WW Domains of Rsp5p Define Different Functions: Determination of Roles in Fluid Phase and Uracil Permease Endocytosis in *Saccharomyces cerevisiae*

Beata Gajewska,* Joanna Kamińska,* Alicja Jesionowska,* Nancy C. Martin,† Anita K. Hopper‡ and Teresa Żołądek*

**Department of Genetics, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, 02-106 Warsaw, Poland, †Department of Biochemistry, University of Louisville, Louisville, Kentucky 40292 and ‡Department of Biochemistry and Molecular Biology, Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033*

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ABSTRACT

Rsp5p, ubiquitin-protein ligase, an enzyme of the ubiquitination pathway, contains three WW domains that mediate protein-protein interactions. To determine if these domains adapt Rsp5p to a subset of substrates involved in numerous cellular processes, we generated mutations in individual or combinations of the WW domains. The *rsp5-w1*, *rsp5-w2*, and *rsp5-w3* mutant alleles complement *RSP5* deletions at 30°. Thus, individual WW domains are not essential. Each *rsp5-w* mutation caused temperature-sensitive growth. Among variants with mutations in multiple WW domains, only *rsp5-w1w2* complemented the deletion. Thus, the WW3 domain is sufficient for Rsp5p essential functions. To determine whether *rsp5-w* mutations affect endocytosis, fluid phase and uracil permease (Fur4p) endocytosis was examined. The WW3 domain is important for both processes. WW2 appears not to be important for fluid phase endocytosis whereas it is important for Fur4p endocytosis. In contrast, the WW1 domain affects fluid phase endocytosis, but it does not appear to function in Fur4p endocytosis. Thus, various WW domains play different roles in the endocytosis of these two substrates. Rsp5p is located in the cytoplasm in a punctate pattern that does not change during the cell cycle. Altering WW domains does not change the location of Rsp5p.

RSP5P is an ubiquitin-protein ligase, an E3 enzyme of the ubiquitination pathway essential for cell growth (Hein *et al.* 1995; Huibregtse *et al.* 1995). It is a member of the family of NEDD4-like proteins that share a common modular structure: they contain C2, WW, and hect domains (Harvey and Kumar 1999). The C2 domain, located at the N terminus, interacts with Ca^{2+}, lipids, and proteins (Nalefski and Falke 1996). Two to four WW domains that are involved in protein-protein interactions follow the C2 domain (Chen and Sudol 1995). Rsp5p has three WW domains. The ubiquitin-protein ligase catalytic hect domain, shared by many other ubiquitin ligases, is located at the C terminus (Huibregtse *et al.* 1995).

WW domains are characterized by two highly conserved tryptophan (W) residues and a proline (P) residue in a stretch of 38–40 amino acids. They interact with polyproline-rich sequences through a small hydrophobic pocket formed by three anti-parallel β-sheets (Macias *et al.* 1996; Ranganathan *et al.* 1997). WW domains can be subdivided into three groups. Group I WW domains interact with core sequence PPXY (Chen and Sudol 1995), group II WW domains interact with stretches of prolines interrupted by leucine (PPLP; Chan *et al.* 1996; Bedford *et al.* 1997; Ermekova *et al.* 1997), and group III WW domains interact with polyproline-rich regions containing glycines and methionines (PGM motif; Bedford *et al.* 1998). Binding of ligands by group I and II WW domains is abolished by the substitution of the second conserved W to phenylalanine (F) or P to alanine (A; Chen *et al.* 1997; Ermekova *et al.* 1997). Recently, phosphoserine/phosphothreonine (pT/pS)-containing proteins and peptides have been shown to interact with particular WW domains, including those in NEDD4 (Lu *et al.* 1999). This binding was phosphorylation dependent and required the second W of the WW domain (Lu *et al.* 1999).

NEDD-like proteins are implicated in many diverse cellular functions (Harvey and Kumar 1999). Mutations of *RSP5* affect transcription (cited in Huibregtse *et al.* 1995), perhaps via modification of the large subunit of RNA polymerase II (Huibregtse *et al.* 1997). Rsp5p influences the turnover of at least three plasma membrane proteins, the general amino acid permease (Hein *et al.* 1995), the uracil permease (Galan *et al.* 1996), and the maltose transporter (Lucero and Lagunas 1997). Ubiquitination of these proteins is a signal for their internalization, endocytosis, and degradation in the vacuole. Rsp5p is also involved in glucose regulation of plasma membrane H^+-ATPase (de la Fuente *et al.* 1997). Furthermore, Rsp5p has been proposed to play a role in minichromosome maintenance (Yashiroda *et*

Corresponding author: Teresa Żołądek, Department of Genetics, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawińskiego 5a, 02-106 Warsaw, Poland. E-mail: teresa@ibb.waw.pl

al. 1996) and mitochondrial inheritance and morphology (Fisk and Yaffe 1999).

We identified *rsp5/mdp1* in a search for mutations that affect mitochondrial/cytoplasmic distribution of Mod5p-I, a tRNA modification enzyme (Żołądek *et al.* 1995). We also showed that some alleles cause a defect in fluid phase endocytosis (Żołądek *et al.* 1997). Suppressor analysis revealed that mutations of *PMA1*, the gene encoding a plasma membrane H^+-ATPase, are able to suppress growth defects caused by Rsp5p hect domain mutations, but do not suppress the Mod5p-I distribution defect. Subsequently, Kamińska *et al.* (2000) showed that *rsp5/mdp1* mutants are pH, paromomycin, and hygromycin B sensitive.

To account for the role of Rsp5p in diverse cellular processes we proposed that the individual WW domains in Rsp5p interact with different proteins and direct it to multiple targets (Żołądek *et al.* 1997). This hypothesis predicts that alterations of individual WW domains will affect a subset of the processes affected by hect domain mutations. To test this hypothesis we created mutations in each of the WW domains of Rsp5p and scored the mutant alleles for their ability to complement pH sensitivity, temperature sensitivity, paromomycin challenge, and endocytosis. As the hypothesis predicts, individual WW domain mutations clearly affect these phenotypes differentially.

MATERIALS AND METHODS

Strains and growth conditions: The *Saccharomyces cerevisiae* strains T8-1D *MAT*α *SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu2-3, 112 his4-519*, MB105-6A *MAT***a** *SUP11 ade2-1 mod5-1 ura3-1 lys2-1 leu2-3,112 trp5 met4* (Żołądek *et al.* 1995), TZ23 *rsp5-13* (Żołądek *et al.* 1997), isogenic to T8-1D, and TZ19 *rsp5-9*, a derivative of MB105-6A, were used. Protease-deficient strain BJ2168 (Jones 1991) was used for cell fractionations. Other yeast strains were derived as described in the text.

YPD, YPG, YPDG, SC, and sporulation media were prepared and genetic manipulations were performed as described by Sherman (1991). 5-Fluoroorotic acid (5-FOA)-containing plates were prepared according to Boeke *et al.* (1987). YPD plates buffered to pH 6.5 or 5.2 with 0.2 M sodium acetate and acetic acid were prepared as described by Kamińska *et al.* (2000). Antibiotic sensitivity was determined on YPD plates containing 1 mg/ml paromomycin or 0.2 mg/ml geneticin. Growth of strains on various plates was compared by a drop test (Kamińska *et al.* 2000).

Plasmids, epitope tagging, and site-directed mutagenesis: The *Eco*RI-*Sph*I 4.9-kb fragment of RB1 (Żołądek *et al.* 1997) was transferred into YCplac33 (*URA3*; Gietz and Sugino 1988) to generate YCp-RSP5. This plasmid complements the temperature-sensitive (ts) phenotype of *rsp5/mdp1*. The tagged version of *RSP5* (*HA-RSP5*) was constructed by inserting the *Not*I fragment from pBF30 (Tyers *et al.* 1992), which encodes a triple-hemagglutinin (HA) epitope, into a *Not*I site introduced by PCR (Hemsley *et al.* 1989). The *Not*I site was located after the ATG initiation codon of the *RSP5* open reading frame (ORF) in the *Sal*I-*Pst*I fragment of *RSP5*. The *Sal*I-*Pst*I fragment of YCp-RSP5 was replaced by a fragment containing an HA tag to generate YCpHA-RSP5. YCpHA-RSP5 fully complements *rsp5/mdp1* mutations. All subsequent constructs employed the tagged *HA-RSP5* gene.

Mutations in *Bam*HI-*Bam*HI and *Kpn*I-*Kpn*I fragments of RB1 cloned in pUC19 were introduced by reverse PCR (Hemsley *et al.* 1989) using gene-specific primers containing mutations to make a new *Dra*I or *Bsa*HI site or destroy the *Bam*HI. All altered DNAs were fully sequenced. The altered DNAs were inserted into YCpHA-RSP5 to replace wild-type sequences. Versions of *RSP5* containing mutations in multiple WW domains were obtained by replacing appropriate wild-type fragments with mutant fragments using *Pst*I and *Age*I restriction sites. The *Eco*RI-*Sph*I fragments of plasmids containing *RSP5* or the mutant genes *rsp5-w1*, *rsp5-w2*, and *rsp5-w3* were also cloned in YCplac111 (*LEU2*; Gietz and Sugino 1988).

A chromosomal deletion of *RSP5* was made in the diploid strain T108 **a**/α *SUP11 mod5-1 ade2-1 ura3 leu2-3, 112 lys2-1 his4-519* obtained by mating T8-1D and a derivative from a cross of T8-1D and MB105-6A. The *Eco*RI-*Sph*I 4.9-kb fragment of RB1 was cloned into pUC19. The *Sal*I-*Mun*I fragment of *RSP5* containing part of the promoter and most of the ORF was replaced by the 1.5-kb *Sal*I-*Eco*RI fragment of *kanMX4* from pFA6a-kanMX4 (Wach *et al.* 1994), after filling in the ends using polymerase, to generate pBG61. A linear 5-kb fragment of pBG61 was isolated by digestion with *Eco*RI and *Kpn*I. This fragment was transformed into T108 and transformants resistant to geneticin were selected. Deletion of one chromosomal copy of *RSP5* was confirmed by PCR in BG1. We obtained two viable and two lethal spores from heterozygous diploid BG1 tetrads. Thus *RSP5* is an essential gene. As expected no kan^R haploid progeny germinated. Viable Δ*rsp5::kan* [YCpHA-RSP5] haploids were obtained by sporulation of the BG1 diploid harboring YCpHA-RSP5. The strain BG1-1C was used in further studies.

The mutant *rsp5-9* allele was amplified by PCR from genomic DNA. The gene was cloned in pUC19 and sequenced. The presence of the *rsp5-9* mutation was confirmed in three independent clones. The *Age*I-*Mun*I fragment containing the *rsp5-9* mutation was substituted for the same fragment in YCpHA-RSP5 to obtain YCpHA-rsp5-9.

Endocytosis assays: Fluid phase endocytosis was monitored employing Lucifer yellow (LY) as a marker (Dulic *et al.* 1991). Cells were grown at 30° and incubated with LY at 30° for 30 min or 1 hr and 30 min. A Nikon Microphot-SA fluorescence microscope was used to observe LY accumulation in the vacuole and vacuolar morphology was viewed by differential interference contrast (DIC) optics.

Uracil uptake was measured in exponentially growing cells expressing Fur4p, a uracil permease, from p195gF containing a *GAL10-FUR4* fusion (Volland *et al.* 1994) at various times after cycloheximide addition. One milliliter of yeast culture was incubated with 5 μM [^{14}C]uracil (Amersham, Little Chalfont, UK) for 20 sec at 30° and then filtered through Whatman GF/C filters, washed twice with ice-cold water, and the radioactivity was measured. At the same time, samples were harvested to analyze Fur4p abundance by Western blotting (see below).

Immunofluorescence: For immunofluorescence cells were grown on glucose selective medium and processed (Boguta *et al.* 1994). The HA-Rsp5p was localized by indirect immunofluorescence using the 16B12 monoclonal anti-HA antibody (Babco) and Cy3-conjugated secondary anti-mouse antibody (Jackson ImmunoResearch Laboratories). Mdm1p was localized using rabbit anti-Mdm1p antibody (McConnel and Yaffe 1992) and anti-rabbit FITC-conjugated secondary antibody. Cells were viewed by fluorescence microscopy using a Microphot-SA (Nikon, Tokyo, Japan). Images were collected using a Photometrix CH350A camera.

Cell fractionation, protein extraction, and immunoblot analysis: Cellular extracts to study Fur4p degradation or Rsp5p

stability were prepared by alkaline lysis (Volland *et al.* 1994). Cellular extracts for nuclear fractionation were prepared from spheroplasts by homogenization. Control organellar and cytosolic fractions were obtained by centrifugation at 300,000 × *g* for 20 min and the nuclear fraction was separated on a Ficoll step gradient according to Dove *et al.* (1998). Cellular fractionation was also performed by differential centrifugation. BJ2168 cells expressing YCpHA-RSP5 or vector alone were grown, washed, and treated essentially as described by Hoffman and Chiang (1996). Spheroplasts were homogenized and lysate was centrifuged at 300 × *g* for 15 min to remove unbroken cells. The 300 × *g* supernatant was fractionated by centrifugation at 13,000 × *g* for 15 min to obtain a pellet (P13K) and supernatant. The 13,000 × *g* supernatant was spun at 100,000 × *g* for 2 hr to obtain a pellet and supernatant. The 100,000 × *g* supernatant was spun again at 200,000 × *g* for 2 hr to yield the pellet and supernatant. Crude mitochondrial fractions were obtained using a published procedure (Glick and Pon 1995). Fractions were analyzed by Western blotting.

To test the nature of interaction of Rsp5p with cellular structures, the 13,000 × *g* pellet (P13K) was suspended in 0.1 M Tris-HCl, 0.15 NaCl, 5 mM EDTA, pH 7.5, and equal portions were treated with buffer alone, 0.1 M Na_2CO_3, pH 11, 2 M urea, or 1% Triton X-100 for 1 hr in ice. Samples were then separated by centrifugation at 13,000 × *g* for 15 min. Each resulting pellet was suspended in the same volume as the original supernatant and the samples were analyzed by Western blotting.

Proteins were resolved by SDS-PAGE or on polyacrylamide SDS-Tricine gels (for Fur4p detection). Mouse monoclonal antibodies 16B12 anti-HA (Babco) and 32D6 for nuclear pore protein Nsp1p (Tolerico *et al.* 1999) and rabbit polyclonal antibodies specific for α-enolase (from M. J. Holland), 60-kD subunit of V-ATPase, *VMA1* gene product (Molecular Probes, Eugene, OR), Mdh1p (from G. Schatz), and uracil permease (Volland *et al.* 1994) were used according to the suppliers' instructions. Primary antibodies were detected with horseradish-peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibodies followed by enhanced chemiluminescence (Amersham).

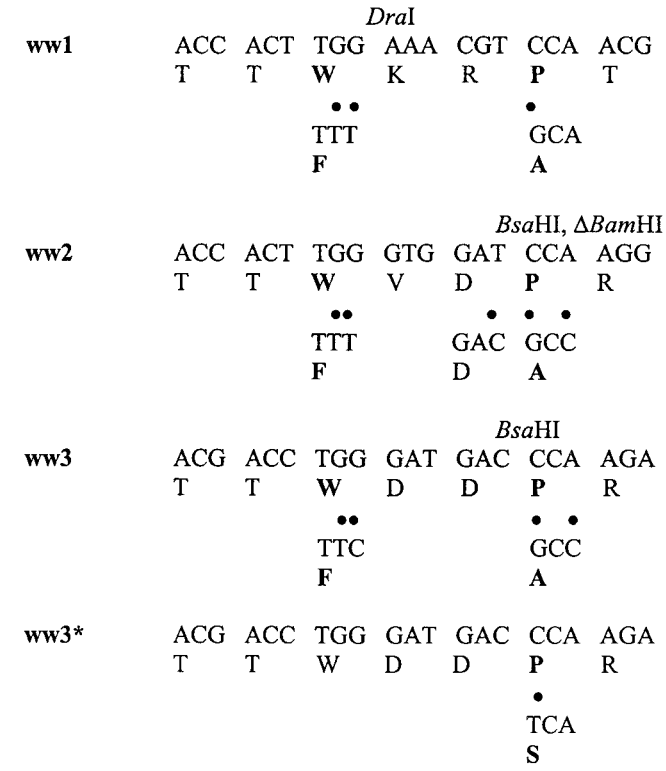

Figure 1.—Mutations in *RSP5* WW domains. The upper line is the nucleotide sequence of the wild-type *RSP5* WW domains. The lower line is the deduced amino acid sequence. The positions of nucleotide changes in *rsp5-w1* (ww1), *rsp5-w2* (ww2), *rsp5-w3* (ww3), and *rsp5-9* (ww3*) are indicated (●). The nucleotides and amino acid substitutions are shown below the wild-type sequence. New restriction sites for *Dra*I, *Bsa*HI, and the position of the destroyed *Bam*HI site are indicated above the wild-type sequence.

RESULTS

Individual WW domains contribute to Rsp5p function, but are not essential: All previously characterized *rsp5/mdp1* mutants have amino acid substitutions in the hect domain (Żołądek *et al.* 1997). We report here that *rsp5-9/mdp1-9* has a mutation in codon 418, causing a replacement of proline (P) to serine (S) in the WW3 domain, and refer to it as *rsp5-9* throughout. We compared the growth of the *rsp5-9* mutant to the *rsp5-13* mutant (previously *mdp1-13*), which has an amino acid substitution G(707)D in the hect domain of Rsp5p (Żołądek *et al.* 1997). Both failed to grow at 37° and were sensitive to paromomycin and to pH 5.2 (not shown). Therefore, mutations of a WW domain can influence Rsp5p function.

To analyze systematically the function of each WW domain of Rsp5p, alterations were introduced in WW domains individually and in combinations. Mutations were designed to abolish the interaction of Rsp5p WW domains with other proteins by altering two conserved amino acids. In each WW domain the conserved W was changed to F and the nearby P was changed to A (Figure 1). W and P of WW domains in other proteins are important for ligand (PPXY or PPLP motif) interactions (Chen *et al.* 1997; Ermekova *et al.* 1997).

Variants of *RSP5*, *rsp5-w1*, *rsp5-w2*, and *rsp5-w3*, *rsp5-w1w2*, *rsp5-w1w3*, *rsp5-w2w3*, and *rsp5-w1w2w3*, were transformed into a ts *rsp5-13*-containing strain. Transformants were tested for growth at various temperatures and on media with varying pH and in the presence of paromomycin. YCpHA-rsp5-w1 complemented the ts phenotype, paromomycin sensitivity, and, partially, the pH sensitivity of the hect domain mutant *rsp5-13*. YCpHA-rsp5-w2 was less effective and YCpHA-rsp5-w3 did not complement these phenotypes (Figure 2A). The double *rsp5-w1w2* mutant complemented the ts phenotype of *rsp5-13* similarly to *rsp5-w2* and the other multiple mutants tested did not (Figure 2B). The *rsp5-9* mutation was complemented by *rsp5-w* mutations very similarly as *rsp5-13*: *rsp5-w1* was most effective, *rsp5-w2* was partially effective, and *rsp5-w3* did not complement ts phenotype (data not shown). The growth of transformants was also similar to growth of the respective strains expressing *rsp5-w* on the deletion *rsp5::kan* background (see below),

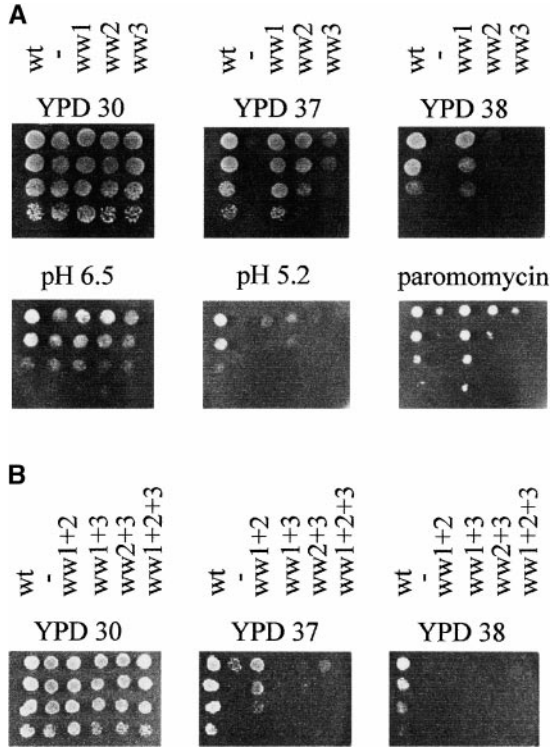

FIGURE 2.—Complementation of *rsp5-13* by *rsp5-w* mutations. (A) The growth of transformants of TZ23 expressing *RSP5* (wt), *rsp5-w1* (ww1), *rsp5-w2* (ww2), or *rsp5-w3* (ww3) from a single-copy plasmid or vector alone (-) on YPD at temperatures indicated and on YPD buffered to pH 6.5 or 5.2 or containing paromomycin at 30° after 2 or 4 days of incubation. (B) The growth of transformants of TZ23 expressing *RSP5* (wt), *rsp5-w1w2* (ww1+2), *rsp5-w1w3* (ww1+3), *rsp5-w2w3* (ww2+3), or *rsp5-w1w2w3* (ww1+2+3) from a single-copy plasmid or vector alone (-) on YPD at temperatures indicated.

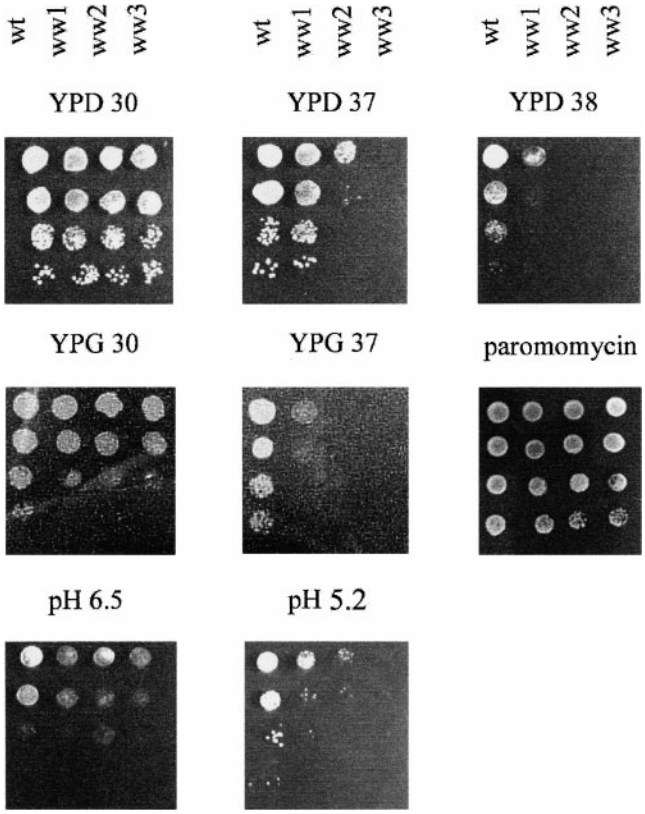

FIGURE 3.—The *rsp5-w* mutants show growth defects and are sensitive to low pH. The growth of BG1-1C strain expressing *RSP5* (wt), *rsp5-w1* (ww1), *rsp5-w2* (ww2), or *rsp5-w3* (ww3) from single-copy plasmid on YPD and YPG at temperatures indicated and on YPD containing paromomycin or buffered to pH 6.5 or 5.2 at 30°.

indicating lack of intragenic complementation among *rsp5-w*, *rsp5-9*, and *rsp5-13*. These results indicate that mutations in WW1 and WW2, like mutations in WW3, alter Rsp5p function.

To assess the contribution of individual WW domains to the essential *in vivo* functions of *RSP5* we tested the ability of the *rsp5-w* mutants to complement *rsp5* deletions by substituting mutant alleles for the wild-type allele in Δ*rsp5::kan* haploids using a standard plasmid shuffle protocol. All double transformants were able to lose the plasmid bearing wild-type *RSP5*. Therefore, individual WW domains of Rsp5p are not essential. The isogenic strains Δ*rsp5::kan* expressing plasmid-encoded wild-type or mutant *rsp5* alleles were tested for growth in various conditions. All strains bearing *rsp5-w* mutations were temperature and pH sensitive and the effect was the most severe for *rsp5-w3* (Figure 3). Similar effects were seen when genomic *RSP5* copy was replaced by *rsp5-w3* in different genetic background (our unpublished data). All mutant strains grew similarly on glycerol-containing medium at the permissive temperature, but slower than wild type, and the mitochondrial distribution appeared the same in all mutants as assessed by 4′,6-diamidino-2-phenylindole (DAPI) staining (data not shown). Sensitivity to paromomycin was similar to wild type for all mutants (Figure 3, *rsp5-9* not shown). This result was surprising since the original *rsp5-9* mutant was paromomycin sensitive and *rsp5-w2* and *rsp5-w3* alleles did not complement paromomycin sensitivity in *rsp5-13* (see Figure 2). We do not have an explanation for this contradiction at this time but it could be related to plasmid copy number.

The *rsp5-w* and *rsp5-9* mutant strains expressed wild-type levels of mutant Rsp5p at 30° (Figure 4, *rsp5-9* not shown). However, less Rsp5p was present in *rsp5-w2* and *rsp5-w3* when cells were incubated at 37° for 2 hr (Figure 4). This effect was most pronounced for Rsp5p-w3. Thus, it is likely that the mutant proteins are either less stable or poorly expressed at the nonpermissive temperature. Lower levels of Rsp5p could account for the ts phenotype of the respective strains, but not for the pH sensitivity observed at the permissive temperature of 30°.

The growth characteristics of strains with mutant WW domains of Rsp5p indicated that individual domains do not play an essential function. However, they contribute differently to the nonessential functions of Rsp5p. WW3

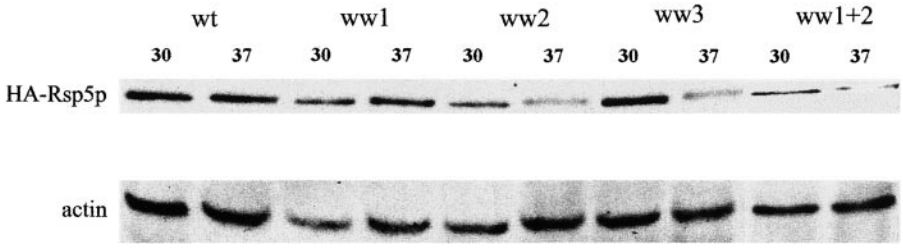

FIGURE 4.—Rsp5p-w2, Rsp5p-w3, and Rsp5p-w1w2 are unstable at 37°. Rsp5p abundance in cells of BG1-1C expressing *RSP5* (wt), *rsp5-w1* (ww1), *rsp5-w2* (ww2), *rsp5-w3* (ww3), and *rsp5-w1w2* (ww1+2) from single-copy plasmid at 30° and after a 2-hr shift to 37°. Actin serves as a protein-loading control.

is the most important for growth at elevated temperatures and growth on media of low pH.

The only WW3 domain is sufficient for Rsp5p essential functions *in vivo*: Since the results showed that individual WW domains of Rsp5p are not essential, we asked if any single WW domain can perform all essential Rsp5p functions and if this ubiquitin ligase requires the function of any WW domain. To address these questions *rsp5* alleles were generated with all possible combinations of two mutant WW and three mutant WW domains. A diploid BG1 strain heterozygous for a deletion of *RSP5* transformed with *rsp5-w1w2*, *rsp5-w1w3*, *rsp5-w2w3*, or *rsp5-w1w2w3* alleles yielded only four viable spores from tetrads derived from YCpHA-rsp5-w1w2-transformed cells. Only two spores were viable in tetrads from cells transformed with the other alleles (not shown). The *rsp5-w1w2* mutant has growth characteristics similar to the *rsp5-w2* mutant when isogenic strains obtained were compared (not shown, compare Figure 2, A and B). These results show that this protein ligase requires at least one WW domain to carry out all essential functions and that the WW3 domain is sufficient for the essential functions, but the WW1 and WW2 domains are not. These results also indicate that the various domains may have separate functions.

Differential effects of individual WW domain mutants on fluid phase endocytosis and internalization of uracil permease: Mutations of *RSP5* affect many cellular processes and, based on growth studies, individual WW domains contribute differently to Rsp5p functions. To determine whether the *rsp5-w* mutations, like the hect domain mutations, affect endocytosis, fluid phase and uracil permease endocytosis were examined. To assay for fluid phase endocytosis, yeast cells were incubated with the fluorescent dye LY at 30° for 30 min or 1 hr and 30 min and observed by fluorescence microscopy. Wild-type strains accumulate LY in yeast vacuoles (Figure 5). Strains with *rsp5-w1*, *rsp5-w2*, or *rsp5-9* mutations accumulate LY in vacuoles to wild-type levels after 1 hr and 30 min of incubation (Figure 5). In contrast, the *rsp5-w3* mutation caused defects in LY endocytosis because no fluorescent dye was observed in vacuoles in these experiments. This block in fluid phase endocytosis was similar to that observed for *rsp5/mdp1* mutations located in the hect domain (ŻOŁĄDEK *et al.* 1997; KAMIŃSKA *et al.* 2000). However, *rsp5-w1* and *rsp5-9* accumulated less dye in vacuoles than the wild-type strain at 30 min (Figure 5), indicating that they are also defective in fluid phase endocytosis. The differences in efficiency of fluid phase endocytosis in *rsp5-w3* and *rsp5-9* indicates that the single P-to-S substitution in the WW3 domain only partially destroys its function. The WW2 domain appears unimportant, whereas WW1 has some role and WW3 is important for fluid phase endocytosis. These results indicate that individual WW domains have different roles in this process.

Ubiquitination by Rsp5p influences the turnover of several plasma membrane transporters, including the uracil permease Fur4p (GALAN *et al.* 1996). The first step of endocytosis, internalization, and the final step after endocytosis, degradation, were examined by following Fur4p in *rsp5-w* and *rsp5-9* strains transformed

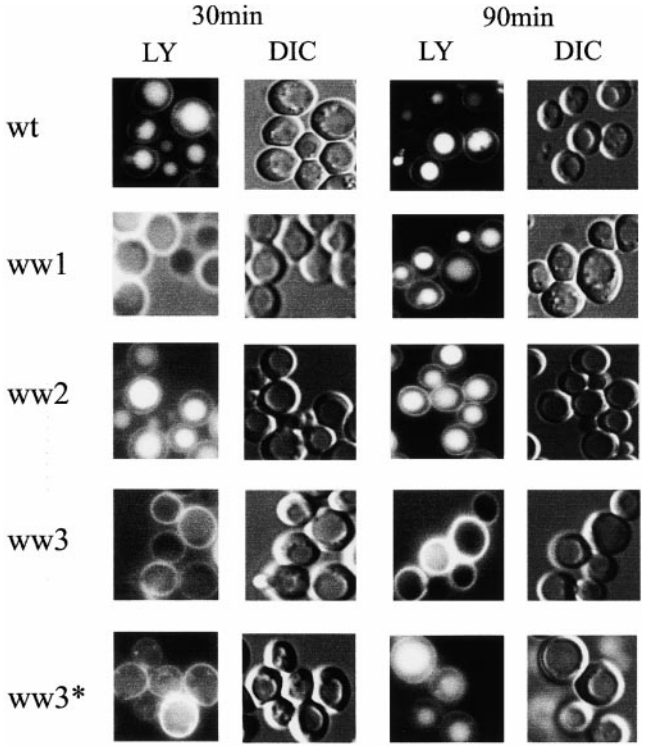

FIGURE 5.—WW1 and WW3 domains are important for fluid phase endocytosis. Lucifer yellow endocytosis assay was performed on BG1-1C expressing *RSP5* (wt), *rsp5-w1* (ww1), *rsp5-w2* (ww2), *rsp5-w3* (ww3), or *rsp5-9* (ww3*) from single-copy plasmids. Cells were incubated 30 min or 1 hr and 30 min with LY, washed, and viewed for epifluorescence and by DIC microscopy to observe vacuoles.

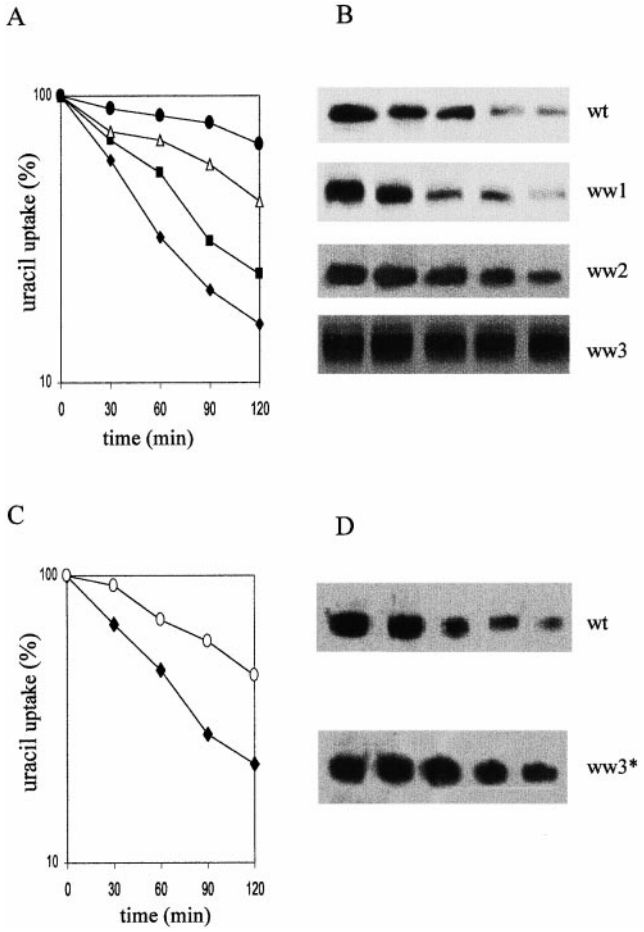

FIGURE 6.—WW2 and WW3 domains are important for Fur4p endocytosis. BG1-1C bearing plasmid YCpHA-RSP5 (◆, wt), YCpHA-rsp5-w1 (■, ww1), YCpHA-rsp5-w2 (△, ww2), YCpHA-rsp-w3 (●, ww3), or YCpHA-rsp5-9 (○, ww3*) and plasmid p195gF were grown at 24° with galactose as a carbon source. Cycloheximide (100 μg/ml) was added to the medium. (A and C) Uracil uptake at times indicated after addition of cycloheximide. (B and D) Western blot of protein extracts prepared at times indicated and analyzed for Fur4p. Representative results of three independent experiments are shown.

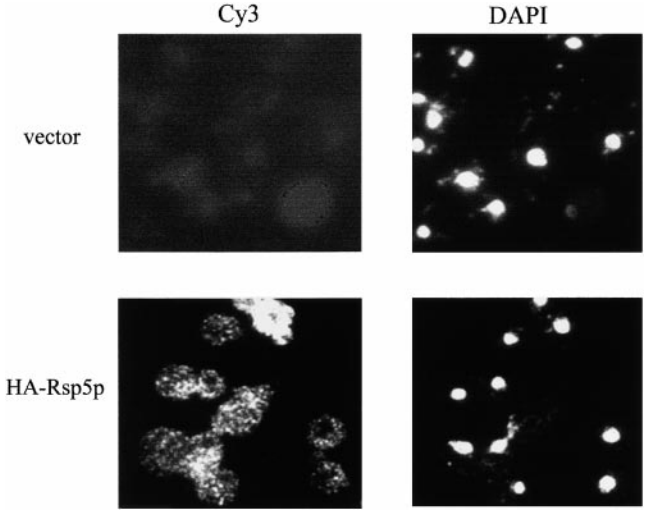

FIGURE 7.—Immunolocalization of Rsp5p. Wild-type cells (T8-1D) expressing plasmid-encoded HA-Rsp5p were processed for immunofluorescence with anti-HA monoclonal antibody and Cy3-conjugated goat anti-mouse secondary antibody and stained with DAPI to detect mitochondrial and nuclear DNA. The same strain with vector only was used as a control.

with p195gF expressing *FUR4* from the *GAL10* promoter. [^{14}C]Uracil uptake measures plasma membrane Fur4p activity while Fur4p degradation was assayed by Western blotting. Both were measured after cycloheximide stress. Mutants *rsp5-w1*, *rsp5-w2*, and *rsp5-w3* showed 1.4-, 2.5- and 6-fold (of $t_{1/2}$) delay compared to wild type in the decrease of uracil uptake after cycloheximide treatment, respectively (Figure 6A), indicating defects in Fur4p internalization. Mutant *rsp5-9* showed a delay in internalization of Fur4p similar to *rsp5-w2* and less pronounced than *rsp5-w3* (Figure 6C), again reflecting the fact that the WW3 domain is not completely defective in this mutant. The slower internalization rate led to a decrease in the rate of Fur4p degradation in *rsp5-w2*, *rsp5-w3*, and *rsp5-9* strains (Figure 6, B and D). We do not observe a decrease in the rate of Fur4p degradation in *rsp5-w1* relative to the wild type, but such a defect could be difficult to detect as the defect in Fur4p internalization is only 1.4-fold. The results from studies of Fur4p endocytosis do not completely parallel the results of fluid phase endocytosis tests. The WW1 domain plays a role in fluid phase endocytosis, but has minimal effect upon Fur4p internalization and no apparent effect upon Fur4p degradation. In contrast, the WW2 domain is important for Fur4p internalization and degradation, but it is not important for fluid phase endocytosis. The WW3 domain is important for both fluid phase and transporter endocytosis. These results underscore the generality that individual WW domains contribute differently to Rsp5p functions.

Cellular location of Rsp5p: The cellular location of Rsp5p was studied by indirect immunofluorescence microscopy of a HA-tagged Rsp5p. Rsp5p was found in the cytoplasm associated with punctate structures similar in appearance to cortical actin patches, but more abundant in number and smaller in size. The pattern of location did not change during the cell cycle nor was it polarized in dividing cells. Rsp5p was not present in the nucleus (Figure 7). Mutant Rsp5p proteins were located similarly to wild-type Rsp5p at permissive temperature (23°) or after exposure to nonpermissive conditions (2 hr at 37°), indicating that WW domains do not play a major role in Rsp5p cellular distribution.

Mutations in *RSP5* were recently found as suppressors of *mdm1* (FISK and YAFFE 1999). Mdm1p forms intermediate filaments and is involved in proper mitochondrial morphology and inheritance. Some *rsp5* mutants have mitochondrial phenotypes similar to *mdm1*. Like Rsp5p, Mdm1p is located in the cytoplasm in punctate struc-

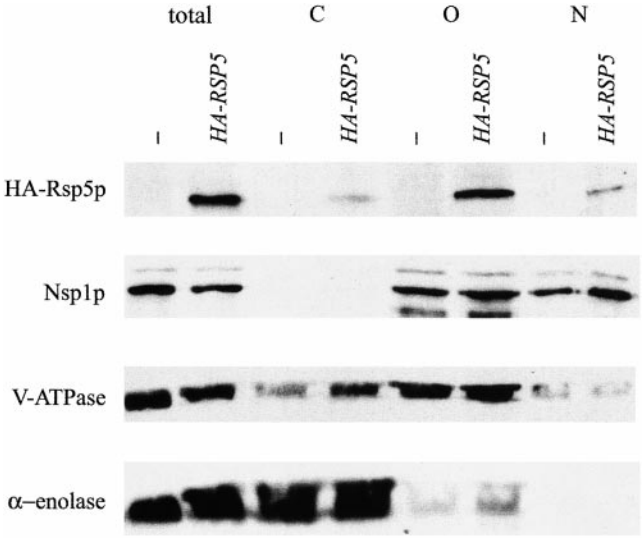

FIGURE 8.—Rsp5p is located in organellar fractions but not in the nucleus. Westen blot of cellular fractions of wild type (T8-1D) expressing the HA-RSP5 or vector only. Total, total extract; C, cytoplasmic fraction; O, membrane and organellar fraction; N, nuclear fraction. Nsp1p, V-ATPase and α-enolase are controls for nuclear, vacuolar, and cytosolic proteins, respectively.

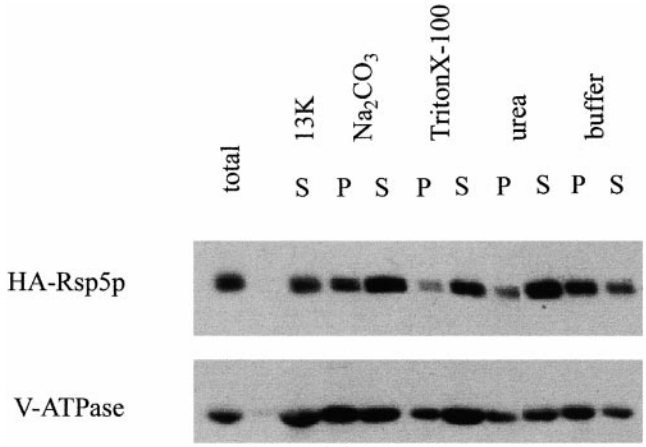

FIGURE 9.—Rsp5p might be a peripheral membrane protein. Western blot of membrane 13K fraction incubated in 0.1 M Na_2CO_3, 1% Triton X-100, 2 M urea, or buffer alone for 1 hr in ice. The samples were separated into supernatants (S) and pellets (P) by centrifugation. V-ATPase is a control.

tures (MCCONNEL and YAFFE 1992). However, when Rsp5p and Mdm1p were localized in the same cells by double indirect immunofluorescence, they did not colocalize. Moreover, the location of Mdm1p did not change in *rsp5-w* mutants at 23° or after a shift for 2 hr to 37° (not shown).

We did cell fractionations to attempt to confirm and define more precisely the cellular location of Rsp5p. Cell lysates were prepared from cells bearing YCpHA-RSP5 or vector alone and organellar and cytosolic fractions were obtained following centrifugation at 300,000 × *g* for 20 min. Aliquots of the lysates were also separated to obtain a nuclear fraction (see MATERIALS AND METHODS). The distribution of Rsp5p was examined by immunoblotting with anti-HA antibody. The results indicate that Rsp5p is either organelle associated or associated with large protein complexes that cofractionate with organelles. Rsp5p does not appear to be nuclear since the amount of Rsp5p found in the nuclear fraction was comparable to the amount of V-ATPase, a known vacuolar and vesicular membrane protein (Figure 8). These results are consistent with the nuclear exclusion of Rsp5p observed by immunofluorescence.

Cellular extracts were also fractionated by differential centrifugation as described by HOFFMAN and CHIANG (1996). Rsp5p was enriched in the 13K pellet, containing endoplasmic reticulum, mitochondria, and other organelles, and in the 100K pellet, containing plasma membrane (not shown). Mitochondrial fractionation experiments showed that Rsp5p was not enriched in the mitochondrial fraction as compared to the control Mdh1p, which was enriched several-fold (not shown). Thus, it seems from microscopy and fractionation studies that Rsp5p might be in multiple locations. Association of Rsp5p with the putative organellar structures appeared to be rather loose since incubation of the 13K pellet fraction with buffer alone solubilized a portion of Rsp5p. Rsp5p was more efficiently released by treatment of the pellet fraction with 1% Triton X-100 or 0.1 M Na_2CO_3. Treatment with 2 M urea caused almost complete dissociation of Rsp5p (Figure 9).

DISCUSSION

We studied the effect of individual WW domain mutations on Rsp5p function to evaluate the hypothesis that they serve as protein modules that recognize different substrates destined for ubiquitination. In support of this hypothesis, we found that individual domains contribute differently to Rsp5p functions. No single Rsp5p WW domain is necessary for its essential functions. The results indicate that WW domains can partially substitute for one another or that they have partially overlapping function(s). Substitution and/or overlapping function is not complete, however, since only WW3 can support all the essential functions of Rsp5p in the absence of the other WW domains. The data indicate that WW3 is the most important Rsp5p WW domain. WW3 contributes to Rsp5p function in growth of cells at elevated temperature, growth on low-pH media, and fluid phase and Fur4p endocytosis. WW1 and WW2 are necessary for growth of cells on low-pH media, but WW2 does not appear to be involved in fluid phase endocytosis and WW1 does not appear to be involved in the turnover of Fur4p (Table 1).

Although our results indicate that the individual WW domains of Rsp5p are not essential, others have reached

TABLE 1

The summary of endocytosis results

Mutant	Fluid phase endocytosis	Fur4p-dependent uracil uptake	Fur4p turnover
wt	++	++	++
rsp5-w1	+/−	+	++
rsp5-w2	++	+/−	+/−
rsp5-w3	−	−	−
rsp5-9	+/−	+/−	+/−

the opposite conclusion. Wang *et al.* (1999) reported that WW2 and WW3 each are needed for Rsp5p essential functions. However, their study investigated complementation of a ts *rsp5-1* allele. Here, we show that lack of growth at 37° is independent of Rsp5p essential functions. At 37° Rsp5p mutant proteins are unstable or poorly synthesized and this could have led to the lack of complementation they observed.

Endocytosis in yeast is a multistep process. Solutes, ligand-bound receptors, and unneeded plasma membrane transporters are internalized via endocytic vesicles. Formation of endocytic vesicles requires coat proteins, certain lipids, and proper functioning of the actin cytoskeleton. That, in turn, depends on actin and many proteins involved in actin cytoskeleton organization. Clathrin and nonclathrin coats mediate plasma membrane vesiculation. Clathrin-coated vesicles only partially contribute to the internalization of the a- and α-factor receptors (Tan *et al.* 1993) and the maltose transporter (Mal61p; Peñalver *et al.* 1999). Clathrin is not required for Fur4p internalization (Gagny *et al.* 2000). Endocytic vesicles are subsequently transported to early and late endosomes. Finally, the cargo is delivered to the vacuole, where proteins are degraded (for review see Kaiser *et al.* 1997; Geli and Riezman 1998). Internalization and degradation of permeases such as Fur4p, Gap1p, Mal61p, Gal2p, and Tat2p require Rsp5p-dependent ubiquitination and ubiquitination precedes internalization (Galan *et al.* 1996; Hicke 1997; Horak and Wolf 1997; Lucero and Lagunas 1997; Springael and André 1998; Beck *et al.* 1999; for review see Rotin *et al.* 2000). Fluid phase endocytosis also depends on Rsp5p (Żołądek *et al.* 1997) even though the cargo is not ubiquitinated. The defect in fluid phase endocytosis underscores a role for Rsp5p in some step(s) of endocytosis that is in addition to tagging plasma membrane proteins for internalization. Involvement of Rsp5p in an internalization step, in addition to protein-cargo (Gap1p) ubiquitination, was also documented recently when Springael *et al.* (1999) showed that Rsp5p lacking the C2 domain is competent for Gap1p ubiquitination but not for its internalization.

Our experiments to study the role of Rsp5p WW domains in fluid phase endocytosis measured the final step of this path, LY accumulation in vacuoles. Therefore, this method cannot discriminate between defects in specific steps of this endocytic pathway. Similarly, an analysis of plasma membrane transporter endocytosis that measures the abundance of Fur4p focuses on the end point of a multistep endocytic process that directs this protein to the vacuole for degradation. The results show that only the WW3 domain of Rsp5p is important for both fluid phase endocytosis and Fur4p degradation. The effect of WW3 domain mutations on Fur4p is very strong, nearly completely abolishing Fur4p degradation, similar to the effect previously observed for vacuolar-protease-defective *pep4* mutants (Galan *et al.* 1996) and for Fur4p missing the PEST sequence necessary for Fur4p ubiquitination (Marchal *et al.* 1998).

The role of WW2 in endocytosis is different from the role of WW3. The effect of the mutation in the WW2 domain on Fur4p degradation is partial. It has no effect on fluid phase endocytosis. If there is only a single path to the vacuole following internalization (Figure 10) then the effect of WW2 upon Fur4p must be at one of the steps that precedes internalization, such as Fur4p ubiquitination or Fur4p sorting (selection) to the budding vesicle. The WW1 domain does not appear to be involved in Fur4p degradation, but it does have some role in fluid phase endocytosis. This discrepancy is not clear at the present time, but it may reflect the limitation of the assay or it may indicate that endocytic routes of Fur4p and LY do not completely overlap.

Studies of Fur4p-dependent uracil uptake test the internalization of a plasma membrane protein and the processes that precede internalization. WW1 seems relatively unimportant for Fur4p internalization. WW2 and WW3 are important in this internalization step and may also influence ubiquitination of Fur4p or Fur4p sorting to the budding vesicle. Studies of Fur4p ubiquitination in *rsp5-w* mutants would clarify that point. Preliminary data indicate that there are multiple higher molecular weight, presumably ubiquitinated, Fur4p species in extracts from *rsp5-w1* and *rsp5-w2*, but not *rsp5-w3* cells. Taken together, our results lead to the model in which we speculate that WW2 and WW3 domains are involved in the processes that precede internalization (Fur4p ubiquitination and sorting) and that WW3, and possibly WW1, are involved in internalization of budding vesicles (Figure 10).

Rsp5p clearly must ubiquitinate substrates that play a role in sorting of transporter proteins to budding vesicles and/or the internalization step of endocytosis. These substrates may be part of the endocytic machinery, including proteins involved in actin cytoskeleton organization. There is a growing list of such proteins in yeast (for review see Geli and Riezman 1998). A role for Rsp5p WW1 and WW3 in actin cytoskeleton dynamics is supported by our studies with latrunculin A, the drug that affects actin cytoskeleton polymerization (Ayscough *et al.* 1997). *rsp5-w1* and *rsp5-w3* strains are

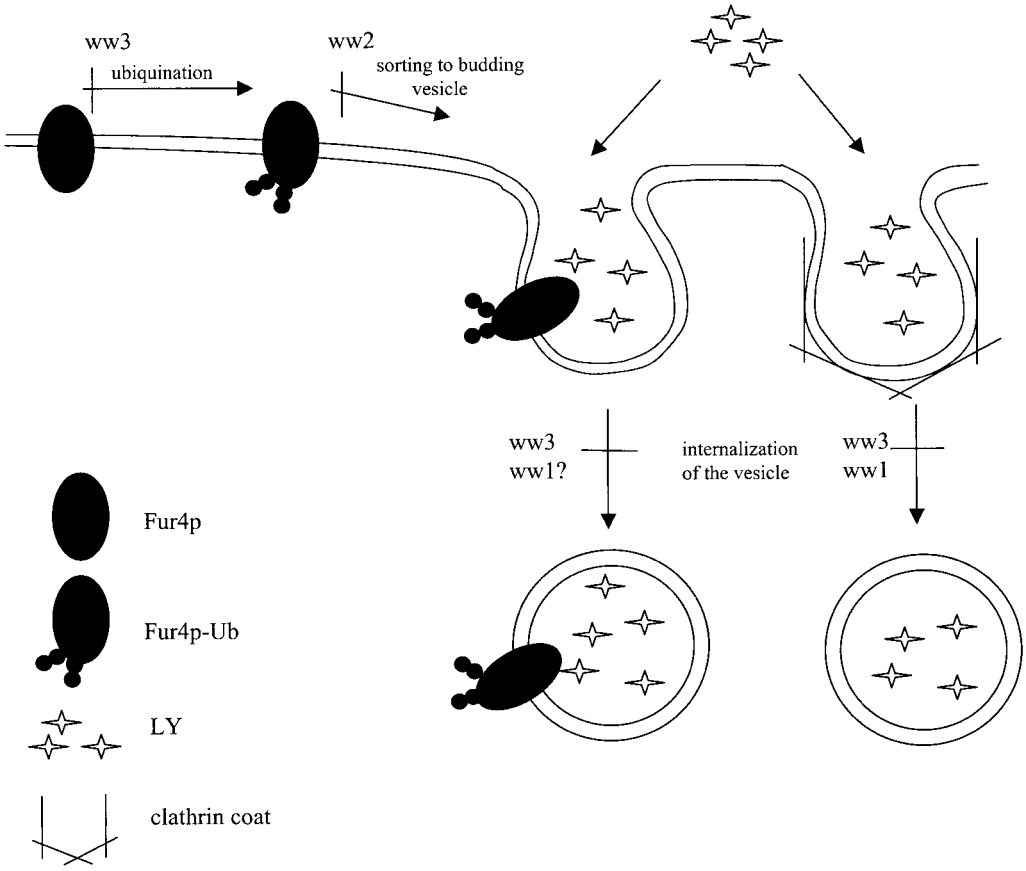

FIGURE 10.—Speculative model for involvement of WW domains of Rsp5p in Fur4p and fluid phase endocytosis.

more resistant to latrunculin A (our unpublished results). This indicates that the actin cytoskeleton is more stable in these mutants and may not function properly. The dynamic action of the actin cytoskeleton is necessary for the budding of all endocytic vesicles.

Very recently, protein ligands that may potentially interact with each Rsp5p WW domain were identified by searching the yeast database using consensus sequences obtained from screening and analyzing phage display combinatorial peptide libraries (CHANG *et al.* 2000). Rvs167p, a protein involved in endocytosis and actin cytoskeleton organization (MUNN *et al.* 1995; LEE *et al.* 1998), was identified as a potential interacting partner for the WW2 domain. Arc15p, the component of the ARP complex that promotes actin nucleation and cross-links actin filaments (for review see MACHESKY and GOULD 1999), was identified as a potential ligand for the WW2 or WW3 domains. These findings fit our results and predictions and validate *rsp5-w* mutant analysis as a tool to study Rsp5p involvement in various cellular processes *in vivo*. These mutants will be very useful in experiments to determine if new potential ligands for interaction actually do interact in yeast cells.

Rsp5p appears to be located in multiple sites in the cell. It was surprising that we could not detect Rsp5p in the nucleus since the large subunit of RNA polymerase II has been documented to be a substrate of Rsp5p (HUIBREGTSE *et al.* 1997; WANG *et al.* 1999). Many transporters and receptors that are substrates for Rsp5p-mediated ubiquitination are located at the plasma membrane. One of them, plasma membrane tryptophan permease Tat2p, is internalized upon nutrient limitation and is transported through the endocytic pathway to the vacuole for degradation. At the same time, internal Tat2p is diverted from the late secretory pathway to the vacuolar pathway (BECK *et al.* 1999). The N-terminal lysines of Tat2p and ubiquitination are required for the degradation of this internal pool of Tat2p (BECK *et al.* 1999). The role of Rsp5p in this latter process has not been tested. However, the pool of Rsp5p associated with internal membranes could play a role in directing Tat2p (and possibly other transporters) from the Golgi to the vacuole.

Our results indicate that individual WW domains adapt Rsp5p to functions in fluid phase and Fur4p endocytosis, as well as the low pH stress response. The results of others document the role of individual WW domains in Rpb1p recognition that affect transcription (WANG *et al.* 1999; CHANG *et al.* 2000). More work is clearly needed to learn how individual WW Rsp5p domains influence the ubiquitination of other plasma membrane transporters and to elucidate their roles in mitochondrial functions and cytoskeletal organization.

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