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Ai Nagayama · Chiaki Kato · Fumiyoshi Abe

The N- and C-terminal mutations in tryptophan permease Tat2 confer cell growth in *Saccharomyces cerevisiae* under high-pressure and low-temperature conditions

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Abstract Tryptophan uptake appears to be the limiting factor in growth of tryptophan auxotrophic *Saccharomyces cerevisiae* strains under the conditions of high hydrostatic pressure and low temperature. When the cells are subjected to a pressure of 25 MPa, tryptophan permease Tat2 is degraded in a manner dependent on ubiquitination by Rsp5. One of the high-pressure growth-conferring genes, *HPG2*, was shown to be allelic to *TAT2*. The *HPG2-1* ($Tat2^{E27F}$) mutation site is located within the ExKS motif in the N-terminus, and the *HPG2-2* ($Tat2^{D563N}$) and *HPG2-3* ($Tat2^{E570K}$) mutation sites are located at the KQEIAE sequence in the C-terminus. The *HPG2* mutations enhance the stability of Tat2 during high-pressure or low-temperature incubation, leading to cell growth under these stressful conditions. These results suggest that the cytoplasmic tails are involved in Rsp5-mediated ubiquitination of Tat2 under high-pressure or low-temperature conditions.

Keywords High-pressure growth · Low-temperature growth · Rsp5 ubiquitin ligase · Tryptophan permease Tat2 · Ubiquitination

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F. Abe (✉)
The DEEPSTAR Group,
Japan Marine Science and Technology Center (JAMSTEC),
2–15 Natsushima-cho,
237-0061 Yokosuka, Japan
E-mail: abef@jamstec.go.jp
Tel.: +81-46-8679678
Fax: +81-46-8679715

A. Nagayama · C. Kato
Department of Biological Information,
Graduate School of Bioscience and Biotechnology,
Tokyo Institute of Technology,
Yokohama, Japan

C. Kato
Department of Marine Ecosystems,
JAMSTEC, Yokosuka, Japan

Introduction

High hydrostatic pressure has inhibitory effects on biological processes employed by organisms inhabiting atmospheric pressure, and deep-sea organisms have developed the ability to survive under such extreme conditions (Yayanos 1995; Abe et al. 1999; Bartlett 1999). Pressures exceeding a few hundred MPa (a few thousand atmospheric pressures) kill microorganisms (Ludwig 2002). In the yeast *Saccharomyces cerevisiae*, a pressure of 150–180 MPa significantly reduces the viability of the cells, and a heat shock protein, Hsp104, and trehalose have protective effects against the pressure (Iwahashi et al. 1997). Recently, the effect of high pressure has been analyzed using yeast DNA microarrays in *S. cerevisiae*, and transcription of numerous genes appeared to be stimulated (Iwahashi et al. 2003).

We have demonstrated that a pressure of 25 MPa inhibits the uptake of tryptophan by cells and induces degradation of the high-affinity tryptophan permease Tat2 in *S. cerevisiae* (Abe and Horikoshi 2000). Tryptophan auxotrophic strains such as YPH499 arrest the cell cycle in G_1 phase as result of such extreme conditions. Overexpression of *TAT2* permits cell growth at high pressure (Abe and Horikoshi 2000). Therefore, tryptophan uptake could be a primary target of increasing pressure in terms of cell growth. Tat2 is also degraded upon starvation or after treatment with the immunosuppressive agent rapamycin, whereas the general amino acid permease Gap1 is induced (Beck et al. 1999). The inactivation process involves ubiquitination, subsequent endocytosis, and the vacuolar sorting mechanism. Ubiquitination of Tat2 occurs on lysines in its cytoplasmic N-terminal domain in a manner dependent on Rsp5 ubiquitin ligase (Beck et al. 1999). Similarly, N-terminal lysines have been shown to be target sites for ubiquitination in Gap1 (Springael and André 1998; Helliwell et al. 2001; Soetens et al. 2001) or the uracil permease Fur4 (Marchal et al. 2000).

We have begun to isolate mutants capable of growth at high pressure, referred to as high-pressure growth (*HPG*) mutants. Phenotypic characterization and cloning of the genes are expected to provide insights into a subset of components involved in the regulation of Tat2 under high-pressure conditions. Analysis of the *HPG* mutants identified four semidominant linkage groups, *HPG1*, *HPG2*, *HPG3*, and *HPG4*, which are involved in cell growth at high pressure (Abe and Iida 2003). *HPG1* was revealed to be allelic to *RSP5*, and the mutation site was located within the catalytic HECT domain of Rsp5 ubiquitin ligase (Abe and Iida 2003). The *HPG1* mutation caused a remarkable accumulation of Tat2 proteins in the plasma membrane, leading to high-pressure growth of the cells (Abe and Iida 2003). In this study, we demonstrate that *HPG2* is allelic to *TAT2* and that the *HPG2* mutation stabilizes Tat2 at high pressure and low temperature, and alters the localization of Tat2 protein in the cells.

Materials and methods

Strains and plasmids

The haploid strain YPH499 was used as a wild-type strain (Sikorski and Hieter 1989). The strains and plasmids used in this study are listed in Tables 1 and 2. pTB355 (*URA3* 2 μ) containing a gene encoding $Tat2^{5K>R}$ protein was kindly provided by M.N. Hall of Basel University, Switzerland (Beck et al. 1999) and was used to generate pTB355c (*URA3*, *CEN4*) (Abe and Iida 2003) . Strain RHY525 containing hemagglutinin (HA)-tagged Pma1 was kindly provided by R. Hirata of Riken, Japan. YPD, SD, and SC media (Guthrie and Fink 1991) were used with slight modifications (Abe and Iida 2003). Cells were grown at 0.1 MPa (atmospheric pressure) and 24 °C, 25 MPa and 24 °C, or 0.1 MPa and 15 °C.

Cloning of the *HPG2* gene

Cloning of the *HPG2* gene was carried out in the same way as that of the *HPG1* gene (Abe and Iida 2003). Genomic libraries containing 10- to 20-kb DNA fragments from *HPG2* strain FAY9D were constructed using the plasmids YCplac33 and YEplac195. The wild-type strain YPH499 was transformed with each of the plasmid libraries, and the transformants capable of growth at 18–25 MPa were selected. Plasmids obtained from the transformants were used for retransformation of the wild-type cells to confirm their ability to grow at high pressure. The *HPG2* alleles were recovered from the eight *HPG2* mutants by the Gap repair method, and the entire coding regions were sequenced. The plasmid carrying 2HA-tagged *HPG2-2* or 2HA-tagged *HPG2-3* was constructed by replacing the *Bam*HI (internal site)/*Eco*RI fragment from p2HA-TAT2c (YCplac33-2HA-TAT2; Abe and Horikoshi 2000) with the corresponding fragment from pHPG2-2c or pHPG2-3c.

Tryptophan uptake assay

Cells from an exponentially growing culture in SC medium were incubated at 0.1 or 25 MPa for 5 h and collected by centrifugation. The tryptophan uptake assay was carried out using L-[5-^{3}H] tryptophan (TRK460, 1.18 TBq/mmol; Amersham Pharmacia Biotech UK, Little Chalfont, UK), as described previously (Abe and Horikoshi 2000; Abe and Iida 2003).

Western blot analysis

To prepare whole-cell extracts for SDS-PAGE and western blot analysis, 1×10^8 cells were collected by centrifugation and washed in ice-cold 10 mM NaN_3/10 mM NaF. The cells were broken using glass beads, and unbroken cells were removed by centrifugation at 500 *g* for 5 min. The supernatant was treated with 4% SDS and 5% 2-mercaptoethanol at 37 °C for 10 min to denature the proteins. Western blot analysis was performed as described previously (Abe and Iida 2003).

Subcellular fractionation

Whole-cell extracts were subjected to centrifugation on a sucrose-density gradient (20–60%) at 100,000 *g* for 18 h as described by Roberg et al. (1997). Fractions were collected from the top and the proteins were precipitated with 7% TCA in the presence of 0.015% deoxycholate.

Results and discussion

HPG2 gene is allelic to *TAT2*

To identify the *HPG2* gene, we transformed the wild-type strain YPH499 with the *HPG2* genomic library and

Table 1 Strains used in this study. All strains are isogenic derivatives of strain YPH499

Strain	Genotype	Reference
YPH499	*MAT* **a** *his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ade2-101 ura3-52*	Sikorski and Hieter (1989)
YPH500	*MAT* **α** *his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ade2-101 ura3-52*	Sikorski and Hieter (1989)
FAY18A	*MAT* **a** *his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ade2-101 ura3-52 HPG1-1*	Abe and Iida (2003)
FAY29E	*MAT* **a** *his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ade2-101 ura3-52 HPG1-4*	Abe and Iida (2003)
FAY3A	*MAT* **a** *his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ade2-101 ura3-52 HPG2-1*	This study
FAY9D	*MAT* **a** *his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ade2-101 ura3-52 HPG2-2*	This study
FAY131E	*MAT* **a** *his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ade2-101 ura3-52 HPG2-3*	This study
FAB158	*MAT* **a** *his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ade2-101 ura3-52 tat2Δ::HIS3*	Abe and Iida (2003)
RHY525	*MAT* ***a*** *his3-D200 leu2-D1 lys2-801 trp1-D1 ade2-101 ura3-52 HA-PMA1*	Hirata; constructed according to dos Santos et al. (2000)

Table 2 Plasmids used in this study

Plasmid	Description	Reference
YCplac33	*URA3 CEN4*	Gietz and Sugino (1988)
YEplac195	*URA3* 2 μm	Gietz and Sugino (1988)
YIplac211	*URA3*	Gietz and Sugino (1988)
pTAT2c	2.6-kb fragment containing*TAT2* promoter-*TAT2* in YCplac33	Abe and Horikoshi (2000)
pTAT2e	2.6-kb fragment containing*TAT2* promoter-*TAT2* in YEplac195	Abe and Horikoshi (2000)
pTAT1e	2.7-kb fragment containing*TAT1* promoter-*TAT1* in YEplac195	Abe and Iida (2003)
pHPG2–1c	2.6-kb fragment containing*HPG2* promoter-*HPG2–1* in YCplac33	This study
pHPG2–2c	2.6-kb fragment containing*HPG2* promoter-*HPG2–2* in YCplac33	This study
pHPG2–3c	2.6-kb fragment containing*HPG2* promoter-*HPG2–3* in YCplac33	This study
p2HA-TAT2c	2.7-kb fragment containing*TAT2* promoter-2HA-*TAT2* in YCplac33	Abe and Horikoshi (2000)
p2HA-HPG2-2c	2.7-kb fragment containing*HPG2* promoter-2HA-*HPG2-2* in YCplac33	This study
p2HA-HPG2-3c	2.7-kb fragment containing*HPG2* promoter-2HA-*HPG2-3* in YCplac33	This study
pTB355c	pAS55c with nucleotide substitutions changing lysine codons 10, 17, 20, 29, and 31 of *TAT2* to arginine codons	Beck et al. (1999), Abe and Iida (2003)

obtained transformants capable of growth at high pressure as described in the case of the identification of *HPG1* (Abe and Iida 2003). As a result, 21 transformants containing three different overlapping plasmids were obtained. The three plasmids carried *TAT2* (chromosome XV), *TAT1-TIP1*-BAP2 (chromosome II), and *TVP18*-YMR067C-YMR068W-YMR069W-*MOT3* (chromosome XIII). Among the nine candidate genes in the three plasmids, the *TAT2*, *TAT1*, and *TVP18* genes, encoding the high-affinity tryptophan permease Tat2 (Schmidt et al. 1994), the low-affinity tryptophan permease Tat1 (Schmidt et al. 1994), and a Tlg2-vesicle integral membrane protein Tvp18, respectively, were revealed to confer high-pressure growth at 25 MPa and low-temperature growth at 15 °C on the wild-type cells in multicopy (data not shown). DNA sequencing of the entire coding regions revealed that a base substitution was only observed in the ORF of the *TAT2* gene, suggesting that *HPG2* is allelic to *TAT2*. To confirm that *HPG2* is allelic to *TAT2*, we integrated the plasmid YIplac-*TAT2* (*URA3*) into each *HPG2* strain. Stable transformants (*MAT***a** *HPG2-X TAT2 URA3*) were crossed with the mating strain YPH500 (*MATα HPG2 TAT2 ura3*), and the segregation of the Hpg^+ phenotype and Ura^+ phenotype was determined for 20 asci. Consequently, only the segregation pattern of Hpg^- Ura^+:Hpg^+ Ura^- = 2:2 was obtained, indicating that the plasmid was integrated into a genomic region tightly linked with *HPG2*. Note that Hpg^- represents the high-pressure growth phenotype. In other words, Hpg^- cells can grow under high-pressure conditions, whereas Hpg^+ cells, namely wild-type ones, cannot. This is genetic evidence that *HPG2* is allelic to *TAT2*.

HPG2 mutation sites are located in the N- and C-terminal cytoplasmic domains of the Tat2 protein

Three mutant alleles, *HPG2-1*, *HPG2-2*, and *HPG2-3* in the *HPG2* mutants, caused amino acid substitutions of Glu27 > Phe (GAA > TTC), Asp563 > Asn (GAC > AAC), and Glu570 > Lys (GAA > AAA), respectively (Fig. 1A). The *HPG2-1* mutation site was located within a gap in a palindromic structure, TTGGATTT-GTAGAATACAAATCCAA, in the *TAT2* ORF which could enable the spontaneous GAA > TTC mutation. The *HPG2-1* mutation site was located within one of two ExKS motifs, similar to the DxKS core of the Ste2 SINDAKSS motif, which is required for ligand-induced Ste2 ubiquitination and internalization (Hicke et al. 1998). The *HPG2-2* or *HPG2-3* mutation site was located adjacent to or within the KQEIAE sequence in the C-terminal cytoplasmic domain of Tat2. The KQEIAE sequence is also observed in Gap1, and the amino acid substitution to KQEIAK has been shown to cause a remarkable stabilization of Gap1 protein upon the addition of ammonium ion (Springael and André 1998). The three *HPG2* mutations resulted in amino acid substitutions on acidic amino acid residues, changing them to either a nonpolar, a polar, or a basic amino acid. These results suggest that the negatively charged amino acid residues within the ExKS motif or the KQEIAE sequence may have a role in Tat2 degradation in response to increasing hydrostatic pressure.

We next examined the effect of *HPG2* mutations on cell growth at high pressure. Although the wild-type cells did not grow at 25 MPa, *HPG2-1*, *HPG2-2*, *HPG2-3*,

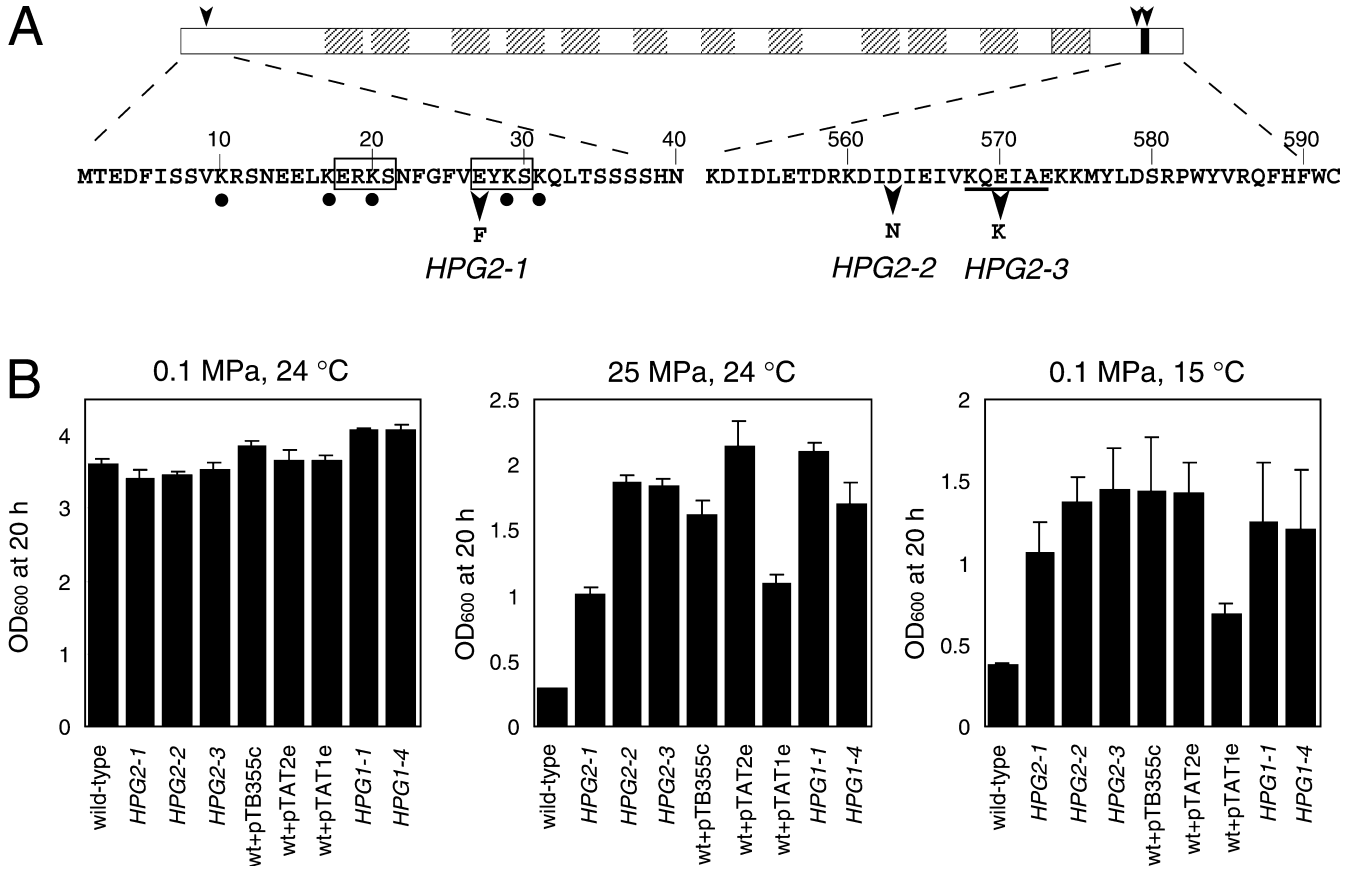

Fig. 1A, B *HPG2* mutation sites and growth profiles of *HPG2* mutants. **A** The three *HPG2* mutation sites were located in the N- or C-terminus of Tat2. *Arrow HPG2* mutation site, *box* the ExKS motif, *underline* the KQEIAE sequence, *dots* five lysines replaced with arginines in $Tat2^{5K>R}$. **B** The mutant forms of Tat2 confer high-pressure growth and low-temperature growth. Cells of the wild-type (YPH499), *HPG2-1* (FAY3A), *HPG2-2* (FAY9D), *HPG2-3* (FAY131E), *HPG1-1* (FAY18A), and *HPG1-4* (FAY29E) carrying empty vector YCplac33, and the cells of the wild-type carrying pTB355c, pTAT2e, or pTAT1e were grown in SC medium under the indicated conditions for 20 h. The data are expressed as mean values with SD from three independent experiments. *wt* Wild-type

and $Tat2^{5K>R}$ cells grew as well at this pressure (Fig. 1B) and as vitally as cells overexpressing Tat2 (Abe and Horikoshi 2000) or cells of the *HPG1* mutants (Abe and Iida 2003). A similar result was obtained with the *tat2*Δ mutant (FAB158) containing *TAT2*, *HPG2-1*, *HPG2-2*, *HPG2-3*, or $Tat2^{5K>R}$ on plasmid YCplac33 (data not shown). Consistent with our previous reports (Abe and Horikoshi 2000; Abe and Iida 2003), there is a good correlation between high-pressure growth and low-temperature growth among the strains (Fig. 1B). Therefore, the high-pressure effect and the low-temperature effect are similar to each other, and the mutant forms of the Tat2 protein could be stabilized or altered in their tryptophan uptake activity at high pressure as well as at low temperature.

Next, we analyzed the tryptophan uptake activity of the wild-type strain and the three *HPG2* mutants using ^{3}H-labeled tryptophan. Cells were cultured at 0.1 or 25 MPa for 5 h, and then the tryptophan uptake assay was performed at 0.1 MPa as described previously (Abe and Horikoshi 2000; Abe and Iida 2003). The uptake activity of the *HPG2-1*, *HPG2-2*, and *HPG2-3* mutants was 1.3-, 1.3-, and 1.9-fold that of the wild-type strain, respectively (Table 3), suggesting that the specific transport activity and/or the steady-state level of Hpg2-1 ($Tat2^{E27F}$), Hpg2-2 ($Tat2^{D563N}$), and Hpg2-3 ($Tat2^{E570K}$) are enhanced. The degree of enhancement of tryptophan uptake at atmospheric pressure was similar to that caused by the *HPG1* (*rsp5*) mutation or deletion of *BUL1* encoding the Rsp5-binding protein Bul1 (Abe and Iida 2003). We have demonstrated that high-pressure incubation downregulates tryptophan uptake in a manner dependent on Rsp5 and Bul1/Bul2 (Abe and Iida 2003). Unlike the wild-type cells, the uptake activity of the *HPG2* mutants remained at high levels after high-pressure incubation (Table 3). These results suggest that the *HPG2* mutation stabilizes Tat2 during high-pressure incubation, probably due to interfering Rsp5-mediated ubiquitination of Tat2. To the best of our knowledge, this is the first evidence that the C-terminus of the Tat2 protein has a role in ubiquitination.

C-terminal mutation stabilizes Tat2 at high pressure

We next analyzed the role of the two C-terminal mutations on the Tat2 protein level under hydrostatic pressure. Whole-cell extracts were prepared from the *tat2*Δ

Table 3 The effect of hydrostatic pressure on tryptophan uptake activity

Strain	Genotype	Tryptophan uptake (pmol 10^7 cells^{-1} min^{-1})				
		0.1 MPa	Fold[a]	25 MPa, 5 h	Fold[b]	Percent[c]
YPH499	Wild-type	4.7 ± 0.6	1.0	1.6 ± 0.3	1.0	34.0
FAY3A	*HPG2-1*	5.9 ± 2.2	1.3	4.0 ± 0.4	2.5	67.8
FAY9D	*HPG2-2*	6.1 ± 0.6	1.3	4.8 ± 1.5	3.0	78.7
FAY131E	*HPG2-3*	9.0 ± 2.6	1.9	6.9 ± 0.8	4.3	76.7

[a]Fold with respect to the wild-type strain incubated at 0.1 MPa
[b]Fold with respect to the wild-type strain incubated at 25 MPa for 5 h
[c]Percent uptake of cells incubated at 25 MPa for 5 h compared with 0.1 MPa

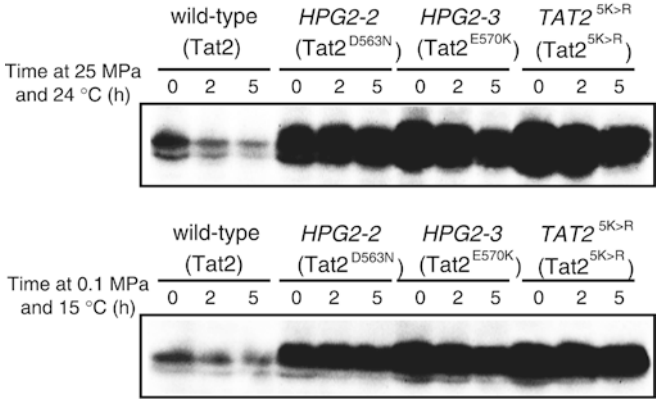

Fig. 2 Stabilization of the C-terminal mutant forms of Tat2 under high-pressure or low-temperature conditions. Cells of the *tat2*Δ mutant (FAB158) carrying p2HA-TAT2c (Tat2), p2HA-HPG2-2c ($Tat2^{D563N}$), p2HA-HPG2-3c ($Tat2^{E570K}$), or pTB355c ($Tat2^{5K>R}$) were grown at 25 MPa and 24 °C or 0.1 MPa and 15 °C. Twenty-five micrograms of the whole-cell extracts were subjected to western blot analysis using anti-hemagglutinin (HA) antibody

mutant expressing 2HA-tagged wild-type Tat2, 2HA-tagged Hpg2-2 ($Tat2^{D563N}$), 2HA-tagged Hpg2-3 ($Tat2^{E570K}$), or 2HA-tagged $Tat2^{5K>R}$. Unlike the wild-type Tat2 protein, the Hpg2-2 and Hpg2-3 proteins as well as $Tat2^{5K>R}$ were stabilized at 25 MPa (Fig. 2). The stable expression of the mutant forms of Tat2 at 25 MPa enables the cells to take up tryptophan efficiently, leading to high-pressure growth. Tat2 appeared to be degraded slightly at the low temperature of 15 °C, but the effect was less remarkable compared with the effect of high pressure (Fig. 2). Since Hpg2-2 and Hpg2-3 proteins were abundant and stable at 15 °C, the *HPG2* mutants could grow at low temperature. The *HPG2-3* mutation site is located in the KQEIAE sequence, and the *HPG2-2* mutation site is close to this motif. The mutation from KQEIAE to KQEIAK of Gap1 is known to cause stabilization of this permease upon the addition of ammonium ion (Springael and André 1998). A similar motif, RQEDEE, is observed in the branched amino acid permeases Bap2 and Bap3 in the C-terminal region. Truncation of the 29 residues containing the RQEDEE sequence has been shown to cause a remarkable stabilization and accumulation of Bap2 in the plasma membrane (Omura et al. 2001). Although the stability of Hpg2-1 ($Tat2^{E27F}$) has not been analyzed, the protein could be less ubiquitinated because the mutation site is adjacent to the 29th and 31st lysines, which are the most probable ubiquitination sites. It is reported that the cytoplasmic tail phosphorylation of Ste2 is required for its ubiquitination and internalization (Hicke et al. 1998). Our results suggest that the C-terminal mutation may interfere with phosphorylation, which has not been shown to occur on the Tat2 protein, ubiquitination, or subsequent endocytosis of this permease through an interaction with the N-terminus or some interacting proteins in response to increasing hydrostatic pressure.

C-terminal mutation alters the intracellular localization of Tat2 protein

As demonstrated previously, the wild-type Tat2 protein is more abundant in the internal membranes overlapping the marker proteins Vps10 (Golgi membrane maker), Pep12 (endosome marker), and ALP (vacuolar membrane marker) than in the plasma membrane (Abe and Iida 2003; Fig. 3). Tat2 accumulates in the plasma membrane upon loss of ubiquitination caused either by the $Tat2^{5K>R}$ mutation (Beck et al. 1999), the *HPG1* (*rsp5*) mutation, or deletion of *BUL1* and *BUL2* encoding Rsp5-binding proteins (Abe and Iida 2003). Likewise, the Hpg2-2 and Hpg2-3 proteins were revealed to be enriched in the plasma membrane (Fig. 3; fraction 9). When a pressure of 25 MPa was applied for 5 h, Tat2 decreased in the plasma membrane in the wild-type strain, whereas Hpg2-2 and Hpg2-3 were maintained in the plasma membrane, like $Tat2^{5K>R}$ (Fig. 3; fraction 9). Under the same pressure condition, Tat2 in internal membranes overlapping with marker proteins, Pep12 and Vps10, in the wild-type and the mutant cells shifted to low density fractions as the occurrence of the similar low-density shifts observed in the two marker proteins (Fig. 3; 25 MPa for 5 h, fractions 1–3).

Although quantitative analysis of the ubiquitination level has not yet been performed, our findings suggest that the ExKS motif and the KQEIAE sequence plays an important role in localization and destabilization of Tat2 at high pressure. Increasing hydrostatic pressure increases the membrane order and reduces the lateral diffusion in both artificial and biological membranes, causing decreased fluidity of the membranes, similar to

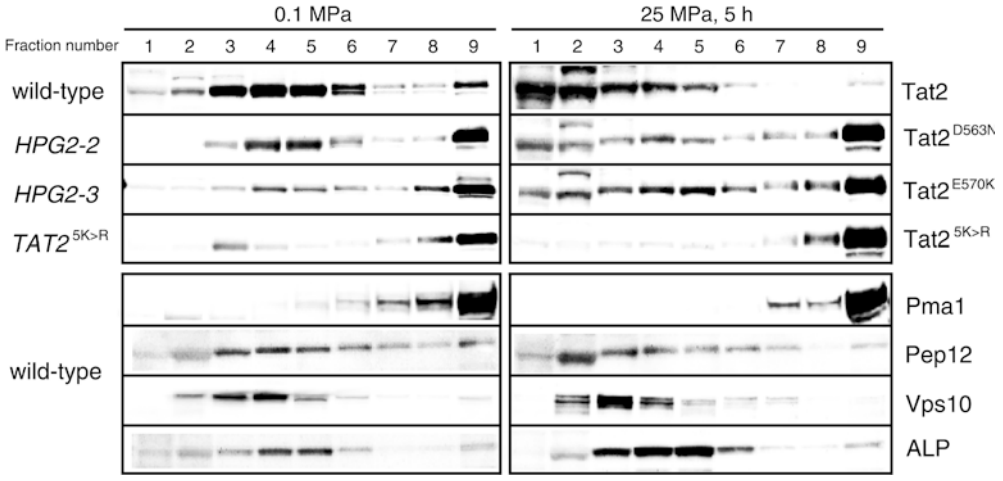

Fig. 3 Subcellular fractionation of the mutant forms of Tat2. Cells of the *tat2*Δ mutant (FAB158) carrying p2HA-TAT2c (Tat2), p2HA-HPG2-2c ($Tat2^{D563N}$), p2HA-HPG2-3c ($Tat2^{E570K}$), or pTB355c ($Tat2^{5K>R}$), and the HA-*PMA1* cells (RHY525) carrying empty vector YCplac33 were grown at 0.1 or 25 MPa and 24 °C. Whole-cell extracts were subjected to centrifugation on a sucrose-density gradient. Nine fractions were collected from the top. Western blotting was performed using membrane marker proteins: the HA-tagged plasma membrane H^+-ATPase Pma1, the endosomal multifunctional yeast syntaxin Pep12, the Golgi membrane protein Vps10, and the vacuolar membrane alkaline phosphatase

the situation at low temperature. Therefore, we hypothesize that hydrostatic pressure would affect the activity of tryptophan permease either directly through changes in the protein conformation or most probably through changes in the lipid bilayer structure. Our recent results indicate that the *HPG1-1*, *HPG1-2*, and *HPG1-3* mutation sites are located within or adjacent to the NPF motif of Rsp5 (Abe and Iida 2003). The NPF motif is proposed to be a sequence interacting with the EH-domain of endocytic components (Santolini et al. 1999; Wang et al. 2001). It is likely that Rsp5-dependent Tat2 ubiquitination on its N-terminus is mediated through the interaction between its C-terminus and endocytic components in the plasma membrane. We have demonstrated that Tat2 localizes in bulk lipids, lipid-disordered domain, within the lipid bilayer (Abe and Iida 2003). Upon loss of ubiquitination caused by the *HPG1* mutation, *bul1*Δ*bul2*Δ double mutation, or $Tat2^{5K>R}$ mutation, Tat2 becomes associated with the lipid rafts, which are lipid-ordered microdomains (Abe and Iida 2003). Consequently, Tat2 in the mutant cells is delivered to the plasma membrane along with rafts like Pma1 (Bagnat et al. 2000) and the other tryptophan permease Tat1 (Abe and Iida 2003). We have speculated that Tat2, which localizes in bulk lipids, could be denatured to a certain degree due to a pressure change or temperature change (Abe and Iida 2003). When the ubiquitin system is intact in the wild-type cells, the denatured form of Tat2 could be ubiquitinated by Rsp5, followed by vacuolar degradation. When ubiquitination defect is present, the denatured form of Tat2 could be associated with rafts. In this sense, rafts may be the place where Rsp5 functions (Abe and Iida 2003). The localization of Tat2 is also controlled by external tryptophan. When cells are transferred to a medium containing a low concentration of tryptophan, Tat2 becomes associated with lipid rafts, followed by the delivery to the plasma membrane (Umebayashi and Nakano 2003). We will examine in future investigations how the C-terminal mutation affects the ubiquitination level and the raft association of Tat2 in response to increasing hydrostatic pressure and external tryptophan.

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