factor, Vps23/Stp22, was originally identified as a tumor susceptibility gene (Li *et al.*, 1999). These provocative findings suggest that proper MVB maturation is critical for normal growth regulation in mammals. This might result from a failure to down-regulate growth factor receptors such as receptor protein tyrosine

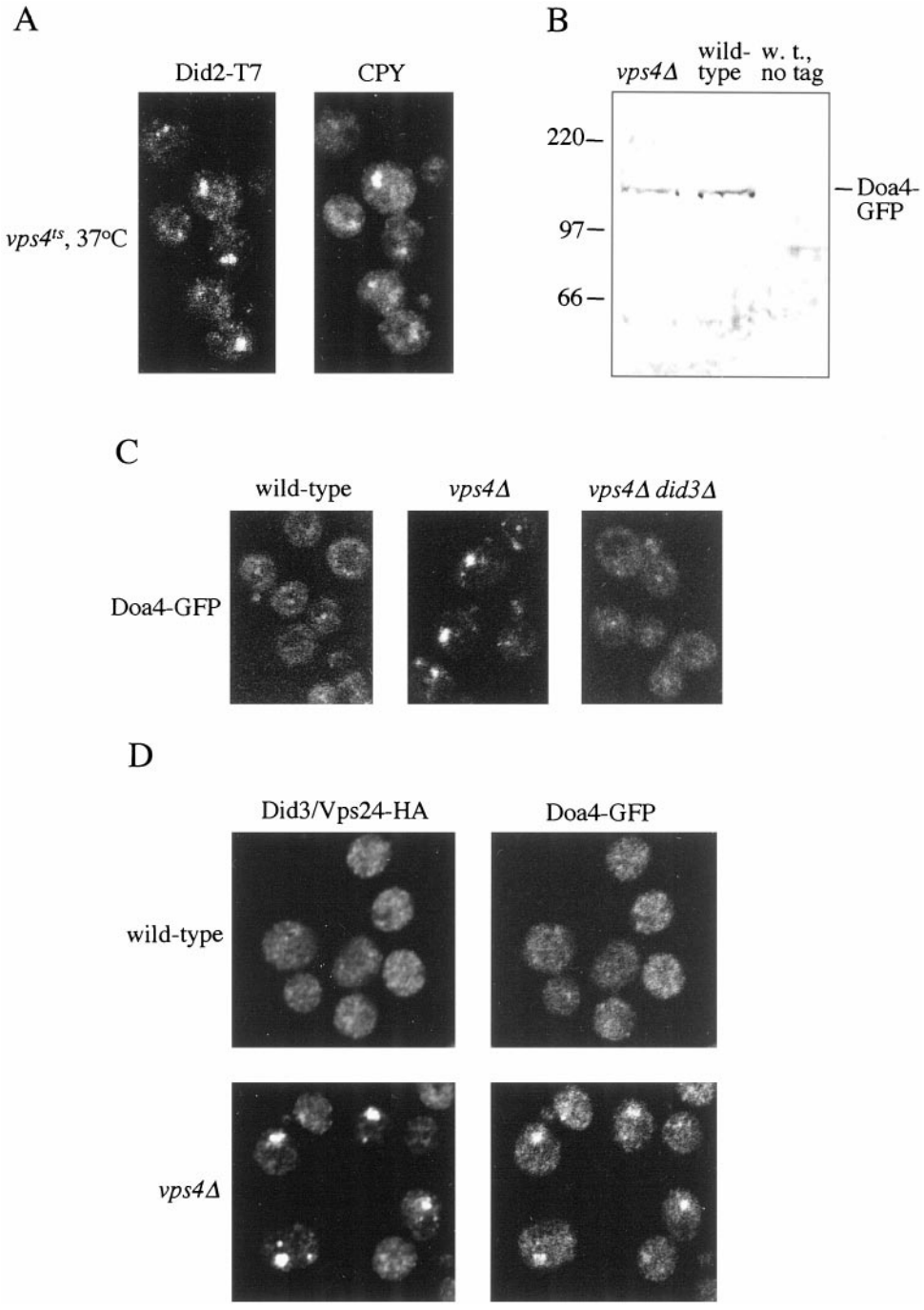

Figure 10. Did2 and Doa4 accumulate in the class E compartment in *vps4* mutants. (A) Cellular distribution of T7-tagged Did2 and CPY analyzed by indirect immunofluorescence and confocal microscopy. Cells were grown at 24°C and then incubated for 30 min at 37°C. (B) Expression of Doa4–GFP in wild-type and *vps4*Δ cells measured by anti-GFP immunoblot analysis. (C) Localization by intrinsic fluorescence of Doa4–GFP in wild-type, *vps4*Δ, and *did3*Δ *vps4*Δ cells. (D) Immunofluorescence colocalization of Doa4–GFP and Did3–HA in wild-type and *vps4*Δ cells by anti-GFP and anti-HA antibodies. All samples were viewed by laser scanning confocal microscopy.

kinases. In yeast, several class E *vps* mutants, including *vps23*, result in a failure to degrade cell surface proteins (Figure 8), and in at least one case, there is partial accumulation of receptors at the plasma membrane, which was suggested to reflect either the recycling of receptors from a late endosome (class E) compartment to the cell surface or a back-up resulting from the downstream block (Davis *et al.*, 1993).

Doa4 and Intracellular Membrane Protein Trafficking

How might ubiquitin and Doa4 participate in these internal membrane-sorting and rearrangement events? Many plasma membrane proteins that are destined for degradation in the vacuole/lysosome are ubiquitinated at the cell surface (Hicke, 1997; Bryant and Stevens, 1998), and they may carry

the ubiquitin tag at least to the late endosome. Some intracellular membrane proteins destined for the vacuole also might require ubiquitination even without passage through the plasma membrane (Beck *et al.*, 1999), a fact that might be relevant to the suppression of *doa4*Δ by *vps45*Δ, which is thought to act only in the Golgi-to-vacuole pathway and not in the endocytic pathway (Piper *et al.*, 1994). The ubiquitinated state of proteins at the late endosome surface might help concentrate them in regions that will invaginate and form the internal vesicles that eventually are delivered to the vacuole interior, perhaps analogous to the function of protein ubiquitination at the cell surface. In this regard, it is interesting that Vps23 has an N-terminal domain related to E2 ubiquitin-conjugating enzymes (but lacking the catalytic Cys residue) (Ponting *et al.*, 1997). Thus, Vps23 might bind the ubiquitin moieties of ubiquitinated membrane proteins and help cluster them on the late endosome/PVC membrane. However, protein deubiquitination of these proteins must generally occur before complete vesiculation and delivery to the vacuole, because the cellular pool of ubiquitin is long-lived (Swaminathan *et al.*, 1999). Based on the data presented here, we propose that Doa4 is responsible for deubiquitination events at the late endosome/PVC surface. Recruitment of the Doa4 enzyme to this compartment appears to depend on Vps factors that assemble on the late endosome and control MVB formation, which would help control the timing and location of membrane protein deubiquitination.

Suppression of *doa4*Δ defects by the class C *vps33* mutant, which is thought to prevent the fusion of MVBs with the vacuole, is more difficult to explain by the model described above, i.e., this block would appear to be too late to allow rescue of ubiquitin from modified membrane proteins. Several explanations can be suggested. By analogy to the accumulation of cell surface receptors in class E *vps* mutants (Davis *et al.*, 1993), there might be a back-up in the VPS pathway caused by the class C mutant block that slows PVC vesiculation. Alternatively, PVC membrane involution and vesiculation might be reversible. A retrograde pathway from the vacuole to the Golgi was recently shown to operate via the late endosome/PVC (Bryant *et al.*, 1998). Interestingly, transit from the PVC to the Golgi in this pathway does not depend on Vps45, a class D factor. The suppression of *doa4*Δ by *vps45*Δ might reflect a retrograde exit of ubiquitinated membrane proteins from the PVC to the Golgi, where they may be deubiquitinated by other Dubs, whereas anterograde rerouting of the ubiquitinated proteins back to the PVC is blocked by loss of Vps45 (Beck *et al.*, 1999).

Recent results suggest the possibility of dynamic ubiquitination/deubiquitination cycles along the endocytic pathway, and these events may help determine whether an endocytosed membrane protein will continue toward the vacuole or be routed to another compartment. In mammalian cells, ubiquitination of the EGF receptor appears to occur at an endosomal compartment (Levkowitz *et al.*, 1998). Components of the endocytic machinery itself may also be targets for reversible ubiquitin additions. For example, Eps15, an endocytosis factor that is required for ligand-induced EGF receptor uptake, is ubiquitinated in response to EGF binding (van Delft *et al.*, 1997), and genetic data strongly implicate the endocytosis factor epsin, which binds Eps15, as a key target of the *Drosophila* fat facets deubiquitinating enzyme (Cadavid *et al.*, 2000). The possibility of Dubs acting early in the endocytic pathway suggests a model for *vps/did* suppression of *doa4*Δ (see below). It also raises the question of whether in yeast there are also internal (re)ubiquitination events necessary for trafficking to the vacuole. Conjugation to Lys-63–linked ubiquitin oligomers enhances but is not absolutely necessary for the endocytosis and degradation of certain membrane proteins (Galan and Haguenauer-Tsapis, 1997; Springael *et al.*, 1999). Free Lys-63–linked chains are synthesized by the Ubc13 E2 isozyme, which requires formation of a complex between Ubc13 and another E2-related protein, Mms2, which, like Vps23, also lacks a catalytic Cys residue (Hofmann and Pickart, 1999). By analogy, Vps23, perhaps with Ubc13 or Ubc4 (Arnason and Ellison, 1994), might help (re)ubiquitinate endosomal membrane proteins or extend their monoubiquitin additions, allowing them to concentrate at PVC invagination sites, after which Doa4 is recruited to cleave off the ubiquitin tag. Unlike wild-type ubiquitin, supplementation of a *doa4*Δ strain with a ubiquitin–K63R mutant fails to rescue many of its defects (Swaminathan *et al.*, 1999).

Mechanisms of Suppression of doa4 Proteasomal Degradation Defects

A major question raised by the present work is how the *did/vps* class E mutations suppress the majority of the *doa4*Δ phenotypic aberrations, particularly its defects in proteasomal degradation. As described previously, loss of Doa4 can inhibit proteasomal degradation by several mechanisms (Swaminathan *et al.*, 1999). A decrease in cellular ubiquitin levels engendered by abnormal degradation of ubiquitin is sufficient to account for the defect in degradation of some substrates, e.g., the Matα2 repressor. However, the defective degradation of other proteins such as N-end rule substrates even when the mutant cells are supplemented with extra ubiquitin indicates an additional point(s) of inhibition. Although the *did* mutations restore ubiquitin to normal or near-normal levels, most of them also suppress the defect in N-end rule substrate proteolysis, which cannot be explained by the increase in cellular ubiquitin. Conversely, the *doa4*Δ defect in Ub-Proβgal degradation was not strongly suppressed by any of the tested *did*Δ mutations, and N-end rule substrate degradation was also not restored in *did2*Δ *doa4*Δ cells. Hence, whatever the bypass mechanism(s), it does not rescue proteasomal degradation completely in the *did*Δ *doa4*Δ double mutants.

As a working hypothesis, we propose that the impairment of the VPS and/or endocytic pathways in *doa4*Δ cells allows another deubiquitinating enzyme(s) to reach many of the targets normally acted on by Doa4, including ubiquitinated proteasomal substrates and protein remnants. The Did factors may normally sequester or otherwise negatively regulate such a Dub. There is accumulating evidence for multiple ubiquitination/deubiquitination steps along the endocytic pathway (see above), and inhibition of deubiquitination at particular points along the pathway may be needed to allow (re)ubiquitination of a receptor target or endocytic factor, e.g., to allow formation of an alternative ubiquitin structure such as a Lys-63–linked ubiquitin oligomer. For instance, a Dub that is recruited earlier in the endocytic pathway might normally be inactivated when specific Vps factors assemble onto the membrane. Failure to form or recycle a normal class

E complex may result in release of the Dub such that it can act on soluble ubiquitinated substrates and possibly even proteasome-bound substrates. The model predicts that inactivation of the putative endocytosis-associated Dub in a *did*Δ *doa4*Δ background will impair proteasome-mediated degradation, but this defect should be reversed by provision of functional Doa4. A genetic screen to test this and related predictions is being developed.

ACKNOWLEDGMENTS

We thank Antony Cooper, William Green, Dan Gottschling, and Pam Silver for antibodies and Rob Piper and Markus Babst for plasmids and strains. This work was supported by National Institutes of Health grant GM53756 and a Fletcher Scholar Award from the Cancer Research Foundation of Chicago to M.H.

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