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

Alexander Y. Amerik,* Jonathan Nowak,† Sowmya Swaminathan,† and Mark Hochstrasser*‡

*Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06520; and †Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, Illinois 60637

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/pervacuolar 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/pervacuolar 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 Hagenauer-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).
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.
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-βgal degradation in the doa-1 mutant MYH11D5-8a (Papa and Hochstrasser, 1993). Seven plasmid-independent revertants were identified from -70,000 colonies. Backcrossing revealed that the suppressor mutations were unlinked to doa-1, and suppression in all cases was recessive. The mutants were sorted into five complementation groups based on matings between different did doa-1 double mutants. When separated from the doa-1 allele, all of the mutants except did3-1 were found to have readily scored recessive defects as well. During several unsuccessful attempts to clone DID5 by suppression of Deg1-βgal degradation in did3-1 doa-1 cells, we also performed control transformations of the doa-1 strain MYH186 with an empty vector. Five additional did suppressors unlinked to doa-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 did3-1 doa-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 restated 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 DID 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 NotI, and subcloned into the CEN/URA3 yeast–E. coli shuttle vector pRS316 (Sikorski and Hieter, 1989). The pRS316-T7-DID2 plasmid was transformed into MYH1234 cells (did2Δ::HIS5). Because the putative DID2/YKR035w-A ORF was completely bracketed by the YKR035w ORF on the opposite strand, selection for 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 YKR035w. 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 MYH606 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 outmost DID4 primers. The resulting DNA fragment contained the LEU2 gene flanked by 400 bp of DID4 upstream and downstream sequences. After transformation of MYH606 with this PCR fragment, the diploid was sporulated and tetrads were dissected as described above.

Plasmids carrying vps27Δ::LEU2 and vps45A::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 YEpplac195 (Gietz and Sugino, 1988). The resulting plasmids were transformed into the various dida 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. ScaI and XhoI restriction sites were built into the 5′ and 3′ primers, respectively, and PCR products were digested with ScaI and XhoI 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 HindIII and KpnI and subcloned into YIp352. The resulting plasmid was cleaved with BglIII, 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.
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 [35S]-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 OD600 ≈ 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 OD600 ≈ 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 MgCl2). Cells were collected by centrifugation, washed with 10 ml of buffer C (0.1 M potassium phosphate, pH 6.8, 0.5 mM MgCl2, 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 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 OD600 ≈ 1, harvested, and resuspended in 200 μl of zymolase buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 1 mM DTT, 1 M sorbitol) supplemented with yeast nitrogen base [YNB plus (NH4)2SO4], glucose, and amino acids. Zymolase 100T (10 μg/2 × 106 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 [35S]-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-
Although the did5-1 doa4-1 cells with the exception of these defects were also partially suppressed in at high temperatures (Papa and Hochstrasser, 1993). Both of active to the arginine analogue canavanine and grows poorly canavanine and temperature sensitivities, the complementing activity in each case except for inserts allowed identification of the genes responsible for the proteasome pathway, the degradation defect was nearly completely sup-

**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 17 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 did2Δ 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
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 isolectric 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 did3Δ, 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-dystichiasis 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...
and ubp14 at 37°C, respectively. Therefore, suppression by the did mutations was specific to doa4 than for the single mutants, most strikingly for overexpression results in little if any suppression of Leu-2 degradation defects caused by mutation of the DID genes. The inability of did2Δ but not other did deletions to suppress the doa4Δ 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 Δ 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.
To investigate this idea, we examined ubiquitin and ubiquitin conjugates in the various mutants by anti-ubiquitin immunoblotting (Figure 6). In all of the doa4Δ didΔ 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 doa4Δ with did1Δ, did3Δ, or did4Δ, these ubiquitinated species were present at very low concentrations. In the doa4Δ did2Δ double mutant, the levels of the small conjugates were slightly but reproducibly higher, a finding congruent with the weaker proteolytic suppression by did2Δ (see above). Interestingly, both didΔ single and doa4Δ didΔ 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 did1Δ and doa4Δ did1Δ 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 mis-sorting of newly synthesized proteins.
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 did2Δ and did4Δ mutants was analyzed by pulse-chase experiments. Spheroplasts were radiolabeled with 35S-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 doa4Δ 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 didΔ mutants, a significant fraction of a p2-like CPY isoform was secreted into the culture medium. Therefore, did2Δ and did4Δ 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 did2Δ and did4Δ 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 didΔ 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 didΔ cells, presumably as a result of ubiquitin limitation, inasmuch as did4Δ has the same effect on the Ste2 receptor (Terrell et al., 1998) and uracil permease (Galan and Hagnauer-Tsapis, 1997). Collectively, these observations establish Did2 and Did4 as novel class E Vps proteins.

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, doa4Δ, and doa4Δ didΔ 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).
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 doa4Δ degradation defect, we constructed doa4Δ vps45Δ, doa4Δ vps33Δ, and doa4Δ ypt1-A136D double mutants and measured Deg1-βgal, Leu-βgal, and/or a2 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 doa4Δ cells (Figure 9A). In contrast, the ypt1-A136D mutation, when introduced into doa4Δ cells, did not change the rate of Deg1-βgal or a2 degradation (Figure 9B). We also attempted to test in doa4Δ 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 doa4Δ, 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 doa4Δ 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 vps4Δ 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 vps4Δ 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 vps4Δ 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-
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 \textit{vps4}\textsuperscript{\Delta} 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 \textit{vps4}\textsuperscript{\Delta}-specific: no relocalization of Doa4–GFP was observed in \textit{did1}\textsuperscript{\Delta} and \textit{did3}\textsuperscript{\Delta} 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 \textit{doa4}\textsuperscript{\Delta} mutant does not have an obvious \textit{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 7.** Vacuolar protein-sorting defect of \textit{did} mutants. (A) Aberrant secretion of a vacuolar enzyme in the \textit{did} mutants. Sorting of the vacuolar hydrolase CPY was determined by pulse-chase analysis. Cells spheroplasts were labeled for 10 min with \textsuperscript{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 \textit{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 \textit{did1} mutant, although this has not been tested. (B) FM 4-64 staining of \textit{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.

**Figure 8.** Ste3 receptor turnover in \textit{doa4}\textsuperscript{\Delta} and \textit{did}\textsuperscript{\Delta} 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 \textmu l) at the indicated times.
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 vps4Δ cells might require other class E factors. To test this, we examined Doa4–GFP localization in did1Δ vps4Δ and did3Δ vps4Δ (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 doa4Δ 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
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 didΔ by vps45Δ, 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 didΔ 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 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 didΔ by vps45Δ 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 didΔ (see below). It also raises the question of whether in yeast there are also internal (re)ubiquitination events necessary for trafficking to the vacuole. Conjunction to Lys-63-linked ubiquitin oligomers enhances but is not absolutely necessary for the endocytosis and degradation of certain membrane proteins (Galan and Hagnenauer-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 didΔ strain with a ubiquitin–K63R mutant fails to rescue many of its defects (Swaminathan et al., 1999).

Mechanisms of Suppression of didΔ Proteasomal Degradation Defects

A major question raised by the present work is how the did/vps class E mutations suppress the majority of the didΔ 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 Mata2 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 didΔ defect in Ub-Proβgal degradation was not strongly suppressed by any of the tested didΔ mutations, and N-end rule substrate degradation was also not restored in didΔ didΔ cells. Hence, whatever the bypass mechanism(s), it does not rescue proteasomal degradation completely in the didΔ didΔ double mutants.

As a working hypothesis, we propose that the impairment of the VPS and/or endocytic pathways in didΔ 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 del ∆ doa4 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.

REFERENCES


