Quality Control of Plasma Membrane Proteins by *Saccharomyces cerevisiae* Nedd4-Like Ubiquitin Ligase Rsp5p under Environmental Stress Conditions

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In *Saccharomyces cerevisiae*, when a rich nitrogen source such as ammonium is added to the culture medium, the general amino acid permease Gap1p is ubiquitinated by the yeast Nedd4-like ubiquitin ligase Rsp5p, followed by its endocytosis to the vacuole. The arrestin-like *Bul1/2*p adaptors for Rsp5p specifically mediate this process. In this study, to investigate the downregulation of Gap1p in response to environmental stresses, we determined the intracellular trafficking of Gap1p under various stress conditions. An increase in the extracellular ethanol concentration induced ubiquitination and trafficking of Gap1p from the plasma membrane to the vacuole in wild-type cells, whereas Gap1p remained stable on the plasma membrane under the same conditions in *rsp5KO* and *Δend3* cells. A 14C-labeled citrulline uptake assay using a nonubiquitinated form of Gap1p (Gap1p<sup>K9R/K16R</sup>) revealed that ethanol stress caused a dramatic decrease of Gap1p activity. These results suggest that Gap1p is inactivated and ubiquitinated by Rsp5p for endocytosis when *S. cerevisiae* cells are exposed to a high concentration of ethanol. It is noteworthy that this endocytosis occurs in a *Bul1/2*-independent manner, whereas ammonium-triggered downregulation of Gap1p was almost completely inhibited in *Δbul1/2* cells. We also found that other environmental stresses, such as high temperature, H<sub>2</sub>O<sub>2</sub>, and LiCl, also promoted endocytosis of Gap1p. Similar intracellular trafficking caused by ethanol occurred in other plasma membrane proteins (Ap1p, Tat2p, and Gnp1p). Our findings suggest that stress-induced quality control is a common process requiring Rsp5p for plasma membrane proteins in yeast.

In eukaryotic cells, there are known to be protein quality-control mechanisms that remove aberrant polypeptides generated as a consequence of errors in transcription, translation, folding, and environmental stresses (1, 2). Misfolded membrane proteins are normally eliminated by endoplasmic reticulum (ER) quality control, in which the misfolded proteins are retrotranslocated into the cytoplasm and ubiquitinated, followed by degradation via the ubiquitin proteasome system (3, 4). In contrast, it is believed that plasma membrane proteins such as permeases and receptors with limited conformational defects can escape ER, localize at the plasma membrane, and be eliminated by lysosomal degradation, which serves as post-ER quality control (5–7). However, it is poorly understood how the post-ER quality control described above is involved in degradation of aberrant plasma membrane proteins generated by environmental stresses.

The budding yeast *Saccharomyces cerevisiae* is a unicellular eukaryote that is exposed to various stresses, including a high concentration of ethanol, freezing, desiccation, and high osmotic pressure during fermentation processes (8). It is important to clarify the quality-control mechanism of yeast plasma membrane proteins because these proteins would be directly exposed to such extracellular stress conditions. In particular, ethanol damages the cell membrane and functional proteins, gradually reducing cell viability and leading to cell death during fermentation (8–10). Therefore, the use of ethanol-resistant yeast strains with enhanced quality control of plasma membrane proteins would enable more efficient production of bioethanol and alcoholic beverages.

In mammalian cells, a variety of ubiquitin ligases recognize different plasma membrane proteins prior to endocytosis (11–13), but yeast endocytosis proceeds mostly through ubiquitination by the HECT-type ubiquitin ligase Rsp5p (14, 15). The essential protein Rsp5p is the only *S. cerevisiae* member of the Nedd4 family; it contains a membrane-binding C2 domain; three substrate-recognizing WW domains, which bind short peptide sequences called PY motifs (XPXY); and the catalytic HECT domain, which ligates ubiquitin to the target protein (16). The general amino acid permease Gap1p has been used as a model of ubiquitin-dependent trafficking of plasma membrane proteins (17). When yeast cells are cultivated on a poor nitrogen source, such as urea, allantoin, or proline, arrestin-like proteins Aly1p and Aly2p promote endosomal recycling of Gap1p, which is localized at the plasma membrane in a stable form (18). In contrast, after addition of a good nitrogen source, such as ammonium, Gap1p is rapidly ubiquitinated by Rsp5p, endocytosed, sorted into the multivesicular endosomes (MVEs), and finally degraded in the vacuoles (19, 20). It should be noted, however, that Rsp5p does not directly bind and ubiquitinate Gap1p and other several permeases that lack PY motifs. Instead, the adaptor proteins containing PY motifs mediate ubiquitination of these proteins by Rsp5p (21, 22). The arrestin-like redundant adaptor proteins Bul1p and Bul2p (*Bul1/2*P) are required for endocytosis of Gap1p in response to nitrogen availability (21). Recently, Merhi and André showed that ubiquitination of Gap1p localized at the plasma membrane is regulated via phosphorylation of Bul1/2p mediated by protein kinase.

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brane and was not ubiquitinated even when the mutant was grown
change at position 401 encoded by
(NH4)2SO4 and distilled water were used as the positive and negative
media (24).
We previously found that Gap1p in rps5Δ401E (i.e., the A-to-E
by incubation at 25°C for indicated periods. For high-temperature
was obtained from pYM-N24 (27) by SacI/XbaI digestion. Plasmid
Npr1p and association of the 14-3-3 protein to the phosphory-
TABLE 1 Yeast strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BY4741</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</td>
<td>Euroscarf</td>
</tr>
<tr>
<td>TSY239</td>
<td>BY4741 rps5Δ401E</td>
<td>24</td>
</tr>
<tr>
<td>TSY262</td>
<td>BY4741 bul1::hphNT1</td>
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</tr>
<tr>
<td>TSY263</td>
<td>BY4741 bul2::natNT2</td>
<td>24</td>
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<tr>
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</tr>
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<td>TSY266</td>
<td>BY4741 end3::natNT2</td>
<td>24</td>
</tr>
<tr>
<td>ESY001</td>
<td>BY4741 gap1::kanMX4</td>
<td>This study</td>
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</tr>
<tr>
<td>ESY003</td>
<td>BY4741 bul2::natNT2 bul3::kanMX4</td>
<td>This study</td>
</tr>
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Npr1p and association of the 14-3-3 protein to the phosphory-
lated site (23). We also proposed a novel mechanism for ubiquiti-
nation of Gap1p, in which Rsp5p is phosphorylated and inactivat-
ated when a poor nitrogen source is provided to the culture medium (24).

We show here that, under severe ethanol stress conditions, Gap1p undergo-
ubiquitinated and degraded under these stress conditions remains unknown.

We show here that, under severe ethanol stress conditions, Gap1p
protein in S. cerevisiae cells under environmental stress condi-
tions.

MATERIALS AND METHODS

Strains, culture media, and plasmids. All of the yeast strains used in
the present study were derivatives of S. cerevisiae BY4741. The strains are
listed in Table 1. Several gene knockout strains were obtained from the
plasmids used to examine localization of plasma membrane pro-
was obtained from pYM-N24 (27) by ScaI/XbaI digestion. Plasmid
pRS416-PGAL1::ccdB-yEGFP (24) was digested with the same restriction
enzymes set and ligated with the PGAL1 fragment. The GAP1, ACP1, GNP1, and TAT2 genes were amplified by PCR from yeast genomic DNA, and Gateway entry vectors were generated by a BP reaction with the am-
plified fragments and pDONR221. LR reactions were performed with the
and entry vectors to construct the plasmids pRS416-PGAL1-GAP1-yEGFP, pRS416-PGAL1-AGP1-yEGFP, pRS416-PGAL1-TAT2-
yEGFP, and pRS416-PGAL1-GNP1-yEGFP. To express Gap1p variants, plasmids pRS416-PGAL1-K16R-yEGFP, pRS416-PGAL1-R181K-
yEGFP, and pRS416-PGAL1-AGP1K16R,yEGFP were constructed using a QuikChange II XL site-directed mutagenesis kit (Stratagene).

To detect ubiquitination of Gap1p, two plasmids (pMK088 carrying
plasmid GAP1-yEGFP fusion gene were treated under various stress con-
ditions as described above and subjected to 40 μM FM4-64 (Biotium) for
min to visualize the vacuole. The cells were then harvested, washed with
medium. The samples were loaded on a 10.5% or an

Measurement of Gap1p activity. The Gap1p activity was measured as the
initial uptake rate of radioactive l-citricline by intact yeast cells (29).
Yeast cells were collected by centrifugation, washed with distilled water, and
suspended in SD medium at OD600 of 0.8. 14C-radioabeled l-citricline was added to the cell suspension at final concentrations of 4 μM. One
hundred microliters of the sample was withdrawn at an interval of 20 s and
filtrated with a glass fiber filter (GC-50, 25 mm diameter; Advantec). After
a washing step with 30 ml of cold distilled water and drying, the $^{14}$C radioactivity was measured by a liquid scintillation counter (LS6500; Beckman Coulter). Permease activities were calculated in pmol per min per OD$_{600}$.

RESULTS

A high concentration of ethanol induces ubiquitination and endocytosis of Gap1p. When a good nitrogen source is provided to the culture medium, Gap1p is ubiquitinated by Rsp5p and finally sorted into the vacuole (20, 30). To examine the localization of Gap1p under ethanol stress condition, we constructed plasmid harboring $y$EGFP-fused GAP1 under the control of the GAL1 promoter. Yeast cells that carry this plasmid were cultivated in SG+Alt medium to express Gap1p. After the expression was shut off in SD+Alt medium, the cells were treated with 50 mM (NH$_4$)$_2$SO$_4$, 15% ethanol, or an equal amount of distilled water as a control for 2 h and observed with the fluorescence microscope for the localization of $y$EGFP-fused proteins. Cell morphology was observed through differential interference contrast (DIC), and vacuolar membranes were stained with FM4-64. Bar, 5 μm. (B) Immunoblot analysis of the $y$EGFP-fused Gap1 proteins. S. cerevisiae wild-type cells harboring pRS416-P$_{GAL1}$-GAP1 were grown in SG+Alt medium to express Gap1p. After the expression was shut off in SD+Alt medium and c-Myc-tagged ubiquitin was ectopically induced from the CUP1 promoter by adding 0.1 mM CuSO$_4$ for 2 h, the cells were treated with 10 or 15% ethanol, and the same conditions as described above for 0.5, 1, 2, or 4 h and were subjected to immunoprecipitation and Western blot analysis with anti-$y$EGFP, anti-c-Myc, and anti-GAPDH antibodies. Whole-cell lysates used as input for the immunoprecipitation are shown (two bottom panels). The cells harboring the empty vector pRS416 (Gap1p-$y$EGFP:−) or expressing nontagged ubiquitin (myc-Ub:−) were used as negative controls (two left lanes). Molecular mass standards in kilodaltons are shown at the right.

FIG 1 Endocytosis and ubiquitination of Gap1p under ethanol stress conditions. (A) Subcellular localization of the $y$EGFP-fused Gap1 proteins. S. cerevisiae wild-type (BY4741), rps5A$^{401E}$, and ∆end3 cells harboring pRS416-P$_{GAL1}$-GAP1, or pRS416-P$_{GAL1}$-GAP1$^{K9/R/K16R}$ were grown in SG+Alt medium to express Gap1p. After the expression was shut off in SD+Alt medium, the cells were treated with 50 mM (NH$_4$)$_2$SO$_4$, 15% ethanol, or an equal amount of distilled water as a control for 2 h and observed with the fluorescence microscope for the localization of $y$EGFP-fused proteins. Cell morphology was observed through differential interference contrast (DIC), and vacuolar membranes were stained with FM4-64. Bar, 5 μm. (B) Immunoblot analysis of the $y$EGFP-fused Gap1 proteins. S. cerevisiae wild-type cells harboring pRS416-P$_{GAL1}$-GAP1-$y$EGFP were grown in SG+Alt medium to express Gap1p. After the expression was shut off in SD+Alt medium and c-Myc-tagged ubiquitin was ectopically induced from the CUP1 promoter by adding 0.1 mM CuSO$_4$ for 2 h, the cells were treated with 10 or 15% ethanol, and the same conditions as described above for 0.5, 1, 2, or 4 h and were subjected to immunoprecipitation and Western blot analysis with anti-$y$EGFP, anti-c-Myc, and anti-GAPDH antibodies. Whole-cell lysates used as input for the immunoprecipitation are shown (two bottom panels). The cells harboring the empty vector pRS416 (Gap1p-$y$EGFP:−) or expressing nontagged ubiquitin (myc-Ub:−) were used as negative controls (two left lanes). Molecular mass standards in kilodaltons are shown at the right.
was shut off by glucose before and during exposure to ethanol. Since the Gap1 expression depends on the nitrogen source is added, and removed from the plasma membrane via endocytosis requiring End3p. Since the Gap1 expression is severely impaired even in the presence of amino-nium (25). In the present study, Gap1p-yEGFP was localized on the plasma membrane of rps5ΔA01E cells under ethanol stress conditions (Fig. 1A), suggesting that endocytosis of Gap1p is also dependent on ubiquitination mediated by Rsp5p in the presence of a high concentration of ethanol. It is known that ubiquitination occurs at two lysine residues at positions 9 and 16 of Gap1p, and the nonubiquitinated Gap1p variant (Gap1pK9R/K16R) is constitutively localized on the plasma membrane, even after shifting to a rich nitrogen source (21). We found that Gap1pK9R/K16R was stable on the plasma membrane even after ethanol treatment, supporting the idea that ubiquitination of Gap1p is required for its endocytosis under ethanol stress conditions. The Δend3 cells carrying a severe defect in endocytosis (32) also showed the plasma membrane localization of Gap1p-yEGFP under ethanol stress conditions.

Furthermore, Western blot analysis detected the ubiquitinated forms of Gap1p-yEGFP in wild-type cells treated with 10% or 15% of ethanol (Fig. 1B), whereas Gap1pK9R/K16R-yEGFP was not ubiquitinated (see Fig. S1 in the supplemental material). Notably, ubiquitinated Gap1p under ethanol stress conditions was detected as a higher-molecular weight band than that observed after (NH₄)₂SO₄ treatment, suggesting that Gap1p is highly polyubiquitinated and/or aggregated after exposure to ethanol. In the presence of 15% ethanol, the low-mobility form of Gap1p appeared faster than in the presence of 10% ethanol. This suggests that severe ethanol stress induces high polyubiquitination or aggregation of Gap1p. Taken together, these data raise the possibility that Gap1p denatured by ethanol is recognized and ubiquitinated by Rsp5p, in a somehow different manner from Gap1p when a rich nitrogen source is added, and removed from the plasma membrane via endocytosis requiring End3p. Since the Gap1p expression was shut off by glucose before and during exposure to ethanol stress, trafficking of newly synthesized Gap1p proteins to the plasma membrane hardly occurred.

**Ethanol causes dysfunction of Gap1p on the plasma membrane.** To demonstrate the quality control process of Gap1p under ethanol stress conditions described above, denaturation or dysfunction of Gap1p on the plasma membrane needs to be confirmed. For cytoplasmic proteins, proteotoxic stresses was reported to lead to quick formation of ER-associated puncta (33). There has been, however, no direct evidence of denaturation of plasma membrane proteins by ethanol in vivo. Therefore, we measured the permease activity to obtain insight into the dysfunction of Gap1p. In S. cerevisiae, a nonstandard amino acid, is reported to be incorporated only through Gap1p in vivo (34). We examined the effect of ethanol stress on Gap1p activity in vivo measured by the incorporation of 13C-labeled l-citrulline. In wild-type cells, the Gap1p activity after the addition of 15% ethanol was dramatically decreased compared to that without stress (Fig. 2A). However, since Gap1p is internalized via endocytosis after ethanol treatment in wild-type cells, the decrease in Gap1p activity might be due to there being less Gap1p on the plasma membrane. Therefore, we next used Δgap1 cells expressing a nonubiquitinated form of Gap1p (Gap1pK9R/K16R) under the control of the GAL1 promoter, because this Gap1p variant remained stable on the plasma membrane even after exposure to ethanol as described above (Fig. 1A). As we expected, before cells were exposed to ethanol stress, l-citrulline uptake was not detected in Δgap1 cells carrying the vector only. Gap1p activity in Δgap1 cells overexpressing the wild-type GAP1 gene was significantly lower than in wild-type cells, suggesting that overexpression of Gap1p caused its rapid endocytosis. In contrast, yeast cells overexpressing GAP1K9R/K16R displayed a Gap1p activity similar to that in wild-type cells in the absence of ethanol, but this activity was markedly decreased after exposure to 15% ethanol for 30 min, as observed in wild-type cells. Even in this case, we could not exclude the possibility that the quantity of the Gap1p variant and/or total cellular activity might affect its permease activity. As shown in Fig. 2B, there were no significant differences in survival rates among yeast cells tested.
with or without ethanol treatment. Furthermore, Western blot analysis showed that the expression level of Gap1pK9R/K16R in the presence of 15% ethanol was almost the same as that under nonstress conditions (Fig. 2C). These results provide evidence demonstrating that ethanol stress directly causes dysfunction of Gap1p on the plasma membrane.

Other environmental stresses also induce endocytosis of Gap1p. Given that ethanol stress inactivated Gap1p and caused endocytosis via ubiquitination in the Rsp5p-dependent manner, we examined the localization of Gap1p-yEGFP under various stress conditions. Figure 3 shows the intracellular localization pattern of Gap1p after exposure to 15% ethanol, high temperature (40°C), 100 mM LiCl, and 0.25 mM H2O2. When (NH4)2SO4 was added to the culture medium, Gap1p-yEGFP rapidly accumulated in the vacuoles in >50% of the cells. In contrast, under ethanol stress conditions, a large quantity of cells showed localization of Gap1p both on the plasma membrane and in the vacuole. Although the percentage of cells in which Gap1p was completely sorted into the vacuoles gradually increased by addition of ethanol, Gap1p-yEGFP certainly accumulated in the intracellular punctate structures. Furthermore, we found that high temperature, H2O2, and LiCl stresses also induced endocytosis of Gap1p. These results indicate that Gap1p is endocytosed in response to various environmental stresses, as well as ethanol stress.

The Bul proteins do not affect endocytosis of Gap1p under ethanol stress conditions. There are two types of Rsp5p-triggered trafficking of Gap1p: one dependent on redundant adaptor proteins Bul1/2p and one independent of them (31). In the present study, we examined the involvement of Bul1/2p and a newly identified Bul family protein Bul3p (35) on endocytosis of Gap1p under both conditions of a rich nitrogen source and ethanol stress. When (NH4)2SO4 was added to the medium, both Δbul1 and Δbul2 cells showed the localization patterns of Gap1p similar to that of wild-type cells, while endocytosis of Gap1p was severely inhibited in Δbul1/2 cells (Fig. 4A). These results suggest the functional redundancy of Bul1/2p, which is consistent with the findings of previous reports (21, 31). In a previous report, it was concluded that both Bul1/2p-dependent and -independent pathways contribute to endocytosis of Gap1p in a rich nitrogen medium that contains Casamino Acids (31). As previously reported (23), our results also indicate that (NH4)2SO4 added to the culture medium induces predominantly Bul1/2p-dependent endocytosis of Gap1p. In addition, in wild-type, Δbul1, and Δbul2 cells, the Δbul3 mutation seemed to derepress endocytosis of Gap1p; disruption of BUL3 enhanced the trafficking of Gap1p to the vacuole with a poor nitrogen source, but did not exhibit any significant effects with (NH4)2SO4 (Fig. 4A). In contrast, disruption of BUL3 promoted endocytosis of Gap1p in Δbul1/2 cells with or without (NH4)2SO4. These results suggest that the Bul3 protein negatively affects Bul1/2p-independent endocytosis of Gap1p, which might occur at a low level under nonstress conditions in wild-type cells.

Under ethanol stress conditions, however, there was no significant difference in the Gap1p localization between wild-type and all bul mutant cells, suggesting that endocytosis of Gap1p proceeds through a Bul1/2p-independent manner in response to ethanol stress. It is known that ubiquitination of the lysine residue at either position 9 or position 16 is sufficient for Bul1/2p-dependent endocytosis (21). To examine the effect of the Bul1/2p-dependent pathway on Gap1p endocytosis under ethanol stress conditions, three types of Gap1p variants—K9R, K16R, and K9R/K16R—were expressed in Δbul1/2 cells (Fig. 4B). The sorting of Gap1pK9R occurred at almost the same level of that of wild-type Gap1p, whereas significant decreases were observed in the sorting of Gap1pK16R and Gap1pK9R/K16R to the vacuoles. These results also support the hypothesis that ethanol triggers Bul1/2p-independent endocytosis of Gap1p. In fact, it was recently shown that the arrestin-related adaptor protein Art1p is involved in Rsp5p-mediated endocytosis of the lysine permease Lvp1p under high-temperature stress conditions (36).

Ethanol also induces endocytosis of other plasma membrane proteins. We wondered whether a high concentration of ethanol induces endocytosis of other plasma membrane proteins, as well as...
as Gap1p. Accordingly, we next analyzed endocytosis of other plasma membrane proteins, Agp1p, Tat2p, and Gnp1p, under ethanol stress conditions (Fig. 5). Agp1p is a low-affinity, broad-specificity amino acid permease and is induced by uncharged amino acids, such as tryptophan and phenylalanine (37). Tat2p is a high-affinity tryptophan permease and is transported from the Golgi apparatus to the vacuole or endocytosed from the plasma membrane at a high concentration of tryptophan (38, 39). Gnp1p is a high-affinity glutamine permease and is constitutively transported to the vacuole in yeast cells expressing the hyperactive variant of Rsp5p (Rsp5p<sup>757L</sup>) (24, 40). Remarkably, all of the plasma membrane proteins described above were sorted into the vacuoles in response to ethanol. Agp1p was strictly delivered to the vacuole compared to Gap1p, whereas Tat2p and Gnp1p were partially internalized similar to Gap1p (Fig. 4A). These results suggest that yeast cells possess common quality control mechanisms for plasma membrane proteins involving ubiquitination, followed by removal from the plasma membrane via endocytosis under various stress conditions.

**DISCUSSION**

Many works have attempted to demonstrate the quality control functions of plasma membrane proteins using temperature-sensitive mutants of transporters and receptors, such as Pma1p (41), α-factor receptors (42), the arginine permease Can1p (5), and cystic fibrosis transmembrane conductance regulator CFTR (6). Recently, Apaja et al. (7) constructed CD4 receptor chimeras containing a temperature-sensitive bacteriophage λ domain in their cytoplasmic region, which is a sophisticated system applicable to various plasma membrane proteins. However, these studies assumed that the quality control mechanism for plasma membrane proteins escaped from the ER quality control involving ER-associated degradation (ERAD). In contrast, we hypothesize here that generation of aberrant proteins under various stress conditions such as a high concentration of ethanol and high temperature directly induces their ubiquitination on the plasma membrane by Rsp5p. Our hypothesis includes the mechanisms that occur after ubiquitination: ubiquitinated plasma membrane proteins are endocytosed and subsequently degraded in the vacuoles, whereas ubiquitinated cytosolic proteins are degraded by the ubiquitin/proteasome system (3, 4). To prove this, we first elucidated that Gap1p on the plasma membrane was ubiquitinated and sorted into the vacuoles in the presence of 10 or 15% ethanol (Fig. 1 and 3). It was confirmed that a high concentration of ethanol induced dysfunction of Gap1p on the plasma membrane, using a nonubiquitinated variant of Gap1p (Fig. 2). We particularly focused on ethanol stress in the present study because it is one of the most serious environmental stresses for yeast cells during fermentation processes involved in alcoholic beverages and bioethanol (8–10). During sake brewing or very high gravity fermentation for bioethanol production, yeast cells are exposed to >15% ethanol, in which other microorganisms cannot survive (8–10). It is noteworthy that yeast cells can still respond to 15% ethanol and change the ubiquitination status of Gap1p (Fig. 1).

Our results also showed that similar phenomena occurred in other plasma membrane proteins, Agp1p, Tat2p, and Gnp1p (Fig. 5), suggesting that the ethanol stress-induced quality control is a general mechanism requiring Rsp5p for plasma membrane proteins in *S. cerevisiae*. This is the first report to examine the quality control mechanism of plasma membrane proteins after exposure.
to severe ethanol stress, which might in part contribute to the improvement of fermentation properties of *S. cerevisiae*. Recently, Keener and Babst (43) reported quality control of the yeast high-affinity uracil importer Fur4p, which is one of the substrates for Rsp5p. They elucidated that Fur4p was localized into the vacuoles after treatment with not only uracil but also high temperature and H$_2$O$_2$. It was suggested that a common mechanism for substrate-induced trafficking and quality control of Fur4p is involved in the loop interaction domain-degron system (43). However, no particular adaptor proteins have been identified for the downregulation of Fur4p.

In addition to ethanol stress, we found that high-temperature, H$_2$O$_2$, and LiCl stresses also induced endocytosis of Gap1p (Fig. 3), suggesting that quality control of Gap1p mediated by Rsp5p is a common process regardless of the type of stress. Further study is necessary for elucidation of the molecular mechanisms, including the adaptor proteins required for endocytosis of Gap1p under these stress conditions. Among the stresses tested in the present study, LiCl treatment caused the largest sorting of Gap1p to the vacuoles. It is probable that high temperature and H$_2$O$_2$ induce protein denaturation due to their physicochemical properties, but the LiCl-triggered endocytosis of Gap1p might be due to the stress responses to cations such as the HOG1 and calcineurin pathways (44). Otherwise, based on a previous report that monovalent cations, including Li$^+$, induce conformational change of glucose oxidase (45), LiCl may directly lead to Gap1p denaturation on the plasma membrane.

Nedd4-family E3 ligases, including Rsp5p, require the adaptor proteins to bind their targets that have no PY motif. Until now, various adaptor proteins, such as arrestin-like Bul proteins and yeast arrestins, have been identified in *S. cerevisiae* (11, 46). It is intriguing to reveal the specificity of these adaptor proteins to-
ward different environments in the quality control mechanism of plasma membrane proteins. There are known to be two types of Rsp5p-triggered endocytosis of Gap1p, one dependent on and another independent of Bul1/2 (31). The Bul1/2-dependent process is involved in the trafficking from the Golgi to the vacuole while the MVEs are delivered to the vacuole through Bul1/2-independent ubiquitination (31). As previously reported, endocytosis of Gap1p from the plasma membrane occurs via both Bul1/2-dependent and independent pathways when Casamino Acids are supplied to the culture medium (31). Here, we determined the localization patterns of Gap1p using fluorescence microscopy and found evidence suggesting that endocytosis of Gap1p proceeds via the Bul1/2-dependent pathway when (NH₄)₂SO₄ was added to the medium but via the Bul1/2-independent pathway under ethanol stress conditions, based on the results for the Δbul1/2 mutant and the K16 variants of Gap1p (Fig. 4).

Another finding in the present study is that disruption of BUL3 led to derepression of endocytosis under negative-control conditions and enhancement of Bul1/2-independent endocytosis observed in ∆bul1/2 cells (Fig. 4A), suggesting a novel physiological role for Bul3p. Bul3p has been identified as a third member of the Bul family proteins based on structural similarities (35). However, the similarity is rather low, and disruption of the BUL3 gene did not confer a significant difference in endocytosis of the known Bul1/2-targeted permeases, such as Smf1p, Gap1p, and Can1p, although the direct interaction of Bul3p with Rsp5p has been observed (35). Given the hypothesis that Bul3p acts as a suppressor of Bul1/2-independent ubiquitination, we propose here a new model for Gap1p trafficking involving interaction with non-Bul adaptor proteins (Fig. 6). When a poor nitrogen source (e.g., allantoin) is provided or under the conditions lacking both Bul1/2p, endocytosis of Gap1p seems to occur at a very low level. Although its physiological role is still unknown, Bul3p is supposed to inhibit such a Bul1/2p-independent process (Fig. 6). Previously reported interaction between Bul3p and Rsp5p (35) might competitively prevent from interaction with the unknown adaptors. If this model is correct, Bul3p might be identified as a novel negative regulator of Rsp5p-dependent ubiquitination. It is also noted that this inhibitory mechanism does not work under ethanol stress conditions, because disruption of BUL3 did not significantly affect endocytosis of Gap1p in any of the tested strains (Fig. 4A).

Together, we have discovered Bul1/2p-dependent and -independent endocytosis of plasma membrane proteins in response to various stresses, namely, the plasma membrane quality-control mechanism. To further elucidate this, (i) phenotypic analysis of the adaptor protein mutants under multiple stress conditions and (ii) biochemical analysis to identify substrate-specific adaptor protein(s) should be intensively performed. Such studies would illuminate the multifunctionality of yeast Nedd4-like ubiquitin ligase Rsp5p and contribute to understanding how specifically and cooperatively the stress response systems are regulated in eukaryotic cells.

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