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Articles

Direct Identification of a G Protein Ubiquitination Site by Mass Spectrometry†

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ABSTRACT: Covalent attachment of ubiquitin is well-known to target proteins for degradation. Here, mass spectrometry was used to identify the site of ubiquitination in Gpa1, the G protein α subunit in yeast *Saccharomyces cerevisiae*. The modified residue is located at Lys165 within the α-helical domain of Gα, a region of unknown function. Substitution of Lys165 with Arg ($Gpa1^{K165R}$) results in a substantial decrease in ubiquitination. In addition, yeast expressing the $Gpa1^{K165R}$ mutant are moderately resistant to pheromone in growth inhibition assays—a phenotype consistent with enhanced Gα signaling activity. These findings indicate that the α-helical domain may serve to regulate the turnover of Gpa1.

Many sensory and chemical stimuli act via cell surface receptors and intracellular G proteins. The primary role of the receptor is to discriminate among external signals, and to transmit the signal to the appropriate G protein. Upon receptor activation, the G protein α subunit releases GDP, binds GTP, and dissociates from the β/γ subunit complex. The dissociated subunits in turn transmit and amplify the signal to effector proteins that produce an intracellular second messenger within the cell. Signaling persists until GTP is hydrolyzed to GDP, and the subunits reassemble (*1*).

In yeast, pheromones act on G protein-coupled receptors to initiate mating—the fusion of **a** and α haploid cells to form the **a**/α diploid. Many of the yeast signaling proteins were identified genetically, through mutations that produce an unresponsive (*sterile* or *STE*) phenotype. These include genes encoding the pheromone receptors (Ste2, Ste3), the G protein β (Ste4) and γ (Ste18) subunits, G protein effectors (Ste5, Ste20, Cdc24), downstream protein kinases (Ste11, Ste7, Fus3), and a transcription factor (Ste12). Yeast lacking the G protein α subunit (Gpa1) are constitutively activated, resulting in pheromone-independent cell cycle arrest and mating (*2*).

There is emerging evidence that G proteins are regulated through posttranslational modifications. All known Gα subunits are myristoylated and/or palmitoylated (*3*). Many mammalian Gα subunits, some Gγ subunits (*3*), and the yeast Gβ (Ste4) are phosphorylated (*3*, *4*). All Gγ subunits are isoprenylated (*5*), and in at least one case (Ste18) is also palmitoylated (*6*, *7*). Such lipid modifications have attracted considerable attention because they are invariably needed for full G protein activity. In some cases, they can be

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regulated by extracellular signals. This has been shown for palmitoylation of mammalian $G_s\alpha$ and $G_q\alpha$ (*8*), and for myristoylation of Gpa1 (*9*).

Finally, it has been demonstrated that Gpa1 can be covalently modified with ubiquitin (*10*), a highly conserved peptide (73 of 76 residues are identical in human and in yeast) that earmarks proteins for degradation (*10*). The rate of degradation can also depend on the N-terminal amino acid of the ubiquitinated substrate, though Gpa1 appears to be an exception to this "N-end rule" paradigm (*10*). Proteolysis of the ubiquitinated substrate is usually carried out by the proteasome (*11*). However, proteolysis of some membrane proteins, including the receptors Ste2 and Ste3, is carried out instead within the vacuole (yeast counterpart to the lysosome) (*12, 13*). Similarly, it was shown recently that several mammalian G protein-coupled receptors are ubiquitinated prior to their endocytosis and degradation (*14, 15*).

Here, we have identified the ubiquitination site of Gpa1. This is the first direct identification of an in vivo ubiquitination site using mass spectrometry.

EXPERIMENTAL PROCEDURES

Strains and Plasmids. Standard methods for the growth, maintenance, and transformation of yeast and bacteria, and for the manipulation of DNA, were used throughout (*16*). The yeast *Saccharomyces cerevisiae* strain YPH499 (*MAT***a** *ura3*−*52 lys2*−*801*^{am} *ade2*−*101*^{oc} *trp1*-Δ*63 his3*-Δ*200 leu2*-Δ*1*) was used for Gpa1 purification and copper induction analysis. Strain YGS5 (*MAT***a** *ura3*-52 *lys2 ade2*^{oc} *trp1 leu2*-Δ1 *gpa1*::hisG *ste11*^{ts}) (*17*) was used for reporter transcription and growth arrest assays (*18*). Strains MHY753 (*MAT***a** *his3*-Δ200 *leu2*Δ1 *ura3*-52 *lys2*-801 *trp1*Δ63 *ade2*-101) and MHY754 (MHY753, *cim3*-1) (*19*) were used for protease sensitivity analysis.

Yeast shuttle plasmids used here are pRS316 (*CEN*, amp^R, *URA3*) (*20*), pRS316-GPA1 which contains *GPA1* under the control of its native promoter (*17*), pAD4M (2 μ, amp^R, *LEU2*, *ADH1* promoter/terminator, from P. McCabe, Onyx Pharmaceutical), pAD4M-GPA1 (*17*), pND187 (2 μ, amp^R, *URA3*, *CUP1* promoter), and pND187-derived plasmids containing ubiquitin (pND186), or myc-ubiquitin (pND747) (*21, 22*). His-tagged *GPA1* was constructed by replacing an internal 0.37 kbp fragment with a PCR product, generated using template *GPA1* (*Xba*I−*Xba*I fragment in pBluescript) and primers flanking the *GPA1 Hin*dIII site (5′-CCC AAG CTT TAC ATC ACC ATC ACC ATC ACC ATC ACG ATT ATA TCA ACG CCA GTG T- 3′) and the multiple cloning site *Sac*I (5′-CCC GAG CTC TCA TAT AAT ACC AAT TTT TTT AA-3′), to yield IHis8-GPA1. The resulting His tag follows codon 125 in the native sequence. The Lys165Arg mutation was generated with the QuikChange mutagenesis kit (Stratagene) and mutagenic oligonucleotide (5′-CAG AGT ACC GGA CGA GCA AAA GCT GCT TTC GAT GAA GAC-3′, plus the complementary oligonucleotide). Reporter transcription assays were carried out using pRS425-FUS1-lacZ (*23*).

Gpa1 Purification from Yeast. S. cerevisiae (YDK499) containing pAD4M-IHis8-Gpa1 and pND747 (myc-ubiquitin) was grown in synthetic complete medium plus 2% dextrose to $OD_{600\ nm} = 0.25$. Copper sulfate (100 μM) was added to induce myc-ubiquitin expression. After 4 h, cells were harvested by centrifugation at 4800*g* for 10 min at 4 °C, resuspended in 10 mM NaN_3, rapidly frozen in liquid N_2, and stored at −80 °C.

The cells were thawed at room temperature in 250 mL of Urea Buffer (6 M urea, 10 mM Tris, pH 8.0, 100 mM sodium phosphate, 10 mM 2-mercaptoethanol) and subjected to glass bead homogenization. The lysate was allowed to rock for 90 min to solubilize proteins, then centrifuged at 3840*g* for 20 min and filtered through Whatman paper no. 1 to remove cellular debris. The sample was mixed with 2 mL of equilibrated TALON Superflow resin (Clontech) for 90 min, then loaded onto a HR 10/10 (Amersham Pharmacia Biotech) column, and washed with 20 mL of Urea Buffer. Gpa1 was eluted using a step gradient consisting of 14 mL each of Urea Buffer plus 0, 8, 20, 28, 40, 48, 60, 68, 80, 88, 100, 120, 140, 160, 200, 240, 280, 320, and 400 mM imidazole. Fractions were analyzed by immunoblotting with polyclonal Gpa1 antibodies, as described previously (*9*). Pooled peak fractions were mixed with 2 mL of equilibrated Q-Sepharose Fast Flow resin (Pharmacia Biotech) for 90 min, washed with 10 mL of Urea Buffer, and eluted with a 20 mL 0−1 M linear NaCl gradient in Urea Buffer at 1 mL/min. Peak fractions were pooled and concentrated using a Millipore Ultrafree centrifugal filter (Biomax-10K MWCO) to a final volume of 250−350 μL. Samples (10−20 μL) were resolved by 16 cm × 0.75 mm sodium dodecyl sulfate−polyacrylamide (8% or 10%) gel electrophoresis (SDS−PAGE)[1] at 50 mA, visualized by Coomassie blue (Bio-Rad) or silver staining, equilibrated in 20% EtOH, 10% glycerol solution, and dried in cellophane.

Mass Spectrometry. Each Coomassie or silver-stained protein band was excised and transferred to a 0.5 mL microcentrifuge tube. The gel band was finely minced, dehydrated in 100 μL of CH_3CN for 10 min, and centrifuged to remove the CH_3CN, and then the step was repeated. The dehydrated gel pieces were dried under vacuum for 15 min after which an equal volume of milli-Q H_2O was added. The gel pieces were allowed to swell for 15 min. The water was removed and CH_3CN added for another round of dehydration. The solution was aspirated, and the gel pieces were dried again under vacuum.

For reduction and alkylation, 60 μL of 10 mM dithiothrietol in 100 mM NH_4HCO_3 was added, and the gel pieces were held at 56 °C for 45 min. After cooling, the solution was aspirated. An equal volume of 55 mM iodoacetamide in NH_4HCO_3 was added, and gel pieces were incubated in darkness for 30 min. Solution was again aspirated; then 60 μL of 100 mM NH_4HCO_3 was added. After 5 min, the solution was removed, and 100 μL of CH_3CN was added. Gel pieces were dehydrated for 15 min. Following aspiration, the dehydrated minced gel band was dried under vacuum for 25 min. Gel pieces were reconstituted with 50 μL of 10 ng/μL modified trypsin (Promega) in 50 mM NH_4HCO_3, held on ice for 40 min and topped with 50 mM NH_4HCO_3, and then transferred to 37 °C for overnight incubation. Gel pieces were centrifuged, and the supernatant was saved. Peptides were extracted first with 50 μL of milli-Q H_2O, then 3 times with 50 μL of 5% HCOOH, 50% CH_3CN. All four

[1] Abbreviations: CID, collision-induced dissociation; LC-MS/MS, liquid chromatography tandem mass spectrometry; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

supernatants were pooled, and the solution was dried under vacuum to almost dryness and then reconstituted in 14 μL of 0.005% heptafluorobutyric acid, 0.4% acetic acid in milli-Q H_2O.

Analysis of peptides by microelectrospray liquid chromatography tandem mass spectrometry (LC-MS/MS) was performed essentially as described by Gygi et al. (*24*). Microelectrospray columns were constructed from 360 μm o.d. × 75 μm i.d. fused silica capillary with the column tip tapered to a 5−10 μm opening. The columns were packed with 200 Å 5 μm C_{18} beads (Michrom BioResources Inc.), a reverse-phase packing material, to a length of 10−12 cm. The flow through the column was split precolumn to achieve a flow rate of 300 nL/min. The mobile phase used for gradient elution consisted of (A) 0.4% acetic acid, 0.005% heptafluorobutyric acid, 5% acetonitrile and (B) 0.4% acetic acid, 0.005% heptafluorobutyric acid in acetonitrile. The gradient was linear from 0.5% to 45% B in 35 min followed by 45−65% B in 5 min. Tandem mass spectra were recorded on a LCQ ion trap mass spectrometer (Thermoquest Corp., San Jose, CA) equipped with an in-house microelectrospray ionization source. Needle voltage was set at 1.6 kV. Ion signals above a predetermined threshold automatically triggered the instrument to switch from MS to MS/MS mode for generating collision-induced dissociation (CID) spectra (data-dependent MS/MS). The CID spectra were searched against a nonredundant yeast protein sequence database using the computer algorithm SEQUEST (*25*). Additional proteins were identified in the upper gel slice (YHNO hypothetical 56.5 kDa protein in dysi-pc15 intergenic region, DCP1 pyruvate decarboxylase 61.5 kDa, YEI1), the upper and middle gel slices (GLYC cytosolic serine hydroxymethyltransferase 52.2 kDa, GLYM mitochondrial serine hydroxymethyltransferase 53.7 kDa, NADM nicotinamide nucleotide adenyltransferase 45.9 kDa, YOR359w), the middle slice (HEM1 5-aminolevulinic acid synthase 59.4 kDa, G6PI glucose 6-phosphate isomerase 61.5 kDa), the middle and lower slices (HS42 heat shock protein 42−42.8 kDa, EF1H elongation factor 1-gamma 2−46.5 kDa), and the lower slice (MT17 *O*-acetylserine sulfhydrylase 48.5 kDa, and EF1A elongation factor 1a 50 kDa).

Pheromone Response Assays. For the pheromone-dependent growth inhibition assay (halo assay), cultures were grown to saturation (2−3 days), and 100 μL was diluted with 2 mL of sterile water, followed by the addition of 2 mL of 1% (w/v) dissolved agar (55 °C). This mixture was then poured onto an agar plate containing selective medium. Sterile filter disks were spotted with synthetic α-factor pheromone and placed onto the nascent lawn. The resulting zone of growth-arrested cells was documented after 3 days.

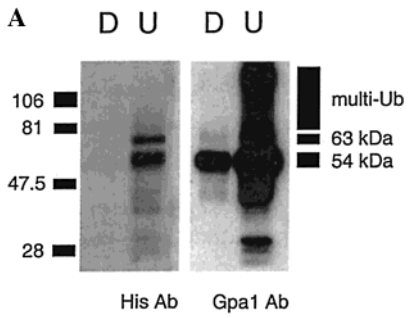
For pheromone-dependent reporter−transcription assays, cells were transformed with pRS425-FUS1-lacZ and grown to mid-log phase. Cultures were then aliquoted (90 μL) onto a 96-well plate and mixed with 10 μL of α-factor, in triplicate. Final α-factor concentrations ranged from 0 to 100 μM. After 90 min at 30 °C, β-galactosidase activity was measured by adding 20 μL of a freshly prepared solution of 83 μM fluorescein di-β-D-galactopyranoside (Molecular Probes, 10 mM stock in DMSO), 137.5 mM PIPES pH 7.2, and 2.5% Triton X-100 and incubating for 90 min at 37 °C. The reaction was stopped by the addition of 20 μL of 1 M Na_2CO_3, and the resulting fluorescence activity was measured with a multi-well plate reader using 485 nm excitation and 530 nm emission. All determinations were carried out at least twice with similar results.

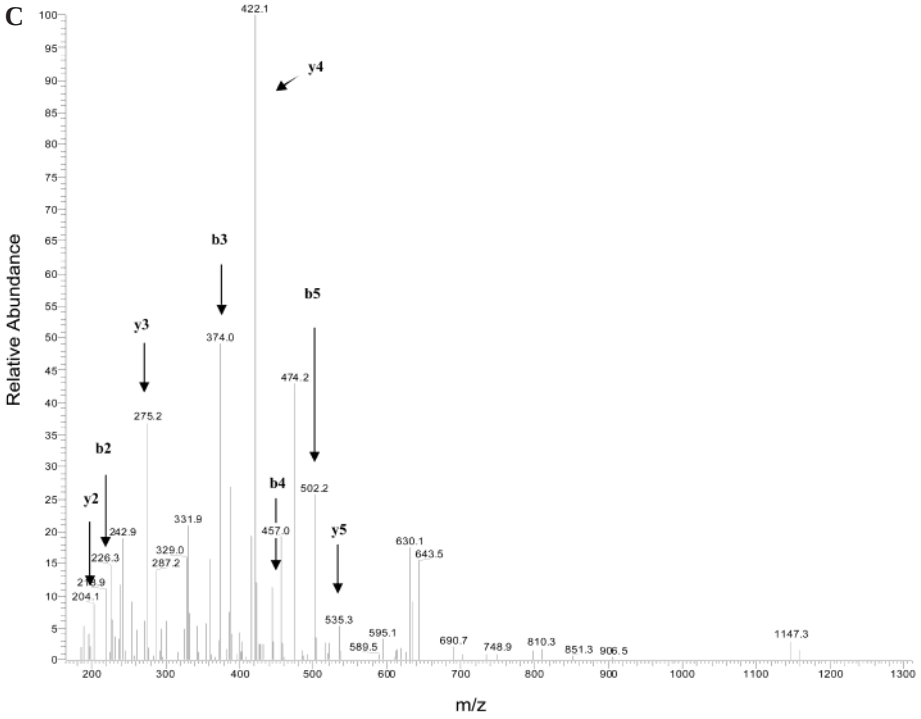
RESULTS

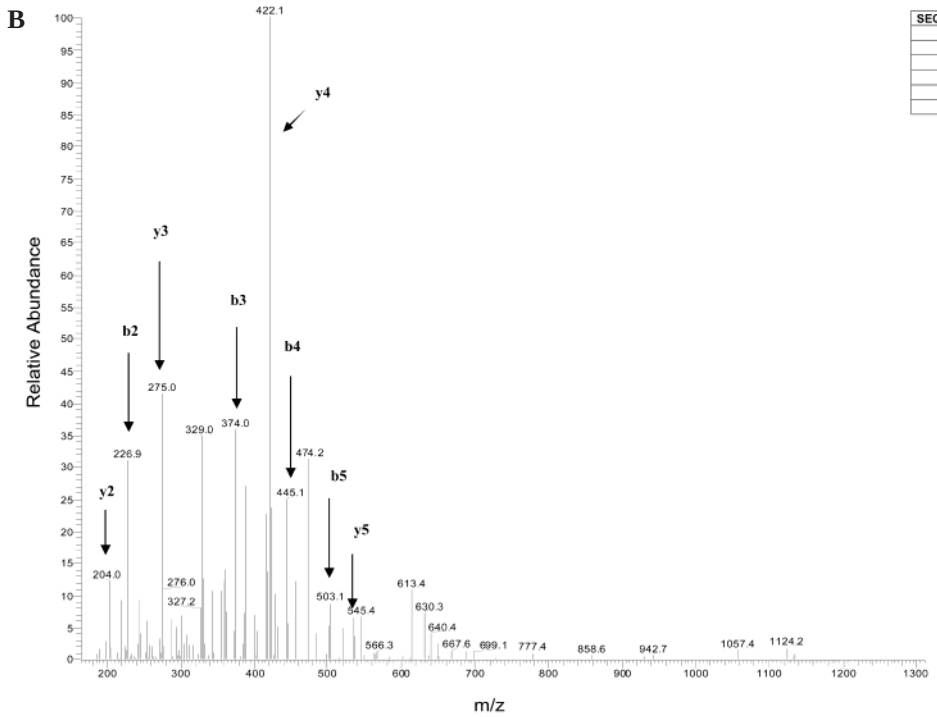
Our primary goal here was to map the site of ubiquitin attachment within Gpa1. Our approach was to purify Gpa1, and analyze the protein by electrospray ionization tandem mass spectrometry. A second aim was to determine if ubiquitination affects G protein function, through characterization of a ubiquitination-resistant mutant in vivo.

A plasmid containing *GPA1* (internally tagged with 8 His codons) was introduced into *gpa1*Δ mutant and wild-type cells. This plasmid could fully restore pheromone-dependent signaling to the *gpa1*Δ mutant strain, indicating that the His-tag does not interfere with Gpa1 function in vivo (data not shown). For purification, wild-type cells were co-transformed with a vector containing an N-terminal myc-tagged ubiquitin under the control of a copper-inducible promoter (*22*). Myc-ubiquitinated proteins are typically poor substrates for proteolysis, so their levels tend to accumulate within the cell (*22, 26*). Cells were lysed in 6 M urea (to fully solubilize Gpa1 and to inhibit proteolysis of the protein) and subjected to cobalt affinity chromatography. Gel electrophoresis and silver staining revealed a prominent band of molecular mass ∼54 kDa, as well as a minor band of ∼63 kDa that corresponds to the predicted size of 8-His-Gpa1 plus ubiquitin. In addition, a series of high molecular mass bands were detected, corresponding to poly- or multi-ubiquitinated forms of Gpa1. The identity of the bands was confirmed by immunoblotting with antibodies against Gpa1, poly-His, and the myc epitope (Figure 1A, and data not shown). These bands were absent in a similarly prepared sample using non-His-tagged Gpa1 (mock purification, data not shown).

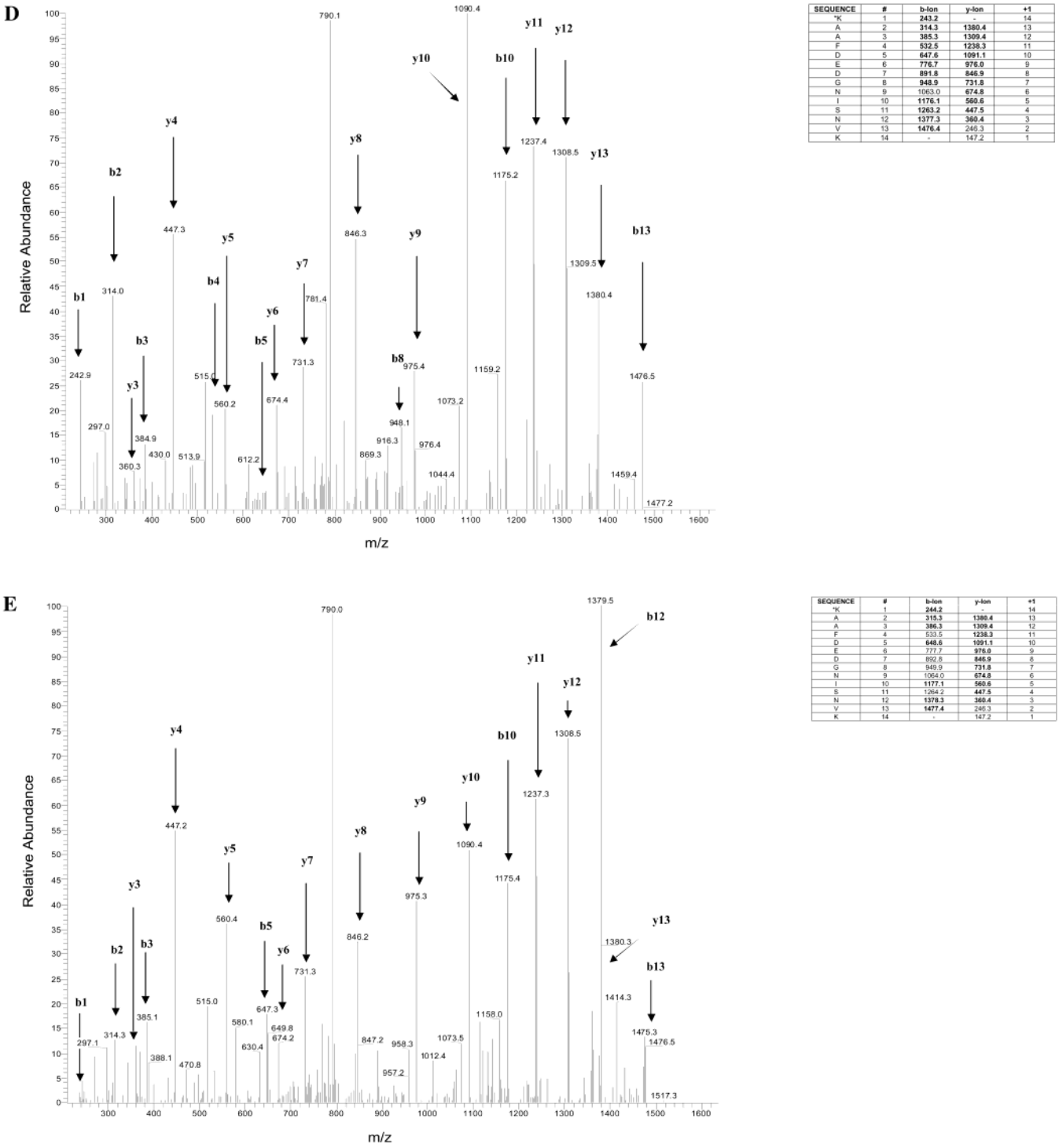
Three gel slices encompassing the region from 54 to 63 kDa were excised, subjected to limited digestion with trypsin, and analyzed by electrospray ionization tandem mass spectrometry. A similar approach was used previously to map the ubiquitination sites on recombinant *Xenopus laevis* calmodulin and purified bovine testis calmodulin, both conjugated in vitro by an enriched source of ubiquityl-calmodulin synthetase from rabbit reticulocytes (*27*). In two of the gel slices ("upper" and "middle"), a tryptic fragment was identified that corresponds to ubiquitin residues 43−48, Leu-Ile-Phe-Ala-Gly-Lys (Figure 1, panels B and C). The same two gel slices yielded a partial tryptic fragment corresponding to Gpa1 residues 165−178 (Lys-Ala-Ala-Phe-Asp-Glu-Asp-Gly-Asn-Ile-Ser-Asn-Val-Lys) plus a 115 Da modification at the Lys-165 position (Figure 1, panels D and E). These data are consistent with the attachment of the ubiquitin C-terminal Gly-Gly fragment (residues 75−76) in isopeptide linkage with the ϵ-amino side chain of Lys. Gly-Gly represents an expected trypsin cleavage product, as it is preceded by an Arg residue. Ubiquitination may have altered trypsin cleavage of Gpa1, since no unmodified peptide of the same sequence (residues 165−178) was detected. Neither ubiquitin nor the ubiquitinated form of Gpa1 was detected in the "low" band. The high probability scores from SEQUEST and the CID spectra corroborate the identity of the modified Gpa1 peptide. The presence of one prominent unassigned peak within the CID spectra at *m*/*z* of 790 can be accounted for







SEQUENCE	#	b-Ion	y-Ion	+1
L	1	114.2	-	6
I	2	**227.3**	**535.6**	5
F	3	**374.5**	**422.5**	4
A	4	**445.6**	**275.3**	3
G	5	**502.6**	**204.2**	2
K	6	-	147.2	1



SEQUENCE	#	b-Ion	y-Ion	+1
*K	1	243.2	-	14
A	2	314.3	1380.4	13
A	3	385.3	1309.4	12
F	4	532.5	1238.3	11
D	5	647.6	1091.1	10
E	6	776.7	976.0	9
D	7	891.8	846.9	8
G	8	948.9	731.8	7
N	9	1063.0	674.8	6
I	10	1176.1	560.6	5
S	11	1263.2	447.5	4
N	12	1377.3	360.4	3
V	13	1476.4	246.3	2
K	14	-	147.2	1

SEQUENCE	#	b-Ion	y-Ion	+1
*K	1	244.2	-	14
A	2	315.3	1380.4	13
A	3	386.3	1309.4	12
F	4	533.5	1238.3	11
D	5	648.6	1091.1	10
E	6	777.7	976.0	9
D	7	892.8	846.9	8
G	8	949.9	731.8	7
N	9	1064.0	674.8	6
I	10	1177.1	560.6	5
S	11	1264.2	447.5	4
N	12	1378.3	360.4	3
V	13	1477.4	246.3	2
K	14	-	147.2	1

FIGURE 1: Purification and mass spectrometry of Gpa1. His-tagged Gpa1 was expressed in YPH499 cells and purified by cobalt affinity and Mono Q-Sepharose chromatography. (A) Two microliters of undiluted ("U") or a 10-fold diluted sample ("D") of pooled and concentrated fractions was subjected to SDS−PAGE (8% acrylamide, 16 cm gel) and visualized using silver or Coomassie stain (not shown), and by immunoblotting using antibodies ("Ab") against Gpa1 or poly-His. Gpa1 migrates predominantly at ∼54 kDa, with minor species at 63 kDa (presumably mono-ubiquitinated) and higher (presumably multi-ubiquitinated), as indicated. Purified protein was excised from the stained gel in three adjoining segments, designated "upper" (contains the 63 kDa species), "lower" (54 kDa species), and "middle" (intervening segment). Each of the gel segments was subjected to limited trypsin proteolysis, and analyzed by LC-MS/MS. CID spectra were acquired by ion trap mass spectrometry for a +2 precursor ion with mass/charge (*m*/*z*) ratio of 649.29 from the "middle" gel slice (panel B, left) and for a +2 precursor ion with *m*/*z* of 648.36 from the "upper" gel slice (panel C), corresponding to the tryptic ubiquitin peptide LIFAGK (residues 43−48). The b and y ions detected are marked with arrows in the spectra and indicated in boldface in the table generated by SEQUEST (panel B, right). X-correlation scores were 1.96 and 1.51, respectively. CID spectra were also acquired for +2 precursor ion with *m*/*z* of 811.83 from the "middle" gel slice (panel D) and for +2 precursor ion with *m*/*z* of 811.6 from the "upper" gel slice (panel E), corresponding to a partial tryptic Gpa1 peptide *KAAFDEDGNISNVK with a Gly-Gly modified Lys residue. The b and y ions detected for the modified Gpa1 peptide are marked by arrows on the spectra and indicated in boldface in the tables generated by SEQUEST (panels D, right, and E, right). X-correlation scores were 2.9738 and 3.2023, respectively. Asterisk indicates modified amino acid residue depicting addition of Gly-Gly from ubiquitin to the peptide. Total Gpa1 sequence coverage was 36%, 36%, and 19% for the upper, middle, and lower samples, respectively.

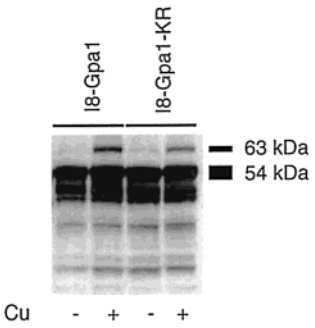


FIGURE 2: Gpa1^{K165R} exhibits a reduction in ubiquitination. To confirm that Lys165 is ubiquitinated in vivo, this residue was replaced with Arg and coexpressed with myc-ubiquitin, expressed under the control of the copper-inducible promoter from *CUP1*. Cells were grown in the presence ("+") or absence ("−") of copper ("Cu"), as indicated, then harvested, and lysed directly in boiling SDS−PAGE sample buffer. The abundance of native (54 kDa) and mono-ubiquitinated (63 kDa) Gpa1 was assessed by 8% SDS−PAGE and immunoblotting, using anti-Gpa1 rabbit polyclonal antibodies. KR, Lys165Arg mutation. I8, internal 8-His tag.

by the peptide Phe-Asp-Glu-Asp-Gly-Asn-Ile resulting from partial fragmentation of the larger modified peptide. This partial fragment was also seen in the CID spectra for the unmodified peptide, Arg.Ala-Lys-Ala-Ala-Phe-Asp-Glu-Asp-Gly-Asn-Ile-Ser-Trp-Val-Lys.

To confirm that ubiquitination occurs at position 165, we compared expression of a Lys165Arg substitution mutant (Gpa1^{K165R}) and the wild-type protein. Arg has a basic side chain but cannot be ubiquitinated. As shown in Figure 2, Gpa1^{K165R} exhibits a substantial reduction in expression of the 63 kDa species, with no change in the 54 kDa form of the protein. These data indicate that Lys165 is a major site of ubiquitination. The remaining 63 kDa species likely represents ubiquitination at a second (unidentified) site, as typically occurs when the primary site is altered (discussed below).

Most ubiquitinated proteins are modified with multiple copies of ubiquitin, and are then degraded by the proteasome. A notable exception is the G protein-coupled receptors Ste2 and Ste3, which are mono-ubiquitinated and degraded in the vacuole (*12, 13*). We were able to detect "laddering" indicative of multi-ubiquitination (Figure 1A). To confirm that Gpa1 is degraded by the proteasome, we monitored expression in a conditional mutant strain deficient in 26S proteasome (*cim3*-1) activity (*19*). As shown in Figure 3, expression of Gpa1 increased in the proteasome-deficient mutants, as did expression of the high molecular weight form of Gpa1. These data indicate that Gpa1 is degraded by the proteasome. A similar pattern was seen with our positive control, a wild-type strain expressing the protease-resistant myc-ubiquitin.

Since overexpression of *GPA1* leads to diminished signaling (*28*), and deletion of *GPA1* leads to sustained release of G$\beta\gamma$ and constitutive signaling, ubiquitin-dependent degradation of Gpa1 could alter the pheromone response (*29, 30*). To determine if the Gpa1^{K165R} mutation alters signaling, we measured pheromone-dependent gene transcription using a reporter−transcription assay (*FUS1* promoter, lacZ reporter, Figure 4A). By this method, we could detect no difference in pheromone sensitivity. Using the growth inhibition plate assay (halo assay), we again could detect little difference in the extent of growth inhibition (halo size, Figure 4B).

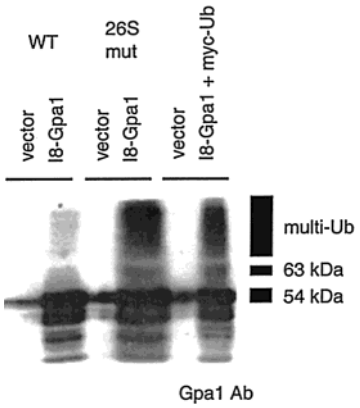


FIGURE 3: Increased Gpa1 ubiquitination in protease-deficient mutants. Gpa1 was expressed in wild-type cells ("WT"), a congenic mutant deficient in *CIM3* (required for 26 S proteasome activity, "26S mut"), or wild-type cells cotransformed with the myc-ubiquitin plasmid ("myc-Ub"). Whole cell lysates were resolved by 10% SDS−PAGE, and subjected to immunoblotting with anti-Gpa1 antibodies. The proteasome mutant and wild-type cells coexpressing a protease-resistant myc-tagged form of ubiquitin each exhibit an increase in high molecular weight (presumably multi-ubiquitinated) forms of Gpa1. I8, internal 8-His tag.

However, cells expressing the Gpa1^{K165R} mutant did exhibit a modest increase in recovery from growth arrest (turbid halos, Figure 4B). These effects indicate an enhanced recovery from pheromone-induced growth arrest, and/or attenuation of long-term signaling.

DISCUSSION

All biological processes are regulated through the modification of existing proteins. With regard to cell signaling, much work has focused on phosphorylation of receptors, and the consequent changes in their expression, localization, and signaling properties. More recently, attention has turned to downstream signaling components, to other types of covalent modification, and to regulation of protein degradation. Ubiquitin in particular is now recognized to be a central regulator of protein turnover. Ubiquitinated substrates include key regulators of cell communication, proliferation, and development (*11, 31*). However, only a handful of G protein signaling components are known to be ubiquitinated. These include several G protein-coupled receptors (*14, 15, 32*), a G protein α subunit (Gpa1) (*10*), and two RGS proteins (*33, 34*).

Most attempts to map sites of ubiquitination have used a process of elimination, through systematic mutation of candidate Lys residues (*26, 35*). This approach may not be practical for proteins with a large number of Lys residues (there are 43 in Gpa1). This approach can also be misleading, since mutation of a ubiquitinated Lys typically leads to modification of a second (nonphysiological) site. Analysis of three known substrates, c-Jun (*36*), the T-cell receptor ζ subunit (*37*), and the encephalomyocarditis virus 3C protease (*38*), revealed no single Lys-to-Arg mutation that blocked ubiquitination. A counter-example is Mdm2, which undergoes auto-ubiquitination of a single Lys residue (*39*), leading to its self-destruction. The inflammatory response regulator IκBα appears to be ubiquitinated at a single site in vivo but not in vitro, probably because of steric blockade of other Lys residues by protein binding partners within the cell (*40*). Thus, it is unclear whether some Lys residues are preferred

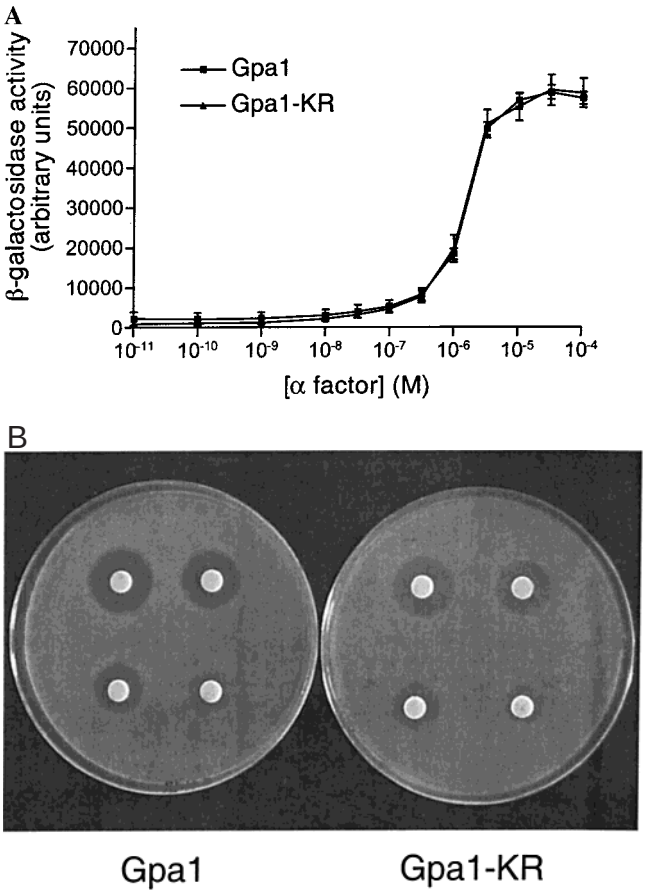


FIGURE 4: Functional consequences of Lys165 ubiquitination. To measure the pheromone-dependent response, *gpa1*Δ mutant cells were transformed with either the wild-type ("Gpa1") or the Lys165Arg mutant ($Gpa1^{K165R}$, "Gpa1-KR"), expressed from a plasmid using the native promoter. (A) Cells were treated with α-factor to induce *FUS1*-lacZ, and the consequent change in *β*-galactosidase activity was measured fluorometrically. The data shown are representative of two independent experiments. Error bars, ±SEM. (B) To measure pheromone-dependent growth arrest, cells were plated on solid medium and exposed to 100, 50, 25, or 12.5 *μ*g of α-factor, spotted onto filter disks. The resulting zone of growth inhibition was documented after 3 days.

sites when others are available. If a consensus sequence exists, direct methods will be needed to determine where ubiquitination occurs in vivo. The method described here should serve the purpose.

Our findings indicate that Gpa1 is ubiquitinated at Lys165 in vivo. Mutation of the ubiquitinated Lys leads to diminished ubiquitination of the substrate protein, but little or no difference in overall G protein expression or G protein-mediated signaling. The absence of any short-term functional effects of the Lys-to-Arg mutation is not surprising, considering the location of the residue within the protein. Crystal structure determination of mammalian Gα proteins has revealed two domains (*1, 41*) (Figure 5), separated by a narrow crevice within which the guanine nucleotide is bound. One domain is structurally similar to Ras ("Ras-like", "GTPase" or "G" domain) and contains all of the conformationally active "switch" regions, as well as all of the known binding determinants for effectors, G*βγ*, and guanine nucleotides. The second consists of a six-helix bundle ("α helical" domain), and has no known protein binding partners and does not appear to have any contact sites for guanine

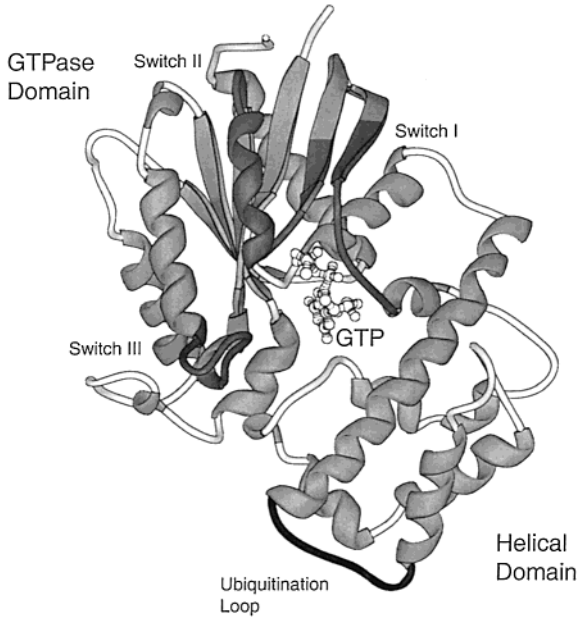


FIGURE 5: Structural localization of the ubiquitination site in Gα. The position of the unique 110 amino acid ubiquitination loop of Gpa1, which includes Lys165, is shown in the context of the $G_t\alpha$ a crystal structure (*40*−*43*). The top is likely oriented toward the lipid bilayer.

nucleotides. Both domains can be expressed separately and reassembled into a functional protein (*42, 43*). Based on structure, there has been speculation that the helical domain contributes to GTP binding affinity (*44*) and to effector recognition (*43, 45, 46*). Interactions between residues across the domain interface appear to be involved in receptor-mediated GTP binding and subsequent receptor−G protein dissociation (*47*). There is also some experimental evidence that the helical domain acts as a tethered GTPase accelerating protein, helping to swing a catalytic Arg (residue 178 in $G_{i1}\alpha$, 201 is $G_s\alpha$) into the substrate binding pocket (*42*). In the absence of any experimental evidence of binding or catalytic activity, however, the true function of the helical domain remains uncertain. Our data suggest that the helical domain of Gpa1 serves as a regulator of protein stability.

A question for the future is whether other Gα proteins are ubiquitinated or otherwise tagged for degradation. Receptor activation has been shown to destabilize Gα proteins in mammalian cells, but it is not known if this involves ubiquitin-dependent degradation of the protein (*48*). Gpa1 differs from other Gα proteins in that it has a half-life of less than 1 h, even when unstimulated (*10, 49*). Gpa1 is also unique in that it contains a large (110 amino acid) insert, encompassing the modified Lys. Although the $Gpa1^{K165R}$ mutant shows a normal rate of degradation (data not shown), deletion of the 110 amino acid insert does result in significant stabilization of the protein (Y. Wang and H. G. Dohlman, manuscript in preparation). Thus, Gpa1 may have evolved to allow rapid turnover, reflecting its transient role in cell signaling prior to mating. Pheromone signaling is required only during the transient developmental stages leading up to cell fusion. Once mating has occurred, the pheromone signal transduction apparatus is no longer needed and must be rapidly dismantled.

In conclusion, this work represents the first direct identification of an in vivo ubiquitination site by mass spectrom-

etry. It is also a rare example of a G protein signaling component that undergoes ubiquitination. This situation is likely to change, however, as improved methods for the identification of ubiquitinated species become available. The approach used here should be broadly applicable to the identification and characterization of other ubiquitinated proteins.

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