Engineering of the yeast ubiquitin ligase Rsp5: isolation of a new variant that induces constitutive inactivation of the general amino acid permease Gap1

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Abstract

Rsp5 is an essential ubiquitin-protein ligase in Saccharomyces cerevisiae. We found previously that the Ala401Glu rsp5 mutant is hypersensitive to various stresses that induce protein misfolding, suggesting that Rsp5 is a key enzyme for yeast cell growth under stress conditions. To isolate new Rsp5 variants as suppressors of the A401E mutant, PCR random mutagenesis was used in the rsp5A401E gene, and the mutagenized plasmid library was introduced into rsp5A401E cells. As a phenotypic suppressor of rsp5A401E cells, we isolated a quadruple variant (Thr357Ala/Glu401Gly/Lys764Glu/Glu767Gly) on a minimal medium containing the toxic proline analogue azetidine-2-carboxylate (AZC). Site-directed mutagenesis experiments showed that the rsp5T357A/K764E cells were much more tolerant to AZC than the wild-type cells, due to the smaller amounts of intracellular AZC. However, the T357A/K764E variant Rsp5 did not reverse the hypersensitivity of rsp5A401E cells to other stresses such as high growth temperature, ethanol, and freezing treatment. Interestingly, immunoblot and localization analyses indicated that the general amino acid permease Gap1, which is involved in AZC uptake, was absent on the plasma membrane and degraded in the vacuole of rsp5T357A/K764E cells before the addition of ammonium ions. These results suggest that the T357A/K764E variant Rsp5 induces constitutive inactivation of Gap1.

Introduction

During fermentation processes, yeast Saccharomyces cerevisiae cells are exposed to various stresses, including a high concentration of ethanol, freezing, desiccation, and high osmolality. Such stresses induce protein denaturation, generate abnormal proteins, and lead to growth inhibition or cell death. Under severe stress conditions, the fermentation ability of yeast becomes rather limited. In terms of industrial applications of yeast, stress tolerance is essential. In general, the accumulation of stress-induced abnormal proteins or misfolded proteins is a serious problem for cells. To overcome it, two strategies can be considered: (1) degradation of the proteins through a ubiquitin–proteasome system or (2) refolding by molecular chaperones, including stress proteins. The addition of some amino acid analogues can induce a transient physiological stress response in cells comparable to that of heat shock or ethanol stress (Ananthan et al., 1986; Trotter et al., 2001). 1-Azetidine-2-carboxylic acid (AZC) is a toxic four-membered ring analogue of proline that causes misfolding of only those proteins into which it is incorporated competitively with proline (Trotter et al., 2001). The accumulation of such abnormal proteins causes growth inhibition and cell death in yeast (Hoshikawa et al., 2003).

We previously isolated an AZC-hypersensitive mutant of S. cerevisiae and identified the RSP5 gene encoding a ubiquitin-protein ligase (E3) required for growth when yeast cells were exposed to AZC (Hoshikawa et al., 2003). The essential ubiquitin ligase Rsp5 is structured in three domains: an amino-terminal C2 domain, three WW domains, and a carboxyl-terminal homologous to the E6-AP carboxyl terminus (HECT) catalytic domain. This enzyme ligates ubiquitin to the target protein through the WW domains, which are the protein interaction modules that bind proline-rich ligands. Rsp5 participates in many biological events through ubiquitination of the target proteins.
endocytosis of plasma membrane permeases (Vandenbol et al., 1987; Galan et al., 1996; Springael & André, 1998), multivesicular body sorting (Katzmann et al., 2004), degradation of the large subunit of RNA polymerase II (Huibregtse et al., 1997), biosynthesis of unsaturated fatty acids (Hoppe et al., 2000), ER-associated degradation (Haynes et al., 2002), and heat-shock element-mediated gene expression (Kaida et al., 2003). In the Rsp5 sequence of the mutant, we found a single amino acid substitution, Ala401Glu, in the WW3 domain (Dunn & Hicke, 2001; Shcherbik et al., 2002). We found that the general amino acid permease Gap1 in rps5A401E cells remained stable and active on the plasma membrane, probably with no ubiquitination, leading to AZC accumulation and hypersensitivity (Hoshikawa et al., 2003). Interestingly, when yeast cells were exposed to stresses that induce protein denaturation, such as toxic amino acid analogues, high growth temperature in a rich medium, ethanol, and heat-shock treatment, rps5A401E cells showed much more sensitivity to these stresses than the wild-type strain (Hoshikawa et al., 2003). These results suggest that Rsp5 is a key enzyme involved in the degradation and repair of stress-induced abnormal proteins for yeast cell growth under stress conditions. In the repair system, the transcription of stress protein genes in rps5A401E cells was significantly lower than that in the wild-type strain when exposed to temperature up-shift or ethanol. Interestingly, the amounts of two major transcription factors required for stress protein gene expression, Hsf1 and Msn4, were remarkably low in rps5A401E cells (Haitani et al., 2006). It was recently found that the mRNAs of HSF1 and MSN2/4 were accumulated in the nucleus of rps5A401E cells after exposure to stresses. These results indicated that, in response to environmental stresses, Rsp5 primarily regulates the expression of Hsf1 and Msn2/4 at the posttranscriptional level, for example the nuclear export of mRNA and tRNA (Haitani & Takagi, 2008).

From the above observations, our objectives in this study were (1) to isolate the Rsp5 variant(s) as suppressors of the A401E mutant and (2) to analyze the stress-tolerant mechanism of the Rsp5 variant(s). The three-dimensional structure of Rsp5 has not yet been determined. Here, we successfully engineered Rsp5 for enhanced AZC-stress tolerance from the error-prone PCR-based mutagenized library. We found that the T357A/K764E variant Rsp5 may induce constitutive degradation of the general amino acid permease Gap1 and may facilitate degradation of other permease(s) or stress-induced abnormal proteins.

Materials and methods

Strains and plasmids

All the S. cerevisiae strains and plasmids used in this study are shown in Table 1. An AZC-hypersensitive mutant CHT81 (the A401E rps5 mutant) was isolated from CKY8 after ethyl methanesulfonate treatment (Hoshikawa et al., 2003). Escherichia coli strain DH5α [F’λ. Φ80lacZAM15A (lacZYA argF) U169 deoR recA1 endA1 hsdR17(rk-mQ) supE44 thi-1 gyrA96] was used to subclone the yeast gene.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype</th>
<th>Background and/or description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CKY8</td>
<td>α ura3-52 leu2-3,112, RSP5</td>
<td>S288C, wild-type (supplied by C. Kaiser)</td>
</tr>
<tr>
<td>CHT81</td>
<td>α ura3-52 leu2-3,112, rps5A401E</td>
<td>S288C, rps5A401E mutant (Hoshikawa et al., 2003)</td>
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<tr>
<td>pAD4</td>
<td>2-μm origin LEU2</td>
<td>Episomal plasmid (supplied by J. Nikawa)</td>
</tr>
<tr>
<td>pAD-RSP5</td>
<td>RSP5 in pAD4</td>
<td>The ADH1 promoter and terminator (Haitani et al., 2006)</td>
</tr>
<tr>
<td>pAD-RSP5-TAKE</td>
<td>rps5A401E in pAD4</td>
<td>This study</td>
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<td>This study</td>
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<td>rps5A401E in pAD4</td>
<td>This study</td>
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<tr>
<td>pRS415</td>
<td>CEN6 LEU2</td>
<td>Centromeric plasmid (Stratagene, La Jolla, CA)</td>
</tr>
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<td>pRS-RSP5</td>
<td>RSP5 in pRS415</td>
<td>The original promoter and terminator, this study</td>
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<td>rps5A401E in pRS415</td>
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<td>rps5A401E in pRS415</td>
<td>This study</td>
</tr>
<tr>
<td>pAD-RSP5-K</td>
<td>rps5A401E in pRS415</td>
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<tr>
<td>pRS415</td>
<td>CEN6 URA3</td>
<td>Centromeric plasmid (Stratagene)</td>
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<td>pRS-GAP1-EGFP</td>
<td>GAP1-EGFP in pRS416</td>
<td>This study</td>
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pAD-RSP5-T, and pAD-RSP5-K were used to overexpress the RSP5 genes under control of the ADH1 promoter and terminator. Plasmids pRS-RSP5, pRS-RSP5-TK, pRS-RSP5-T, and pRS-RSP5-K were used to express the RSP5 genes under control of the original promoter and terminator.

**Culture media**

The media used for growth of *S. cerevisiae* were a nutrient YPD medium (2% glucose, 1% Bacto yeast extract, and 2% Bacto peptone), a synthetic complete (SC) (Rose et al., 1990) consisting of 2% glucose, 0.67% Bacto Yeast Nitrogen Base without (NH₄)₂SO₄ and amino acids]. The SD medium contained 0.5% (NH₄)₂SO₄ (SD), 1% Bacto Yeast Nitrogen Base without (NH₄)₂SO₄ (Difco Laboratories, Detroit, MI), drop-out mix lacking l-leucine (SC-Leu), and a synthetic dextrose (SD) [2% glucose, 0.67% Bacto Yeast Nitrogen Base without (NH₄)₂SO₄ and amino acids]. The SD medium contained 0.5% (NH₄)₂SO₄ (SD+Am), 0.1% l-proline (SD+Pro), or 0.1% allantoin (SD+Alt) as the sole nitrogen source. Yeast strains were also cultured on SD+Am agar plates containing AZC, l-canavanine (l-arginine analogue), and l-norleucine (l-methionine analogue), l-β-hydroxy-norvaline (l-valine analogue). All of the analogues were obtained from Sigma-Aldrich (St. Louis, MO). The *E. coli* recombinant strains were grown in Luria–Bertani (LB) medium. The full-length Rsp5 variant that induces constitutive inactivation of Gap1 was added as a template to a solution containing 50 µg mL⁻¹. If necessary, 2% agar was added to solidify the medium.

**PCR-random mutagenesis**

The full-length *rps5*³⁴⁰₁E gene was amplified from plasmid p366-A401rps5 (Hoshikawa et al., 2003) by error-prone PCR with oligonucleotide primers RSP5-A401 (+) and RSP5-A401E (−) (Table 2). Ten nanograms of p366-A401rps5 was added as a template to a solution containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100, 0.5 mM dATP, 0.5 mM dTTP, 0.2 mM dGTP, 0.5 mM dCTP, 1.0 µM each of the two primers, 0.25 µM of *Taq* DNA polymerase (5 U µL⁻¹), and enough distilled water to bring the total volume to 50 µL. Twenty-five cycles (94 °C for 1 min, 65 °C for 1.5 min, and 72 °C for 1.5 min) of PCR were carried out. The unique 2.4-kb amplified band was digested with HindIII and SacI and ligated to the HindIII and SacI sites of pAD4. The ligated DNA was used to transform *E. coli* DH5α on LB solid medium containing ampicillin, and plasmid DNAs prepared from the ampicillin-resistant colonies (c. 375 000) were used to isolate mutant Rsp5 as the mutagenized plasmid library.

**Site-directed mutagenesis and construction of plasmids for expressing the RSP5 gene**

Plasmids pAD-RSP5-TAKE, pAD-RSP5-TKE, pAD-RSP5-TAK, pAD-RSP5-TK, pAD-RSP5-T, and pAD-RSP5-A were constructed for overexpressing the *rps5*³⁴⁰₁E, *rps5*³⁴⁰₁E³⁵⁷A, *rps5*³⁴⁰₁E³⁵⁷A⁻⁷⁶₇G, *rps5*³⁴⁰₁E³⁵⁷A⁻⁷⁶₇G⁻⁷₆⁷A, *rps5*³⁴⁰₁E³⁴⁰₁G⁻⁷⁶₇G, and *rps5*³⁴⁰₁G genes, respectively. These point mutations were introduced using a QuikChange II XL Site-directed Mutagenesis Kit (Stratagene) with pAD-RSP5 and oligonucleotide primers RSP5 (Table 2). The PCR products were digested with HindIII and SacI and ligated to the HindIII and SacI sites of pAD4. The ligated DNA was used to transform *E. coli* DH5α on LB solid medium containing ampicillin, and plasmid DNAs prepared from the ampicillin-resistant colonies (c. 375 000) were used to isolate mutant Rsp5 as the mutagenized plasmid library.

**Table 2. Oligonucleotide primers used in this study**

<table>
<thead>
<tr>
<th>Name</th>
<th>DNA sequence</th>
<th>Underlined sequence</th>
<th>Corresponding site</th>
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</thead>
<tbody>
<tr>
<td>RSP5-A401 (+)</td>
<td>5'-GG CCC AAC GTT AGC CCT TCA TCC ATG GCC GTG TCA -3'</td>
<td>HindIII site</td>
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<tr>
<td>RSP5-A401E (+)</td>
<td>5'-CTT TAT TGT GAG CTC AAA TAT AAA ACA -3'</td>
<td>SacI site</td>
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<tr>
<td>RSP5-T357A (+)</td>
<td>5'-GAC CAT AAT ACT AGA ACA ACC ACT TTT GTG GTG CCA AGG A -3'</td>
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<tr>
<td>RSP5-T357A (−)</td>
<td>5'-TCTT GCC TCG ACC CCA AGT GGC TGT TCT ATG ATT GTG GCC -3'</td>
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<td></td>
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<tr>
<td>RSP5-A401G (+)</td>
<td>5'-ATG AGA TGG ACC AAT AGC GGA CTG GTA TAT TTC GTT GAC C -3'</td>
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<tr>
<td>RSP5-A401G (−)</td>
<td>5'-G CTG ACC GAA AAT ACA TAC AGC TCG ACC GTT CTT AGT GAA AAT CTG -3'</td>
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<tr>
<td>RSP5-K764E (+)</td>
<td>5'-AGA AGA TCG TTC ATT AGA GAA GTT GGA GAA GTA CAA CCA TCA -3'</td>
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<tr>
<td>RSP5-K764E (−)</td>
<td>5'-A TGG TGG TAC TCT ACC AGC TTC ATT AGT GAA AAT GCT TCT -3'</td>
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<td></td>
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<tr>
<td>RSP5-E767G (+)</td>
<td>5'-C ACT ATT GAA AAA GAT GGT GGA GTA CAA CAA TTG CCA AAA -3'</td>
<td>Mutation site</td>
<td></td>
</tr>
<tr>
<td>RSP5-E767G (−)</td>
<td>5'-TGG CAA TGG TAT TCC ACC AGC TTT ACT AAT AGT G -3'</td>
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<tr>
<td>RSP5 (−)</td>
<td>5'-A CGT GGA ATG TCG TTA TGA TCT CTA TCA GTA TTA ACT ACC CCA TCA -3'</td>
<td>EcoRI site</td>
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<tr>
<td>RSP5 (−)</td>
<td>5'-ACC GGA ATT CCG TTT CAA GTA TAT ACC TCA -3'</td>
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<tr>
<td>RSP5 (−)</td>
<td>5'-TTG GTG CCT CTC GTG TGG ATG-3'</td>
<td>BamHI site</td>
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<tr>
<td>RSP5 (−)</td>
<td>5'-TTG CTA TAC TGA TTT TTT CCA CCA AAA TTA TTA TCA TCA TCA -3'</td>
<td>Sall site</td>
<td></td>
</tr>
</tbody>
</table>
construct pRS-RSP5. Plasmids pRS-RSP5-TK, pRS-RSP5-T, and pRS-RSP5-K for expressing the \( rsp5^{7357A/K764E} \), \( rsp5^{K757A} \), and \( rsp5^{K764E} \) gene, respectively, were constructed based on pRS-RSP5 using the same method as described above. The nucleotide sequences were confirmed by DNA sequencing.

**RNA extraction, reverse transcription-PCR, and real-time PCR**

Yeast cells were grown to the exponential growth phase (\( \text{OD}_{600\text{nm}} \) of 1.0) in SD+Pro+Ura medium at 25 °C, and 10 mM (NH\(_4\))\(_2\)SO\(_4\) was added to the culture medium. After cultivation for 0–10 min, the cells were harvested and washed, and the whole-cell extracts were prepared by vortexing the cells with glass beads. Total RNA from *S. cerevisiae* was isolated by the RNaseasy Mini Kit (Qiagen) and incubated with the RNAse-free DNase set (Qiagen). Two micrograms of total RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA) following the supplier’s guidelines. cDNA were amplified with the GAP1 gene-specific primers GAP1 (+) and GAP1 (-) (Table 2) and analyzed by real-time quantitative PCR performed with a 7300 Real-Time PCR System (Applied Biosystems). The mRNA level of the target gene was normalized to that of ACT1, which encodes β-actin, in the same sample. The cycle threshold \( (\text{Ct}) \) value for each reaction was determined using the 7300 real-time PCR system software package (Applied Biosystems). \( \text{Ct} \) values were used to calculate the mean fold change of the reactions via the 2\(^{-\Delta\Delta\text{Ct}} \) method for each sample in triplicate, for which 1 indicates no change in abundance (Livak & Schmittgen, 2001). For each gene tested, the nontranscript level measured in wild-type cells was arbitrarily set to 1.0 and all other values were represented relative to this standard.

**Western blot analysis**

Yeast cells were cultured to the exponential growth phase in SD+Pro+Ura medium, and 10 mM (NH\(_4\))\(_2\)SO\(_4\) was added to the culture medium. After cultivation for 0–60 min, the cells were harvested and washed, and the whole-cell extracts were prepared by vortexing the cells with glass beads in 1 M Tris-HCl buffer (pH 7.5) containing 0.5 M EDTA, 1 M MgCl\(_2\), 1 M KCl, 5% glycerol, and 2% sodium dodecyl sulfate (SDS) (Sambrook & Russell, 2001). The membrane-enriched fraction was collected by centrifugation for 45 min at 15 000 g, suspended in 0.1 M Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl, 5 mM EDTA, and 5 M Urea, kept on ice for 30 min, and sedimented as above. The resulting pellets were resuspended in 0.1 M Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl, 5 mM EDTA, and 2% SDS, and boiled for 10 min. The supernatant (30 μg of solubilized proteins) after centrifugation (10 min at 15 000 g) was boiled for 5 min and loaded on a 7.5% SDS-polyacrylamide gel. Rsp5, Gap1, and Pma1 were detected using an ECL plus Western blotting Detection System (Amersham Biosciences), anti-Rsp5 polyclonal antibody at 1:1 000 dilutions (supplied by J. Huibregste), anti-Gap1 polyclonal antibody at 1:3 000 dilutions (supplied by B. André), and anti-Pma1 monoclonal antibody at 1:4 000 dilutions (Funakoshi, Tokyo, Japan). Protein concentrations were determined using a Bio-Rad Protein Assay Kit (Hercules, CA) with bovine serum albumin as the standard protein.

**Fluorescence microscopy and vacuole staining**

The DNA fragment of 700 bp containing the 5′-upstream and 3′-downstream regions of the GAP1 gene was PCR-amplified using genomic DNA from the strain CKY8 and oligonucleotide primers GAP1-EGFP (+) and GAP1-EGFP (-) (Table 2). The PCR products were digested with Sall and BamHI and then ligated into the Sall–BamHI sites of pRS416 to construct pRS-GAP1. Plasmid pEGFP-N1 (Clontech, Mountain View, CA) was digested with BamHI and NolI, and the DNA fragment containing the EGFP gene was ligated into the BamHI–NolI sites of pRS-GAP1 to construct pRS-GAP1-EGFP. The nucleotide sequences were confirmed by DNA sequencing.

Yeast cells harboring pRS-GAP1-EGFP were grown to the exponential growth phase in SD+Alt medium at 25 °C and subjected to 5 mM Cell Tracker Blue CMAC (10 mM stock solution in dimethyl sulfoxide; Molecular Probes, Eugene, OR) for 15 min to visualize the vacuole. The cells were then harvested, washed with SD+Alt medium, and concentrated by a factor of 10 by centrifugation. Cells were viewed immediately, without fixation, under a fluorescence microscope (Axiovert 200 M, Carl Zeiss, Oberkochen, Germany), and images were captured with an HBO 100 Microscope Illuminating System (Carl Zeiss) digital camera and processed using Adobe Photoshop Elements 2.0 (Adobe Systems, San Jose, CA). The results presented are based on our observations of > 50 cells.

**Intracellular content of AZC**

Yeast cells were cultured to the exponential growth phase in SD+Pro+Ura medium, and AZC (a final concentration of 500 μg mL\(^{-1}\)) was added to the culture medium. After cultivation for 30 min, 5 mL of the cell suspension was removed, washed twice with 0.9% NaCl, and suspended in 0.5 mL of distilled water. The 1.5-Ml microcentrifuge tube containing cells was transferred to a boiling water bath, and intracellular amino acids were extracted by boiling for 10 min. After centrifugation (5 min at 12 000 g), each supernatant was subsequently quantitated with an amino acid analyzer JLC-500/V (model JLC-500/V; JEOL, Tokyo,
Prediction of the tertiary structure of the Rsp5 HECT domain

The three-dimensional structure was predicted using the computer program 3DJIGSaw (http://www.bmm.icnet.uk/~3djigssaw/), an automated system for construction of three-dimensional models of proteins based on homologues of known structures. The crystal structure of the human E6AP HECT domain-UbcH7 complex [PDB code 1C4Z (Huang et al., 1999)] was used as a template. Predicted structure was visualized using the computer program PYMOL (v0.99) (http://pymol.sourceforge.net/).

Results

Design of engineering Rsp5

Our previous works indicated that the stress hypersensitivity of rps5\textsuperscript{A401E} cells mainly results from the reduced induction of stress protein genes (Haitani et al., 2006) and that rps5\textsuperscript{A401E} cells are defective in the nuclear export of mRNAs of two major transcription factors required for stress protein gene expression: Hsf1 and Msn2/4 (Haitani & Takagi, 2008). Additionally, it is probable that rps5\textsuperscript{A401E} cells fail to degrade stress-induced abnormal proteins through specific recognition and ubiquitination (Hoshikawa et al., 2003). We therefore thought that it would be of interest to determine whether Rsp5 is involved in the degradation of abnormal proteins via the proteasome or the vacuole pathway.

In Rsp5, Ala401 in the WW3 domain is a nonconserved residue and is situated in the turn-like or the polar region (Sudol et al., 1995). An Ala401Glu substitution in Rsp5 is believed to impair the specific interaction with the targeted protein (Hoshikawa et al., 2003). Replacement of Ala401 (GCA) by the large polar residue Glu (GAA) may cause structural changes because of steric hindrance or may influence the electrical environment with the adjacent residue Arg402. Thus, if random mutagenesis is first introduced in the rps5\textsuperscript{A401E} gene, second-site reversions in the A401E mutant Rsp5 that at least partially restore function could be isolated as ‘global’ suppressors (Shortle & Lin, 1985), which may have multiple different alleles that display phenotypic suppression of the Rsp5-deficient characters. Subsequently, introduction of the second-site replacements by site-directed mutagenesis in the wild-type Rsp5 is expected to improve the enzymatic functions. To isolate new Rsp5 variants as suppressors of the A401E mutant, PCR random mutagenesis in the rps5\textsuperscript{A401E} gene was used as described in Materials and methods.

The Rsp5 variants that can grow at a high temperature were isolated by PCR random mutagenesis

The rps5\textsuperscript{A401E} gene-based mutagenized plasmid library was introduced into the rps5\textsuperscript{A401E} mutant CHT81, and transformants were selected on SC-Leu medium at 37 °C. Transformants harboring pAD4 (the empty vector) and pAD-RSP5 (the wild-type RSP5 gene) were prepared as negative and positive controls, respectively. As a result, we obtained 68 colonies capable of growing at 37 °C as well as colonies carrying pAD-RSP5. Plasmids prepared from these colonies were then shuttled into E. coli DH5α and back into rps5\textsuperscript{A401E} cells to retest high-temperature resistance. Of the 68 plasmids, 24 were proved to confer high-temperature resistance, confirming that this phenotype was caused by mutation(s) on the plasmid. The RSP5 genes in the plasmids were sequenced, and 16 types of amino acid substitutions were identified (data not shown). It is noteworthy that all clones contained a common amino acid change replacing Glu (GAA) with Gly (GGA) at position 401 within the WW3 domain, which is the target protein interaction module that binds proline-rich ligands. We considered that this substitution is required for phenotypic suppression of rps5\textsuperscript{A401E} cells, because a Gly residue at position 401 is conserved in the WW1 and WW2 domains (Fig. 1a). When we introduced pAD-RSP5-A carrying the rps5\textsuperscript{A401G} into rps5\textsuperscript{A401E} cells, the transformed cells were capable of growing to the level of the wild-type cells on agar plates with various stresses, such as high growth temperature, ethanol, LiCl, and H2O2, but not AZC (Fig. 2).

The Rsp5 variants with higher AZC resistance were isolated by PCR random mutagenesis

In terms of industrial applications, ethanol tolerance is one of the most important traits for yeast cells. However, it was technically difficult for us to control the concentration of the volatile ethanol on agar plates. Based on a report that AZC treatment can mimic ethanol treatment in the cell to induce the same spectrum of gene expression changes (Trotter et al., 2002), we attempted to obtain the Rsp5 variants with enhanced tolerance to the nonvolatile AZC instead of ethanol. We then transformed an AZC-hypersensitive mutant CHT81 with the mutagenized plasmid library and incubated the transformants on SD+Ura agar plates containing 2 μg mL\textsuperscript{-1} AZC at 30 °C for several days. As a result, we obtained 25 Leu\textsuperscript{+} colonies capable of growing on the AZC-containing medium as well as the colonies carrying pAD-RSP5. After secondary screening by spotting the candidates on SD agar plates containing 10 μg mL\textsuperscript{-1} AZC, three plasmids were recovered from three transformants that were resistant to AZC. When the plasmid was used to retransform
strain CHT81, all of the transformants were found to grow on the AZC-containing medium.

The \( \text{rsp5}^{A401E} \) genes in the plasmids were sequenced, and only one type of quadruple amino acid substitution, Thr357Ala/Glu401Gly/Lys764Glu/Glu767Gly (a single-base change from A to G at positions 1069, 1202, 2290, and 2300), was identified. Interestingly, the T357A/E401G/K764E/E767G Rsp5 variant showed a much greater tolerance to AZC than the wild-type Rsp5, suggesting that this quadruple mutation alters the function of Rsp5 (Fig. 3a). It should be noted that the E401G variant Rsp5 did not reverse the AZC hypersensitivity of \( \text{rsp5}^{A401E} \) cells (Fig. 2). To further investigate the degree of contribution to the AZC resistance of each substitution (T357A, K764E, and E767G) in the multiple-substitution variant, plasmids carrying triple, double, and single mutations were constructed by site-directed mutagenesis. The resultant plasmids were introduced into strain CHT81, and the growth phenotypes of transformants on the AZC-containing plates were examined. As shown in Fig. 3a, \( \text{rsp5}^{A401E} \) cells expressing the T357A/K764E variant Rsp5 were more tolerant than those expressing the wild-type enzyme. More interestingly, when we introduced pAD-RSP5-TK into wild-type strain CKY8, the transformed cells were more tolerant to AZC than those harboring the vector only (Fig. 3b). However, it is noteworthy that no single mutation alone reversed the stress hypersensitivity of \( \text{rsp5}^{A401E} \) cells (Fig. 3a). Thr357 within the WW2 domain is a conserved residue among the domains and Lys764 is located in the vicinity of a catalytic residue Cys777 within the HECT domain (Fig. 1a). These results suggest that double mutations of T357A and K764E in Rsp5 have a significant influence on the mechanism of AZC tolerance.

We also tested the growth phenotype of \( \text{rsp5}^{A401E} \) cells, which express the Rsp5 variants using the centromere plasmid pRS415 (Fig. 3c). Most of the transformants exhibited a growth pattern similar to those possessing high-copy-number plasmids under stress conditions. Unexpectedly, the single T357A mutation conferred AZC tolerance, although overexpression of the T357A variant did not reverse the hypersensitivity of \( \text{rsp5}^{A401E} \) cells (Fig. 3a).

Expression and stability of the T357A/K764E variant Rsp5 in \( \text{rsp5}^{A401E} \) cells

The protein level of Rsp5 was identified by Western blot analysis using the Rsp5 antibody (Fig. 4). We found that the protein amounts of Rsp5 (wild type and T357A/K764E variant) were reasonably detected, in proportion to the copy number of the \( \text{RSP5} \) gene. However, the protein levels of Rsp5 in the soluble fractions prepared from whole-cell extracts were almost the same among the different cell types tested (data not shown). Although the reason remains unclear, it is likely that a large portion of the overexpressed proteins passes into the insoluble membrane fraction. We further examined the stability of the Rsp5 proteins in whole-cell extracts. The protein amounts of Rsp5 were unchanged after exposure for 30 min at 37°C, suggesting that the wild-type and the T357A/K764E variant Rsp5 proteins are stable in the cells (Fig. 4).

Overexpression of the T357A/E764K variant Rsp5 did not reverse the hypersensitivity of \( \text{rsp5}^{A401E} \) cells to other stresses

The \( \text{rsp5}^{A401E} \) cells (CHT81 (pAD4)) also showed hypersensitivity to stresses that induce protein denaturation, such as...
The Gap1 proteins in \textit{rsp5}^{T357A/K764E} cells were absent on the plasma membrane and degraded in the vacuole before the addition of (NH₄)₂SO₄

The addition of ammonium ions to yeast cells growing on proline as the sole nitrogen source induces rapid inactivation and degradation of the general amino acid permease Gap1 through a process requiring Rsp5 (Springael & André, 1998). However, we found that Gap1 in \textit{rsp5}^{A401E} cells remained stable and active in the plasma membrane probably with no ubiquitination, leading to AZC accumulation and hypersensitivity (Hoshikawa et al., 2003). For the cell to acquire high tolerance to AZC, two strategies can be considered: (1) blocking of AZC uptake by rapid degradation of Gap1 or (2) degradation of the AZC-induced abnormal protein in the vacuole or the proteasome via ubiquitin-mediated pathways.

Thus, we examined the effect of the Rsp5 variant on the mRNA levels and the protein amounts of Gap1, because the toxic AZC enters yeast cells partially via Gap1 based on the result that the gap1-disrupted strain CKY8 grew on the AZC-containing medium and accumulated smaller amounts of intracellular AZC than strain CKY8 (Hoshikawa et al., 2003). We introduced high-copy-number plasmid pAD-\textit{RSP5}-TK harboring the \textit{rsp5}^{T357A/K764E} gene into \textit{rsp5}^{A401E} cells (CHT81). The transformant cells were grown in SD+Pro, and (NH₄)₂SO₄ was added to the culture medium. Real-time PCR analysis showed that the mRNA levels of \textit{GAP1} were almost the same between three strains CKY8 (pAD4) and CHT81 (pAD-\textit{RSP5}) expressing the wild-type and the T357A/K764E variant Rsp5, respectively (Fig. 6a). It appears that the gene dosage effect of \textit{RSP5} may affect the mRNA level of \textit{GAP1} in strain CHT81 (pAD-RSP5). It was also found that the transcription of \textit{GAP1} was significantly repressed by the addition of ammonium ions in these strains. Although we do not know the mechanistic basis for this reduction, Rsp5 may regulate the amount of \textit{GAPI} gene transcription in the presence of a good nitrogen source. In Western blot analysis using Gap1 antibody, Gap1 in strains CKY8 (pAD4) and CHT81 (pAD-RSP5) expressing the wild-type Rsp5 was removed from the plasma membrane after the addition of ammonium ions, whereas Gap1 in strain CHT81 (pAD4) expressing the A401E mutant Rsp5 remained stable with little degradation (Fig. 6b), as occurred in the previous reports (Springael & André, 1998; Hoshikawa et al., 2003). Interestingly, the Gap1 proteins in \textit{rsp5}^{T357A/K764E} cells were hardly detectable on the plasma membrane even in the absence of NH₄⁺ (Fig. 6b). To further determine whether the T357A/K764E variant Rsp5
influences the sorting of Gap1, the subcellular localization of Gap1 was examined (Fig. 7). We constructed plasmid pRS-GFP-EGFP containing the EGFP-fusion gene of GAP1 and introduced it into wild-type and rsp5A401E cells. When cultured in SD+Am, Gap1 is distributed both in the plasma membrane and in the vacuole because of nitrogen catabolite repression (NCR) (data not shown). To avoid NCR, we then used allantoin, which supports high-level GAP1 expression (Tate et al., 2002), as well as proline, as the sole nitrogen source. In both strains CKY8 and CHT81 expressing the wild-type Rsp5, Gap1 was mainly localized to the plasma membrane. However, we found that Gap1 was clearly observed in the lumen of the vacuole in rsp5T357A/K764E cells [CKY8 (pAD-RSP5-TK) and CHT81 (pAD-RSP5-TK)]. These results indicate that the Gap1 proteins were absent on the plasma membrane and degraded in the vacuole of rsp5T357A/K764E cells before the addition of ammonium ions.

We also measured the relative amount of intracellular AZC (Table 3). In rsp5A401E cells [CHT81 (pAD4)], the AZC content at 30 min after addition of 5 mM AZC was approximately twofold higher than that in wild-type cells [CKY8 (pAD4)]. On the other hand, extremely small amounts of AZC were detected in rsp5T357A/K764E cells [CHT81 (pAD-RSP5-TK)], in agreement with the Gap1 levels on the plasma membrane. These results indicate that the T357A/K764E variant Rsp5 confers AZC resistance to yeast cells by preventing the Gap1-mediated import of AZC. This is probably due to the Gap1 inactivation caused by constitutive degradation even in the absence of NH₄⁺ or by the failure of intracellular sorting in rsp5T357A/K764E cells.
Discussion

In this study, we attempted to screen the phenotypic suppressors that have second-site mutation(s) in the \( \text{rps}^{5A401E} \) gene with PCR random mutagenesis. As a result, we newly isolated an Rsp5 variant (T357A/K764E) with increased AZC-stress tolerance when overexpressed. A multicopy-suppressor strategy can be a good way to improve the protein function in a process of interest, but it is also possible that the mutations identified represent artifacts that are not really involved in the function. As shown in Fig. 3c, the single T357A mutation unexpectedly conferred AZC tolerance. As described above, it appears that the T357A/K764E variant Rsp5 remains stable but the T357A variant might be aggregated or mislocalized in cells when overexpressed. A revertant-type variant (E401G) was also isolated at a high growth temperature (37 °C) as a multicopy suppressor of \( \text{rps}^{5A401E} \) cells. It seemed slightly strange that this variant failed to counter the AZC hypersensitivity of

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**Fig. 4.** Expression and stability of the T357A/K764E variant Rsp5. Saccharomyces cerevisiae strains CKY8 (wild type) harboring an empty vector (pAD4), and CHT81 (\( \text{rps}^{5A401E} \) mutant) harboring pAD4, a wild type (pAD-RSP5), or a T357A/K764E variant (pAD-RSP5-TK) RSP5 gene were cultured to the logarithmic growth phase in SD+Pro+Ura medium at 25 °C and subjected to a temperature up-shift (from 25 to 37 °C, 30 min). Whole-cell extracts were prepared and analyzed by Western blot analysis using anti-Rsp5 polyclonal antibody. The 3-phosphoglycerate kinase Pgk1 was used as a protein-loading control.

**Fig. 5.** Effect of the T357A/K764E variant Rsp5 on yeast growth phenotypes. After cultivation in liquid SD+Am+Ura medium at 30 °C for 48 h, c. \( 10^6 \) cells of Saccharomyces cerevisiae CKY8 (wild type) or CHT81 (\( \text{rps}^{5A401E} \) mutant) harboring an empty vector (pAD4), a wild type (pAD-RSP5), or a T357A/K764E variant (pAD-RSP5-TK) RSP5 gene and serial dilutions of \( 10^{-1} \) to \( 10^{-6} \) (from left to right) were spotted and incubated on SD+Am+Ura medium containing the toxic analogue, ethanol, sorbitol, or LiCl, SD+Am+Ura medium after freezing treatment, or SC-Leu medium containing H$_2$O$_2$ at 25 °C (or 37 °C) for 3 days.
A401E cells. This may suggest that a nonpolar Ala residue rather than a polar Gly residue at positions 401 in the WW3 domain of wild-type Rsp5 is required for nitrogen-regulated degradation of Gap1, due to a hydrophobic interaction with side chain(s) of neighboring residue(s).

Our results showed that two amino acid substitutions at positions 357 and 764 in the WW2 domain and the HECT domain, respectively, are crucial for higher tolerance to AZC. The three WW domains are protein–protein interaction motifs with an affinity for proline-rich ligands (Macias et al., 2002) and, in some cases, they mediate direct substrate binding (Huibregtse et al., 1997; Ciechanover, 1998). In particular, the WW2 domain is essential for the interaction of Rsp5 with its substrate Rps1, the largest subunit of RNA polymerase II (Wang et al., 1999). WW domains are presumably located at cellular membranes and fold into three antiparallel β-sheets (Macias et al., 1996). As shown in Fig. 1a, Thr357 in the WW2 domain is a conserved residue and is situated in the turn-like or polar region (Sudol et al., 1995). The mutation at position 357 is novel because the point mutations of conserved residues, Trp359Phe and Pro362Ala, resulted in temperature-sensitive growth defects due to the failure of normal receptor-mediated endocytosis (Dunn & Hicke, 2001) and proper nuclear export of mRNA (Rodriguez et al., 2003).

On the other hand, the HECT domain encodes an activity by which ubiquitin is ligated to a protein in the process of targeted protein degradation (Ciechanover, 1998). In the Rsp5 protein, catalytic Cys777 forms a ubiquitin-thioester intermediate, accepting ubiquitin from the ubiquitin-conjugating enzyme (E2) (Scheffner et al., 1995). Substrates are then ubiquitinated by transfer from the HECT domain to the substrate (Salvat et al., 2004). Mutations within the HECT domain, such as active-site mutation (Cys777Ala) and the rsp5-1 allele (Leu733Ser) that disrupts or impairs formation of the ubiquitin-thioester and/or the ability to catalyze transfer of ubiquitin to substrates, abrogate the in vivo function of Rsp5 (Wang et al., 1999). In addition, several rsp5 mutants, such as mdp1-1 [Pro784Thr (Zoladek et al., 1997)], rmc3 [Pro772Ser (Harkness et al., 2002)], spa1-1 [Phe748Val (Estrella et al., 2008)], and HPG1-4 [Pro514Thr, Cys517Tyr/Phe, and Ala799Thr (Abe & Iida, 2003)], are known to exhibit defects in growth at an elevated temperature, in chromatin assembly, in turnover of low-affinity phosphate transporters, and in pressure-induced differential regulation of tryptophan permeases, respectively. Interestingly, Lys764 is a nonconserved residue among the HECT domain family of proteins. Based on the crystal structure of the human E6AP HECT domain (the Rsp5 homologue)—the UbcH7 E2 complex (Huang...
Rsp5 variant that induces constitutive inactivation of Gap1

et al., 1999), the Rsp5 HECT domain was predicted to consist of two lobes packed across a small interface (Abe & Iida, 2003). It should be noted that the mutation site of K764E is mapped in a β-strand of a small C-terminal lobe, which is probably at the interface of the HECT domain (Fig. 1b). The transfer of ubiquitin from the HECT domain to the substrate likely proceeds through an isopeptide bond formation between ubiquitin and the substrate. This would require the HECT domain, which has a catalytic cysteine, and the WW domain that binds to the substrate to be in close proximity. Although the tertiary structure has not been determined for the WW domain, it is conceivable that a significant conformational change would result in the juxtaposition of the HECT and WW domains during ubiquitin transfer. This may involve substantial movement or rotation of the C-terminal lobe (VanDemark & Hill, 2002). It is
Table 3. Intracellular AZC content of yeast strains expressing the RSP5 gene

<table>
<thead>
<tr>
<th>Strain (plasmid)</th>
<th>AZC content (% of dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CKY8 (pAD4)</td>
<td>1.7 (100)</td>
</tr>
<tr>
<td>CHT81 (pAD4)</td>
<td>3.9 (217)</td>
</tr>
<tr>
<td>CHT81 (pAD-RSP5)</td>
<td>1.3 (76)</td>
</tr>
<tr>
<td>CHT81 (pAD-RSP5-TK)</td>
<td>0.28 (17)</td>
</tr>
</tbody>
</table>

The intracellular AZC content was measured 30 min after addition of 5 mM AZC in SD+Pro+Ura medium. Values in parentheses are relative contents. The data are means from three independent experiments.

Currently unclear why double mutations in the separated domains altered the Rsp5 function, but the dramatic changes in terms of charge and polarity (K764E and T357A, respectively) may enhance the interaction between the two residues.

As a possible mechanism by which overexpression of the rsp5\textsuperscript{T357A/K764E} gene confers AZC resistance to yeast cells, we noted that the T357A/K764E variant Rsp5 might induce constitutive degradation or impair intracellular trafficking of Gap1. Upon addition of NH\textsubscript{4}\textsuperscript{+}, Gap1 is endocytosed and targeted to the vacuole, where it is degraded. This downregulation requires ubiquitination of the permease in an Rsp5-dependent manner (Springael & André, 1998). With respect to the regulation of Gap1 activity and trafficking, the Npr1 kinase controls both the transport of neosynthesized Gap1 from the Golgi to the plasma membrane and the stabilization of Gap1 at the plasma membrane (De Craene et al., 2001). It is possible that an altered Rsp5 with enhanced ubiquitin ligase activity can recognize and bind to a dephosphorylated (inactivated) form of Gap1 in the sorting pathway or at the plasma membrane. In addition to the above mechanisms, our results raise the possibility that the T357A/K764E variant Rsp5 may efficiently inactivate other permease(s) involved in AZC import, such as Put4, Agp1, or Gnp1 (Hellwell et al., 2001; Andréasson et al., 2004), or may degrade the AZC-induced misfolded proteins, or both. We must further analyze the rps5\textsuperscript{T357A/K764E} cells isolated here to clarify the mechanism.

Ubiquitination of proteins in many cases targets abnormal proteins or short-lived proteins for proteasome-mediated degradation (Hochstrasser, 1996; Varshavsky, 1997) or, as has been shown for some membrane proteins, for internalization and degradation via the vacuolar pathway (Galan et al., 1996; Hicke & Riezman, 1996). The ubiquitin system consists of the concerted action of three classes of enzymes: ubiquitin–activating enzymes (E1), E2, and E3. First, ubiquitin is activated by E1 in an ATP-dependent reaction through the formation of a high-energy thioester intermediate between E1 and ubiquitin. Ubiquitin is then transferred to one of several E2s. Finally, ubiquitin is transferred to a substrate protein by E2 alone or in conjunction with E3. This final step plays a major role in substrate recognition. Therefore, once the function of an E3 such as Rsp5 can be improved as described here, it may be possible to confer stress tolerance to yeast cells. Similarly, we have already shown enhancement of stress resistance in S. cerevisiae by co-overexpression of Rsp5 and the appropriate E2 (Hiraishi et al., 2006). In other cases, overexpression of two transcriptional factors, Kin28 and Pog1, isolated as multicy copressors of rps5\textsuperscript{A401E} cells, was suggested to enhance the protein refolding and degradation ability on the specific pathway between Rsp5 and the candidate genes or through Rsp5-independent pathways (Demae et al., 2007). These results imply that a combination of mutagenesis of the target gene(s) involved in the ubiquitin system and selection of the desired phenotype under stress conditions is a useful method for improved stress tolerance.

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