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Differential Regulation of G Protein α Subunit Trafficking by Mono- and Polyubiquitination*

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Previously we used mass spectrometry to show that the yeast G protein α subunit Gpa1 is ubiquitinated at Lys-165, located within a subdomain not present in other Gα proteins (Marotti, L. A., Jr., Newitt, R., Wang, Y., Aebersold, R., and Dohlman, H. G. (2002) *Biochemistry* 41, 5067–5074). Here we describe the functional role of Gpa1 ubiquitination. We find that Gpa1 expression is elevated in mutants deficient in either proteasomal or vacuolar protease function. Vacuolar protease *pep4* mutants accumulate monoubiquitinated Gpa1, and much of the protein is localized within the vacuolar compartment. In contrast, proteasome-defective *rpt6/cim3* mutants accumulate polyubiquitinated Gpa1, and in this case the protein exhibits cytoplasmic localization. Cells that lack Ubp12 ubiquitin-processing protease activity accumulate both mono- and polyubiquitinated forms of Gpa1. In this case, Gpa1 accumulates in both the cytoplasm and vacuole. Finally, a Gpa1 mutant that lacks the ubiquitinated subdomain remains unmodified and is predominantly localized at the plasma membrane. These data reveal a strong relationship between the extent of ubiquitination and trafficking of the G protein α subunit to its site of degradation.

Many sensory and chemical stimuli act via cell surface receptors and intracellular G proteins. In yeast, G protein-coupled receptors initiate a signaling cascade that leads to morphological changes, new gene transcription, cell cycle arrest, and eventually, mating. Mating, the process by which **a** and α haploid cells fuse to form the **a**/α diploid, is initiated by cell type-specific peptide pheromones. Haploid **a**-type cells secrete **a**-factor pheromone, which binds to specific receptors found only on α-type cells, whereas α-type cells secrete α-factor that acts exclusively on **a** cells (1).

Most components of the pheromone-signaling cascade in yeast have been identified through the isolation of gene mutations that produce an unresponsive sterile (*ste*) phenotype. Genes required for mating include the receptors for α-factor (*STE2*) and **a**-factor (*STE3*), the G protein β (*STE4*) and γ (*STE18*) subunits, G protein effectors (*STE5*, *STE20*, *CDC24*), and downstream protein kinases (*STE20*, *STE11*, *STE7*, *FUS3, KSS1*) as well as a transcription factor (*STE12*). The G protein α subunit serves primarily to regulate the levels of free Gβγ. Cells lacking the Gα subunit gene (*GPA1*) cannot sequester Gβγ and so are permanently activated (2, 3). The Gα subunit can also modulate signaling indirectly through binding to the polyribosome-associated protein Scp160 (4).

As with other G protein systems, pheromone binding to its receptor promotes the exchange of GTP for GDP on the Gα subunit followed by dissociation of Gα from the Gβγ subunit complex (5). The dissociated subunits in turn transmit and amplify the signal to effector proteins that produce an intracellular response. Signaling persists until GTP is hydrolyzed to GDP and the subunits reassemble. Given their position as intermediaries between signal detectors (receptors) and signal transmitters (effectors), G proteins are well positioned to serve as targets of regulation. Particularly important in this role is the regulator of G protein signaling protein Sst2, which attenuates the pheromone signal by accelerating Gpa1 GTP hydrolysis and thereby reducing the lifetime of the activated G protein (6, 7).

There is growing evidence that G protein signaling components are also regulated by post-translational modifications (8). Recent attention has focused on ubiquitination, the process by which a ubiquitin polypeptide is covalently attached to specific target proteins (9). Once a protein is ubiquitinated, ubiquitin can itself be ubiquitinated, resulting in the formation of a polyubiquitin chain. Polyubiquitinated substrates are then captured by the proteasome protease complex and rapidly degraded (10). Of the pheromone signaling components in yeast, ubiquitination has been reported for the Gα protein Gpa1 (11, 12), the regulator of G protein signaling protein Sst2 (13) and the effector kinase Ste7 (14, 15). Ubiquitination of both Ste7 and Sst2 are induced by pheromone, and these modifications are thought to represent feedback loops leading to pheromone desensitization and resensitization, respectively (13–15).

Ubiquitination has also been described for the pheromone receptors Ste2 and Ste3 (16, 17). However, in this instance the proteins are monoubiquitinated instead of polyubiquitinated and degraded by the vacuole (the yeast counterpart to the lysosome) instead of by the proteasome (16–19). This discovery led to the hypothesis that ubiquitination is primarily a protein-trafficking signal, and monoubiquitination promotes degradation only by allowing the substrate to undergo internalization and delivery to the vacuole compartment (20).

Recently we used mass spectrometry to identify the ubiquitination site of Gpa1 *in vivo* (11). The site of modification (Lys-165) lies within the α-helical domain of the G protein and, more specifically, within a 110-residue subdomain not found in other

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Gα subunits. Here we show that the Gpa1 subdomain undergoes both mono- and polyubiquitination. Monoubiquitinated Gpa1 is targeted to the vacuole, whereas polyubiquitinated Gpa1 is delivered to the proteasome. These findings reveal that G protein trafficking can function independently of substrate targeting signals and, instead, depends solely on the extent of substrate ubiquitination.

MATERIALS AND METHODS

Strains and Plasmids—Standard methods for the growth, maintenance, and transformation of yeast and bacteria and for the manipulation of DNA were used throughout (21). Yeast *Saccharomyces cerevisiae* strains used in this study were BY4741 (*MAT***a** *leu2*Δ *met15*Δ *his3*Δ *ura3*Δ), BY4741-derived mutants lacking *PEP4* or *UBP12* (disrupted using the KanMX G418-resistance marker, from Research Genetics, Huntsville, AL), MHY753 (*MAT***a** *his3*-Δ200 *leu2*Δ1 *ura3*-52 *lys2*-801 *trp1*Δ63 *ade2*-101), MHY754 (MHY753, *cim3*-1), CRY1 (*MAT***a** *ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1^{oc} can1-100*), CB007–1Δ (CRY1, *pep4-2*::*HIS3 prb1*::*LEU2*) (provided by Linda Hicke, Northwestern University) (22), and YGS5 (*MAT***a** *ura3*-52 *lys2 ade2^{oc} trp1 leu2*-1 *gpa1*::hisG *ste11^{ts}*) (23). *STE2* was disrupted in BY4741 and BY4741-derived *pep4*Δ mutant strains by single-step gene replacement with *ste2*::*HIS3* (this work).

Yeast shuttle plasmids used here were pRS316 (*CEN*, amp^R, *URA3*), pRS406 (amp^R, *URA3)* (24), pRS316-GPA1, which contains *GPA1* under the control of its native promoter (23), pAD4M (2 μm, amp^R, *LEU2*, *ADH1* promoter/terminator, from Peter McCabe, Onyx Pharmaceutical), pAD4M-GPA1 (23), pND747 (2 μm, amp^R, *URA3*, Myc-tagged ubiquitin under the control of the *CUP1* promoter, from Nicholas Davis, Wayne State University) (25), and pRS425-FUS1-lacZ (26). pRS316-*GPA1*$^{\Delta 128-236}$ was constructed by replacing the 1.5-kbp HindIII-HindIII fragment with a 1170-bp PCR product, generated using *GPA1* as template. The forward primer 5′-CCC AAG CTT TAA TTC ACG AAG ACA TTG CTA AGG CAA TAA AGC AAC TTT GG-3′ disrupts and regenerates the *GPA1* HindIII site; the reverse primer 5′-AGG TCG ACG GTA TCG ATA AGC-3′ flanks the multiple cloning site HindIII upstream of the gene insert within pRS316. pAD4M-GPA1$^{\Delta 128-236}$ was constructed by PCR amplification of *GPA1*$^{\Delta 128-236}$ using primers 5′-ACG CGT CGA CAT GGG GTG TAC AGT GAG TAC GCA AAC AAT A-3′ and 5′-CGA GCT CTC ATA TAA TAC CAA TTT TTT TAA GGT TTT GCT-3′, engineered with SalI and SacI sites, and subcloning into the SalI/SacI sites within the multiple cloning site of the pAD4M vector. Note that positions 127 and 236 both encode Leu. A triple-FLAG epitope tag was placed at the C terminus of Ubp12 (UBP12-FLAG) by PCR amplification and subcloning into pYES2.1/V5-His-TOPO (2 μm, *URA3*, *GAL1* promoter, *CYC1* terminator) (Invitrogen) to yield plasmid pYES-UBP12-FLAG. PCR primers were 5′-CCC AAG CTT CCA GAA TGG GTT CTT CAG ATG TTT CAA GTC-3′ and 5′-G TTA CTT GTC ATC GTC ATC TTT ATA ATC CTT GTC ATC GTC ATC TTT ATA ATC CTT GTC ATC GTC ATC TTT ATA ATC AAG CTT TTC TGG CGA TTC TAG TGT CAC-3′.

The Gpa1-green fluorescent protein (GFP)[1] fusion was constructed by PCR amplification of the *GPA1* gene, digested with XbaI and ClaI, and subcloned into the corresponding sites of yeast-enhanced GFP-containing plasmid pUG35 (*CEN*, *URA3*, *MET25* promoter, *CYC1* terminator) (27). PCR primers used were 5′-CCT GCA GCC CGG GGG ATC CAC TAG TCT AGA-3′ (forward) and 5′-ACA TCG ATT ATA ATA CCA ATT TTT TTA AGG TTT TGC TGG ATC-3′ (reverse) and included unique XbaI and ClaI restriction sites. A cassette containing the promoter *GPA1*-GFP and terminator was digested with SacI and KpnI and subcloned into the corresponding sites of integrating vector pRS406 to yield pRS406-GPA1-GFP. The resulting plasmid was linearized with HindIII (a site unique to *GPA1*) to direct gene integration. *GPA1*$^{\Delta 128-236}$-GFP was constructed as described for *GPA1*-GFP except for the use of a different forward primer (5′-GGT CTA GAC ATG GGG TGT ACA GTG AGT ACG-3′) and *GPA1*$^{\Delta 128-236}$ as the template.

Growth, Transcription, Degradation, and Ubiquitination Bioassays—The pheromone-dependent growth inhibition (halo) and reporter-transcription assays were conducted as described previously (26). Gpa1 expression and ubiquitination was monitored as described previously (11). To monitor the loss of Gpa1, cultures were treated with cycloheximide (10 μg/ml in 0.1% ethanol, final concentrations) for up to 120 min, as described previously (28). Growth was stopped at mid-log phase ($A_{600\ nm} \sim 1$) by the addition of 10 mM NaN_3 and transferred to an ice bath. Cells were centrifuged and washed with 10 mM NaN_3, and the cell pellet was resuspended directly in boiling SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 1% 2-mercaptoethanol, 0.0005% bromphenol blue) for 10 min. The samples were then subjected to glass bead homogenization, clarified by microcentrifugation, resolved by SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose. The membrane was probed with antibodies to Gpa1 at 1:1,000 (29), Ste4 at 1:2,000 (from Duane Jenness, University of Massachusetts), or Pgk1 at 1:75,000 (from Jeremy Thorner, University of California Berkeley). Immunoreactive species were visualized by enhanced chemiluminescence detection (Pierce) of horseradish peroxidase-conjugated anti-rabbit IgG (Bio-Rad).

Immunoprecipitation—The association of Gpa1 and Ubp12 was examined by immunoprecipitation of FLAG-tagged Ubp12 and immunoblotting with anti-Gpa1 antibodies. Cells (50 ml) co-transformed with plasmids pAD4M-GPA1 and pYES-UBP12-FLAG were grown to $A_{600\ nm} \sim 1$, harvested, and resuspended in 550 μl of lysis buffer (50 mM $NaPO_4$, pH 7.5, 400 mM NaCl, 0.1% Triton X-100, 10% glycerol, 0.5 mM dithiothreitol, 25 mM NaF, 25 mM glycerophosphate, 1 mM sodium orthovanadate, 10 mM *N*-ethylmaleimide, 5 mM phenylmethylsulfonyl fluoride, and one pellet of complete EDTA-free protease inhibitor mixture (Roche Applied Science)). This and all subsequent manipulations were carried out at 4 °C. Cells were subjected to glass bead vortex homogenization for 30 s, repeated 8 times, and centrifuged twice at 6000 × *g* for 5 min and 25 min. Lysates were incubated for 2 h with a bead volume of 10 μl of anti-FLAG M2 affinity resin (Sigma) equilibrated in lysis buffer. Immunoprecipitates were collected by centrifugation at 1000 × *g* for 30 s, and pellets were washed with 1 ml of lysis buffer for 3 min, repeated 4 times before final resuspension in 30 μl of 2× SDS-PAGE sample buffer. Each sample was resolved by 7.5% polyacrylamide gel electrophoresis and immunoblotting with anti-Gpa1 polyclonal antibodies at 1:1000 or anti-FLAG monoclonal antibodies at 1:2000.

Microscopy Analysis—Cells expressing single-copy, integrated GFP-tagged gene fusions were visualized by differential interference contrast (DIC) and fluorescence microscopy using a Nikon eclipse E600EN, photographed with a Hamamatsu digital camera, and analyzed with MetaMorph Version 5.0 software.

RESULTS

Many cellular proteins are polyubiquitinated and degraded by the proteasome. However, a handful of proteins are instead monoubiquitinated and delivered to the vacuole. This alternative pathway was first documented for the yeast G protein-coupled receptors Ste2 and Ste3 (16, 17, 19). Both of these receptors are monoubiquitinated in response to prolonged treatment with pheromone, and once modified they are rapidly endocytosed and degraded within the vacuole compartment (16–18). Gpa1 is also monoubiquitinated (11, 12), suggesting that this protein is likewise degraded by the vacuole. To test this supposition we examined the fate of Gpa1 in *pep4* mutant cells, which lack vacuolar protease function (30). Pep4, also known as proteinase A, is a saccharopepsin aspartyl protease required for activation of degradative enzymes within the vacuole. Cell lysates from wild-type and *pep4* mutant cells were resolved by gel electrophoresis and immunoblotting, and the abundance of Gpa1 was monitored using anti-Gpa1 antibodies. Gpa1 normally migrates as a doublet of 54 and 56 kDa (representing myristoylated and non-myristoylated species, respectively) (29, 31). As shown in Fig. 1, steady state levels of Gpa1 were ~2-fold higher in the vacuolar protease-deficient *pep4* mutant strain. Expression of Ste4 (Gβ) was also elevated in these mutants. We then measured the abundance of the G protein α and β subunits in a temperature-sensitive *cim3*-1 mutant, which at the restrictive temperature has severely impaired proteasomal protease activity (22). Cim3 (also known as Rpt6) is a subunit of the proteasome 19 S regulatory particle, which confers specificity for ubiquitinated proteins for presentation to the proteasome proteolytic subunits (32). Gpa1 (but not Ste4) expression was enriched in the *cim3*-1 mutant, suggesting that the Gα protein can also be degraded by the pro-

[1] The abbreviations used are: GFP, green fluorescent protein; DIC, differential interference contrast; UBP, ubiquitin-specific processing protease.

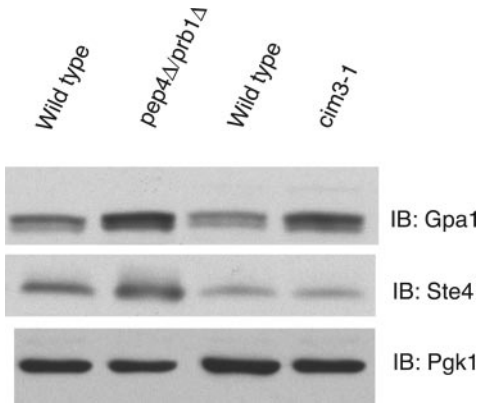

FIG. 1. **Gpa1 is degraded by both the proteasomal and vacuolar protease pathways.** Whole cell extracts were prepared from a vacuolar protease mutant (*pep4-2, prb1*::*LEU2*, or *pep4*::KanMX with similar results; data not shown), a temperature-sensitive proteasomal protease defective mutant (*cim3*-1) grown at the restrictive temperature of 37 °C for 4 h, and isogenic wild-type strains. Samples were resolved by 7.5% SDS-PAGE and detected by immunoblotting (*IB*) with antibodies against Gpa1 (Gα), Ste4 (Gβ), or Pgk1 (loading control), as indicated.

teasome (Fig. 1) (11). These findings suggest that Gpa1 can be degraded either by the vacuole or the proteasome, whereas Ste4 is degraded primarily by the vacuole.

Whereas degradation by the proteasome typically requires substrate polyubiquitination, vacuolar degradation of Ste2 is triggered by monoubiquitination (16). If Gpa1 is degraded by the vacuole, defects in this pathway should result in an accumulation of the monoubiquitinated form of the protein. Conversely, if Gpa1 is degraded by the proteasome, defects in that pathway should result in an accumulation of polyubiquitinated Gpa1. To test this we overexpressed Gpa1 in mutant strains deficient in either vacuolar protease or proteasome activity. Overexpression was necessary for this experiment in order to detect the minor ubiquitinated species. In wild-type cells, Gpa1 migrated at 54 and 56 kDa (Fig. 2). In the vacuolar-protease-deficient *pep4*Δ strain an additional monoubiquitinated species migrated near 63 kDa (Fig. 2*A*) (11, 12). In contrast, the proteasome-deficient *cim3*-1 strain accumulated a ladder of high molecular weight bands representing polyubiquitinated Gpa1 (Fig. 2*B*) (11, 12). These results support the suggestion that Gpa1 is degraded by two routes; one entails monoubiquitination and delivery to the vacuole, whereas the second requires polyubiquitination and delivery to the proteasome.

The vacuolar degradation pathway is used by the pheromone receptor and, as shown here, by the G protein α subunit Gpa1. Monoubiquitination of the receptor is enhanced by pheromone. Because the receptor and G protein bind to one another, we investigated whether monoubiquitination and/or degradation of both substrates occur in a coordinated manner. We first examined whether monoubiquitination of Gpa1 was affected by prolonged exposure to pheromone and consistently found no difference (data not shown) (11). Second, we tested whether monoubiquitination of Gpa1 was affected by deletion of the receptor gene *STE2* and again found no difference (compare *pep4*Δ and *ste2*Δ/*pep4*Δ mutants, Fig. 2, *A* and *C*). These data indicate that Gpa1 monoubiquitination as well as vacuolar sorting occurs independently of receptor binding or pheromone occupancy.

It was shown previously that the pheromone receptor accumulates in the vacuole of cells deficient in vacuolar protease activity (16, 18). To determine whether Gpa1 is likewise delivered to the vacuole we compared the distribution of the protein (expressed as a chromosomally integrated GFP fusion) in wild-type and *pep4*Δ mutant strains. As shown in Fig. 3, Gpa1 in wild-type cells was detected at the plasma membrane. In contrast, Gpa1 in the *pep4*Δ mutant was visible at the plasma membrane and also within the large vacuolar compartment, which is easily identified as the most prominent organelle within the cell. Taken together these data indicate that Gpa1, like Ste2, is monoubiquitinated and degraded by the vacuole. Whereas the receptor is delivered to the vacuole upon pheromone stimulation, the G protein is delivered in a constitutive or pheromone-independent manner.

Most cytoplasmic proteins are polyubiquitinated and delivered to the proteasome complex. Thus, we anticipated that polyubiquitinated Gpa1 would not be targeted to the vacuole. To test this prediction we examined the subcellular distribution of Gpa1-GFP in the proteasome-defective *cim3*-1 mutant strain, which accumulates the polyubiquitinated form of the substrate. As shown in Fig. 3, Gpa1 in the *cim3*-1 mutant was predominantly localized in the cytoplasm and, in contrast to *pep4*Δ, was largely excluded from the vacuole. We presume that the cytoplasmic staining represents Gpa1 associated with or en route to the proteasome complex. Taken together these data suggest that Gpa1 is delivered to the proteasome when polyubiquitinated but not when monoubiquitinated. Conversely, Gpa1 is delivered to the vacuole when monoubiquitinated but not when polyubiquitinated.

To further corroborate these results, we asked whether expression of both the mono- and polyubiquitinated forms of Gpa1 might direct the protein to both the vacuolar compartment and the cytoplasm. To this end we used two methods in combination, one to stabilize the monoubiquitinated form of Gpa1 and a second designed to stabilize the polyubiquitinated form of the protein. The first approach was to overexpress Myc-tagged ubiquitin through the use of a copper inducible promoter (from *CUP1*). Myc-ubiquitinated substrates are degraded more slowly and, therefore, can be detected more easily *in vivo* (33). Copper-induction of Myc-ubiquitin promoted the accumulation of monoubiquitinated Gpa1, as shown previously (11, 12) (Fig. 4*A*). The second approach was to delete the ubiquitin-specific processing protease (UBP) that removes ubiquitin from Gpa1. This approach is similar to one used in our earlier analysis of the effector kinase Ste7 (14, 15). By screening mutants that lack each of 16 ubiquitin-processing proteases (or UBPs) we had found one (*ubp3*Δ) that resulted in the accumulation of polyubiquitinated Ste7. We conducted a similar screen of the UBP deletion mutants and found another, *ubp12*Δ, that specifically accumulated ubiquitinated Gpa1 (Fig. 4*A* and data not shown). Moreover, immunoprecipitation of Ubp12 resulted in the co-purification of Gpa1, further suggesting that Gpa1 ubiquitination is regulated directly by Ubp12 (Fig. 4*B*). By overexpressing Myc-ubiquitin in a *ubp12*Δ strain, we could achieve an increase in both mono- and polyubiquitinated forms of Gpa1 (Fig. 4*A*, *fourth lane*).

We then asked how mono- and polyubiquitination affects Gpa1 localization. To this end we examined the subcellular distribution of Gpa1-GFP in *ubp12*Δ mutant cells after induction of Myc-ubiquitin. Whereas Gpa1 is normally localized at the plasma membrane, overexpression of Myc-ubiquitin in the *ubp12*Δ strain led to a redistribution of Gpa1 to the vacuolar compartment as well as to the cytoplasm (Fig. 5). Thus, under conditions where Gpa1 is polyubiquitinated and monoubiquitinated in the same cell, the protein is directed to both the vacuolar compartment and the cytoplasm. Stated differently, the distribution of Gpa1 in this case is an amalgamation of the distribution seen in *pep4*Δ and *cim3*-1 cells.

We then wished to determine the fate of Gpa1 that is not ubiquitinated. Initially we examined the localization of $Gpa1^{K165R}$, which lacks the primary site of *in vivo* ubiquitination (11). However, we found the subcellular distribution of

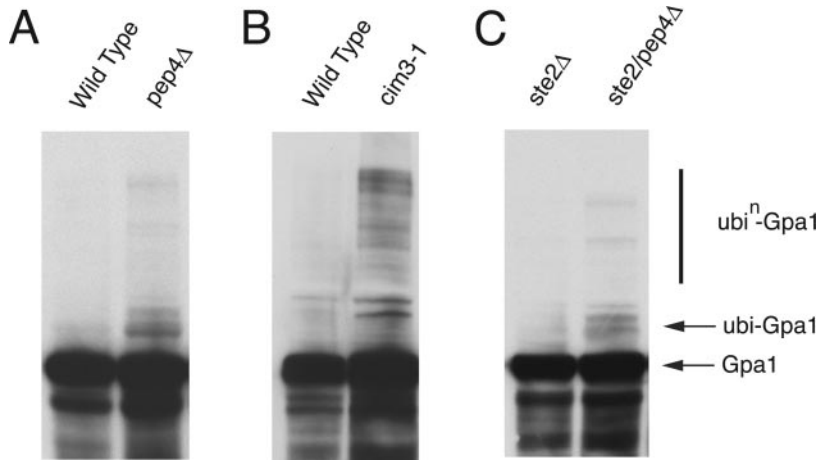

FIG. 2. **Accumulation of ubiquitinated Gpa1 in vacuolar and proteasomal protease-deficient mutant strains.** *A*, a vacuolar protease-deficient (*pep4*::KanMX) mutant and the isogenic wild-type strain were transformed with a plasmid containing wild-type *GPA1* (pAD4M-GPA1). Plasmid-borne overexpression of Gpa1 was required to detect the minor ubiquitinated species. Whole cell extracts were resolved by 7.5% SDS-PAGE and immunoblotting with anti-Gpa1 antibodies. *B*, a proteasomal protease defective (*cim3*-1) mutant and the isogenic wild-type strain were transformed with plasmid pAD4M-GPA1, grown at the restrictive temperature of 37 °C for 4 h, and analyzed by immunoblotting with anti-Gpa1 antibodies as described above. *C*, the same strains as in *panel A*, except lacking the α-factor receptor gene *STE2*. *Arrows* indicate the mobility of unmodified Gpa1, monoubiquitinated Gpa1 (*ubi-Gpa1*), and polyubiquitinated Gpa1 (*ubi^n-Gpa1*).

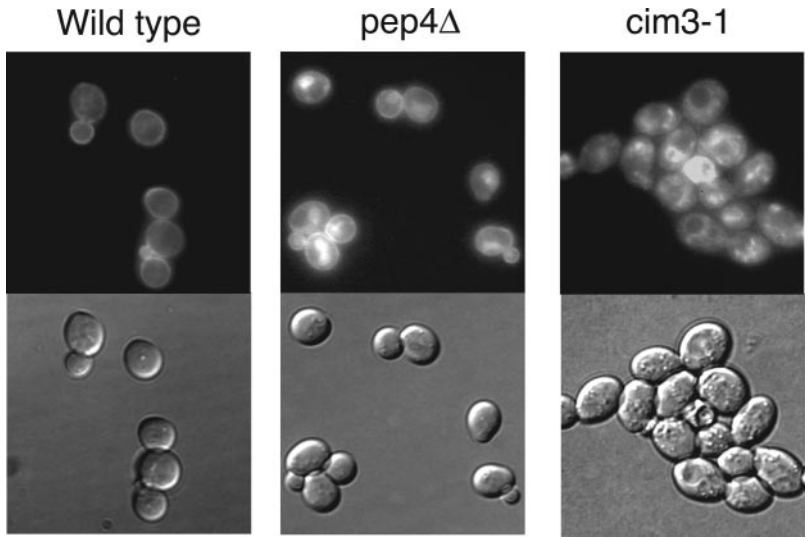

FIG. 3. **Mislocalization of Gpa1 in vacuolar and proteasomal protease-deficient mutant strains.** Vacuolar protease-deficient (*pep4*::KanMX), proteasomal protease-deficient (*cim3*-1), and the isogenic wild-type strains were transformed with an integrating plasmid (pRS406-GPA1-GFP) containing the native *GPA1* promoter and gene fused to the gene encoding yeast-enhanced green fluorescent protein. Cells were grown to mid-log phase at 30 °C and visualized by DIC and fluorescence microscopy. Cells expressing Gpa1 alone exhibited negligible background autofluorescence (data not shown).

Gpa1^{K165R} was similar to that of the wild-type protein (data not shown). We presume that one or more secondary sites are modified when Lys-165 is altered, and this accounts for the residual ubiquitination as well as the unaltered subcellular localization of the mutant protein. We also reasoned that any secondary sites are likely to be clustered within the same 110-residue subdomain that contains Lys-165. To test this possibility we constructed and analyzed a mutant form of the protein that lacks the ubiquitinated subdomain altogether (Gpa1$^{\Delta 128-236}$). The position of the internal deletion was chosen based on a sequence alignment of Gpa1 with $G_i\alpha$ and $G_t\alpha$, two mammalian proteins that lack the subdomain and for which crystal structures are available (34, 35).

We first compared the ubiquitination of wild-type Gpa1 and Gpa1$^{\Delta 128-236}$. Once again we expressed Myc-ubiquitin in order to improve detection of the ubiquitinated product. The wild-type protein migrated as a doublet of 54 and 56 kDa as a monoubiquitinated species of 63 kDa and as a higher molecular mass polyubiquitinated species near the top of the gel. Copper-induced expression of Myc-ubiquitin further increased detection of the monoubiquitinated species (Fig. 6). In comparison, Gpa1$^{\Delta 128-236}$ migrated as a doublet of 42 and 44 kDa, close to the size predicted for the unmodified mutant product. In this case, however, there were no additional bands after copper induction and no specific bands of the size expected for either monoubiquitinated or polyubiquitinated forms of the protein (Fig. 6). These results suggest that ubiquitination is largely blocked by deletion of the 110-residue subdomain in Gpa1.

We then asked whether the ubiquitination subdomain is required for Gpa1 function *in vivo*. Initially we measured pheromone sensitivity using a standard growth arrest assay. In this method a nascent lawn of cells is exposed to a point source of α-factor. As the lawn develops, a zone of growth inhibition appears, the size of which reflects the sensitivity of the cells to pheromone-induced growth arrest. As shown in Fig. 7*A*, cells expressing Gpa1$^{\Delta 128-236}$ exhibited a smaller and more turbid zone of growth inhibition compared with the wild-type protein, indicating a diminished pheromone response. We also compared Gpa1$^{\Delta 128-236}$ and Gpa1 activity using a reporter-transcription assay consisting of the pheromone-inducible *FUS1* promoter driving expression of lacZ (β-galactosidase) (26). As shown in Fig. 7*B*, cells expressing Gpa1$^{\Delta 128-236}$ exhibited a ~30% reduction in the maximum pheromone response, consistent with the reduced growth arrest response described above. Thus, deletion of the ubiquitination subdomain appears to enhance the ability of Gpa1 to block the Gβγ-mediated signal,

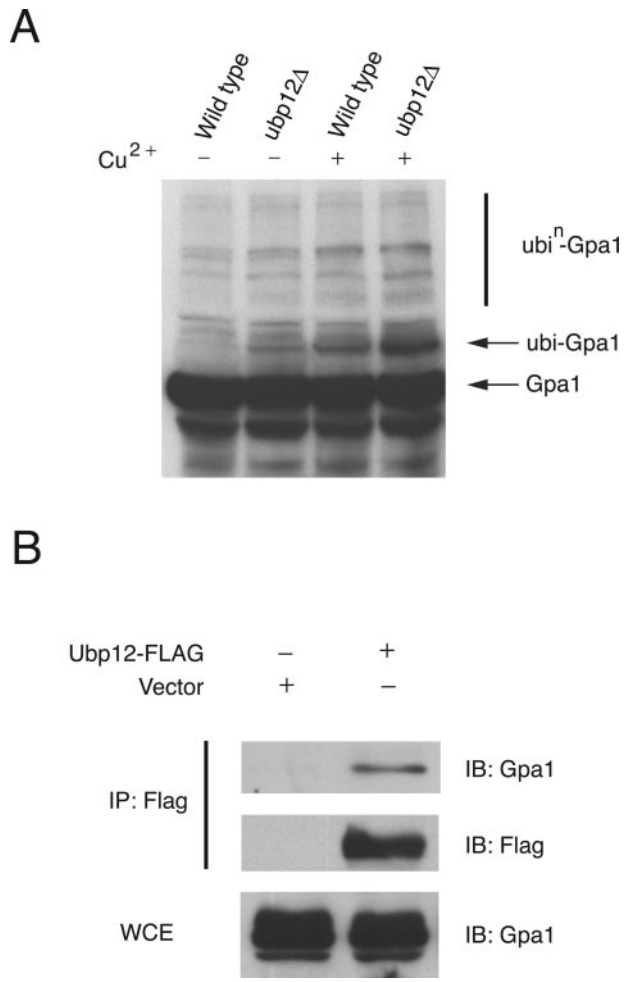

FIG. 4. **Accumulation of ubiquitinated Gpa1 in ubiquitin-processing protease-deficient mutant cells.** A, a ubiquitin-processing protease-deficient (*ubp12*::KanMX) mutant and isogenic wild-type strain were co-transformed with plasmids containing Gpa1 (pAD4M-GPA1) and Myc-ubiquitin under the control of the copper-inducible *CUP1* promoter (pND747). Mid-log phase cells were treated with 100 μM $CuSO_4$ for 4 h as indicated and then collected and resolved by 7.5% SDS-PAGE and immunoblotting using anti-Gpa1 antibodies. *Arrows* indicate the mobility of unmodified Gpa1 as well as the monoubiquitinated (*ubi-Gpa1*) and polyubiquitinated (*ubi^n-Gpa1*) species. *B*, wild-type cells were co-transformed with plasmids containing Gpa1 (pAD4M-GPA1) and FLAG epitope-tagged Ubp12 (pYES-UBP12-FLAG) or the empty parent vector. Ubp12-FLAG was immunoprecipitated (*IP*) with M2 anti-FLAG resin, and the copurification of Gpa1 with Ubp12 was detected by immunoblotting (*IB*) with anti-Gpa1 and anti-FLAG antibodies. An immunoblot of whole cell extracts (*WCE*) is also shown to confirm equal expression of Gpa1.

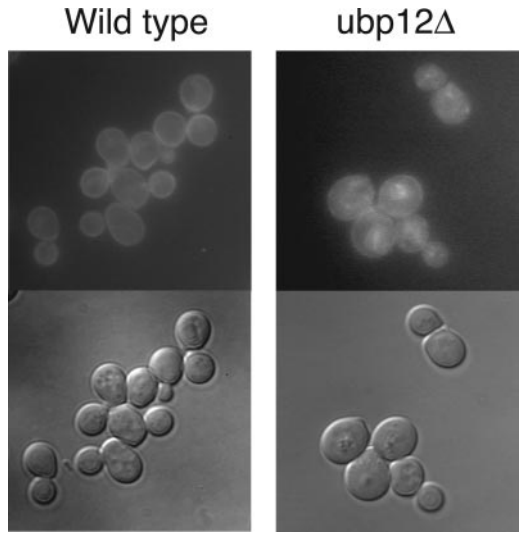

FIG. 5. **Accumulation of Gpa1 within the cytoplasm and vacuole of ubiquitin-processing protease-deficient mutant cells.** A ubiquitin-processing protease deficient (*ubp12*::KanMX) mutant and isogenic wild-type strain were transformed with an integrating plasmid (pRS406-GPA1-GFP) encoding Gpa1 fused to GFP and a plasmid containing Myc-ubiquitin under the control of the copper-inducible *CUP1* promoter (pND747). Cells were treated with $CuSO_4$ for 4 h and visualized at mid-log phase by DIC and fluorescence microscopy. Cells expressing Gpa1 alone exhibited negligible background autofluorescence (data not shown).

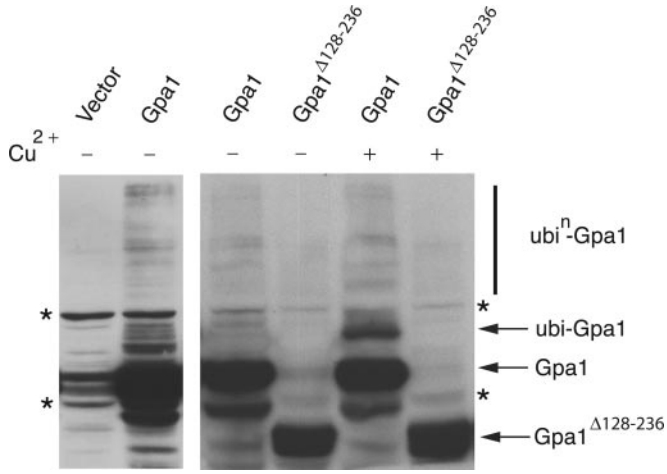

FIG. 6. **Removal of the 110 amino acid subdomain eliminates Gpa1 ubiquitination *in vivo*.** Wild-type cells (BY4741) were co-transformed with a plasmid (pAD4M) containing wild-type *GPA1* or a *GPA1* mutant lacking the ubiquitination subdomain ($Gpa1^{\Delta 128-236}$) and a plasmid containing Myc-ubiquitin under the control of the copper-inducible *CUP1* promoter (pND747). Mid-log phase cells were treated with $CuSO_4$ for 4 h, then collected and resolved by 7.5% SDS-PAGE and immunoblotting using anti-Gpa1 antibodies. *Arrows* indicate the mobility of unmodified Gpa1 as well as the monoubiquitinated (*ubi-Gpa1*) and polyubiquitinated (*ubi^n-Gpa1*) species. Other bands (*) appear to be non-specifically recognized (*i.e.* cross-react with anti-Gpa1 antibodies), since they are also present at equal levels in extracts from cells that express only the endogenous Gpa1 (*left panel*) or lack Gpa1 altogether (*e.g.* diploid cells, data not shown), and none is induced by copper treatment. The *left panel* was exposed longer to show bands that are non-specifically recognized by the Gpa1 antibody.

resulting in diminished growth arrest and transcription-induction responses. Stated differently, the $Gpa1^{\Delta 128-236}$ mutant exhibits a moderate gain-of-function phenotype.

$Gpa1^{\Delta 128-236}$ was expressed at slightly higher steady state levels than wild-type Gpa1. This difference persisted long after treatment with the protein synthesis inhibitor cycloheximide (Fig. 7*C*). However expression of the mutant did diminish over time, suggesting that some degradation can occur even in the absence of ubiquitination. Apparently any reduction in Gpa1 degradation is sufficient to cause an increase in steady state expression but not large enough to be easily detected after exposure to cycloheximide.

Because either mono- or polyubiquitination promotes a redistribution of Gpa1 away from the plasma membrane, the absence of ubiquitination would most likely favor Gpa1 localization to the plasma membrane. Indeed, $Gpa1^{\Delta 128-236}$ was present almost exclusively at the plasma membrane even when expressed in the protease-deficient *pep4*Δ and *ubp12*Δ mutants (Fig. 8). Because overexpression of Gpa1 diminishes $G\beta\gamma$-mediated signaling, increased plasma membrane localization could easily account for the diminished pheromone response in cells that express $Gpa1^{\Delta 128-236}$. This seems more likely than an alteration in subunit interaction since the ubiquitinated subdomain lies within the helical domain of the protein, and this

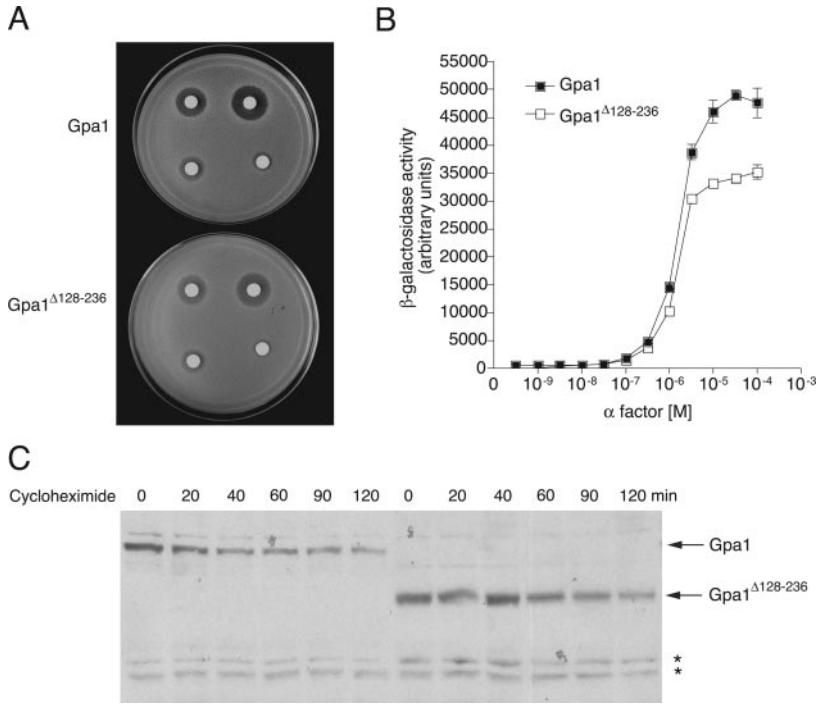

FIG. 7. **Non-ubiquitinated Gpa1 exhibits an increased ability to regulate Gβγ-mediated signaling.** Strain YGS5 (*gpa1*Δ *stell*^{ts}) was transformed with a single-copy plasmid (pRS316) containing either the wild-type *GPA1* promoter and gene (*Gpa1*) or a mutant lacking the ubiquitination subdomain (*Gpa1*$^{\Delta 128-236}$) as well as a plasmid (pRS425-FUS1-lacZ) containing the pheromone-induced *FUS1* promoter and *lacZ* reporter. *A*, cells were plated onto solid medium and exposed to paper discs containing α-factor pheromone (*clockwise* from *bottom right*: 1, 5, 15, and 45 μg) for 48 h. *B*, cells in mid-log phase of growth were treated with the indicated concentrations of α-factor, and the resulting β-galactosidase activity was measured spectrofluorimetrically. Data shown are representative of three independent experiments performed in triplicate. *Error bars*, ±S.E. *C*, to measure protein stability the cells were grown to mid-log phase and treated with the protein synthesis inhibitor cycloheximide for the indicated times. Cells were then collected and resolved by 7.5% SDS-PAGE and immunoblotting using anti-Gpa1 antibodies. *, non-specifically recognized species.

domain has no contact with the guanine nucleotide or Gβγ subunits and does not undergo any conformational change upon GTP hydrolysis (34). Taken together, these data suggest that intracellular targeting of Gpa1 depends on the extent of ubiquitination. Whereas non-ubiquitinated Gpa1 is retained at the plasma membrane, monoubiquitinated Gpa1 is delivered to the vacuole, and polyubiquitinated Gpa1 is delivered to the proteasome.

DISCUSSION

Ubiquitin is widely recognized as a key regulator of protein stability. Less understood is how the extent of ubiquitination affects the behavior of a given substrate. Most known substrates are polyubiquitinated and degraded by the proteasome. Other substrates are monoubiquitinated, and those substrates that are integral-membrane proteins are typically delivered to the vacuole or lysosome. These generalizations hold true for components of the G protein-signaling cascade. Known targets of polyubiquitination include mammalian and yeast regulator of G protein-signaling proteins (13, 36, 37), G protein α subunits (Gpa1, transducin) (12, 38), a G protein γ subunit (39, 40), and downstream protein kinases such as Ste7 and MEKK1 (14, 15, 41, 42). In contrast the pheromone receptors in yeast (16, 17) and at least one G protein-coupled receptor in mammals (43) are monoubiquitinated and delivered to the vacuole or lysosome. Many receptor-tyrosine kinase growth factor receptors are likewise monoubiquitinated and degraded within the lysosome. Ubiquitin-dependent endocytosis has also been exploited by some viruses to down-regulate key molecules of the host immune system and thereby evade clearance by the host organism (20).

Together these observations led to the suggestion that ubiquitination is a protein trafficking signal first and a degradation signal second (20). Whereas monoubiquitination results in trafficking and delivery to the vacuolar compartment, polyubiquitination results in trafficking to the proteasome. Degradation in either case depends on delivery to the proper cellular location. Rigorous proof of this hypothesis would require the ability to manipulate the extent of ubiquitination of a single substrate and in so doing determine its route of degradation. In support of this model we present evidence that Gpa1 is delivered to the proteasome when polyubiquitinated and is delivered to the vacuole when monoubiquitinated. If the pool of cellular Gpa1 is monoubiquitinated and polyubiquitinated, the protein is delivered to both sites. If Gpa1 cannot be ubiquitinated it is not delivered to either destination and instead remains at the plasma membrane.

Our analysis benefited greatly from several unique features of the experimental system. First, our previous mass spectrometry analysis had revealed that Gpa1 is ubiquitinated at Lys-165 *in vivo* (11). This allowed us to design a mutant form of Gpa1 (Gpa1$^{\Delta 128-236}$) that does not undergo ubiquitination. Another important advantage was the available crystal structure of mammalian $G_t\alpha$ and $G_i\alpha$ proteins, which closely resemble yeast Gpa1 in both sequence and function (34, 35). Thus, our Gpa1$^{\Delta 128-236}$ mutant could be designed in a rational manner based on precise knowledge of the position of the ubiquitinated subdomain within the predicted folded structure of the protein. Because the ubiquitinated subdomain is not required for G protein function, it could be deleted without altering known sites of GTP binding, subunit interaction, receptor coupling, or membrane association.

Another unique feature of our experimental system is the ability to manipulate the extent of ubiquitination through the use of mutants that selectively stabilize mono- or polyubiquitinated substrates. Myc-ubiquitinated proteins are poor substrates for proteolysis and, therefore, accumulate to higher-than-normal levels within the cell (33). *pep4* mutants disrupt vacuolar protease function and have been successfully used to enrich monoubiquitinated substrates (16, 17, 30). *cim3* mutants disable proteasome protease activity and, therefore, result in the accumulation of polyubiquitinated substrates (11, 14, 22, 44, 45). Although less commonly applied, *ubp* mutants can also be very effective in preserving short-lived changes in protein ubiquitination. This approach is analogous to using specific phosphatase inhibitors to preserve transient increases in protein phosphorylation.

The cellular function of most UBPs is unknown (46), and none is essential for viability (47). One of the best-characterized UBPs is Ubp14, the yeast counterpart of mammalian isopeptidase T (48, 49) and *Drosophila* UbpA (49). This enzyme

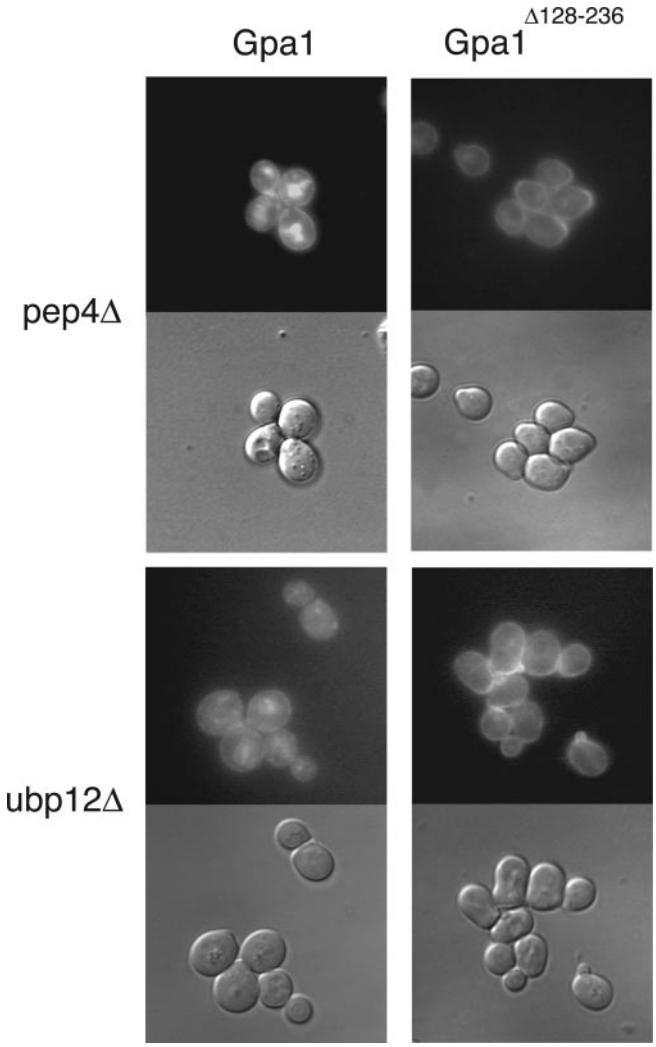

FIG. 8. **Non-ubiquitinated Gpa1 accumulates at the plasma membrane.** The indicated strains were transformed with an integrating plasmid encoding Gpa1 fused to GFP (pRS406-GPA1-GFP) or the corresponding mutant lacking the ubiquitination sub-subdomain (pRS406-GPA1$^{\Delta 128-236}$-GFP). Cells were grown to mid-log phase and visualized by DIC and fluorescence microscopy. Cells expressing Gpa1 alone exhibited negligible background autofluorescence (data not shown).

acts primarily on unanchored ubiquitin chains generated as intermediates in substrate degradation (50). A *ubp14*Δ mutant exhibits a defect in ubiquitin-dependent proteolysis and an accumulation of unanchored polyubiquitin chains (48, 51, 52). Another well characterized UBP is Doa4 (Ubp4) (53), which serves to remove ubiquitin from substrate intermediates during the course of proteasomal proteolysis (54). Cells lacking *DOA4* accumulate small polyubiquitinated peptide fragments, and the consequent depletion of free ubiquitin leads to impaired ubiquitination and stabilization of some substrates (54–57). Depletion of cellular ubiquitin through disruption of *DOA4* has been used to document proteasome-dependent degradation of the effector kinase Ste11 (58) and vacuole-dependent degradation of the **a**-factor transporter Ste6 (59). A third family member, Ubp3, is the only UBP isoform that regulates the pheromone-induced growth arrest and transcription-induction responses. We previously used a *ubp3*Δ mutant to detect the ubiquitinated form of the effector kinase Ste7 (14, 15). Ubp3 also regulates the ubiquitination of β′-COP (a subunit of the COPI coat protein complex) and Sec23 (a subunit of the COPII complex), which mediate transport between the endoplasmic reticulum and Golgi compartments (44, 45). Finally, we have shown here that Ubp12 binds specifically to Gpa1 and regulates Gpa1 ubiquitination. Ubp12 has not been characterized previously, but a related protein in *Schizosaccharomyces pombe* was shown to de-ubiquitinate Pop1. Pop1 is a subunit of the COP9 signalosome, a multiprotein complex whose role is to remove the ubiquitin-related peptide NEDD8 (Rub1) from members of the cullin family of ubiquitin ligases (60).

Of the pheromone-signaling proteins shown to undergo ubiquitination, nearly all are modified in response to pheromone treatment. Documented examples include the pheromone receptors Ste2 and Ste3, the regulator of G protein-signaling protein Sst2, and the effector mitogen-activated protein kinase kinase Ste7. In contrast, Gpa1 is ubiquitinated in a constitutive manner independent of pheromone stimulation. Gpa1 abundance is still regulated by pheromone however, since transcription is strongly pheromone-induced (2, 61). Moreover, once mating has occurred, transcription of *GPA1* stops altogether. Thus, although Gpa1 ubiquitination is unaffected by pheromone, Gpa1 expression is nevertheless pheromone-regulated. We believe that a high rate of Gpa1 turnover permits the cell to tightly regulate expression and rapidly eliminate Gpa1 once pheromone is removed or mating has occurred successfully.

Another question is why Gpa1 has evolved two separate mechanisms for degradation. One possibility currently being tested is that Gpa1 is polyubiquitinated when misfolded, misassembled, or mislocalized. Indeed it has been estimated that as much as 30% of newly synthesized proteins are incorrectly made and quickly degraded (62). Conversely, Gpa1 monoubiquitination may occur as part of the normal protein-clearance process. Stated differently, polyubiquitination could represent a mechanism for quality control whereas monoubiquitination places a time limit on the lifetime of the functional protein.

Thus, we have exploited the unique advantages of our experimental system to determine how the extent of ubiquitination can influence the degradation fate of a single protein. Based on our data we can propose a model in which Gpa1 is degraded by two pathways. The first pathway, used by the receptor and G protein, involves monoubiquitination, endocytosis, and delivery to the vacuole. The second pathway, also used by Ste7, involves polyubiquitination and delivery to the proteasome. This strong relationship between the extent of ubiquitination and trafficking of the G protein α subunit to its site of degradation establishes a new mechanism of G protein regulation. More generally, the discovery of a protein that is degraded by either of two pathways, depending only on the extent of ubiquitination, is to our knowledge unprecedented in any system.

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