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Loop 5 region is important for the activity of the long-chain base transporter Rsb1

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Running title: Important region of the long-chain base transporter Rsb1

Abbreviations: DHS, dihydrosphingosine; FA, fatty acid; 3xHA, triple HA, LCB, long-chain base; LCBP, long-chain base 1-phosphate; PHS, phytosphingosine; SC, synthetic complete; SPH, sphingosine.

Summary

Intracellular lipid amounts are regulated not only by metabolism but also by efflux. Yeast Rsb1 is the only known transporter/floppase of the sphingolipid components long-chain bases (LCBs). However, even fundamental knowledge about Rsb1, such as important amino acid residues for activity and substrate recognition, still remains unclear. Rsb1 belongs to the Rta1-like family. To date, it has not been determined whether all family members share a common ability to export LCBs. Here, we revealed that within the Rta1-like family, only Rsb1 suppressed the hypersensitivity of the mutant cells lacking LCB 1-phosphate-degrading enzymes, suggesting that LCB-exporting activity is specific to Rsb1. Rsb1 contains a characteristic region (loop 5), which does not exist in other proteins of the Rta1-like family. We found that deletion of this region caused loss of Rsb1 function. Further mutational analysis of loop 5 revealed that the charged amino acid residues E223, D225, and R236 were important for Rsb1 activity. In addition to LCBs, Rsb1 facilitated the export of 1-hexadecanol, but not palmitic acid, which suggests that Rsb1 recognizes the C1 hydroxyl group. Thus, our findings provide an important clue for understanding the molecular mechanism of LCB export.

Keywords: Lipid, Long-chain base, Plasma membrane, Sphingolipid, Transporter

Introduction

Sphingolipids are components of the eukaryotic plasma membrane and are essential for cell viability in all known eukaryotes (1, 2). The sphingolipid backbone ceramide contains two hydrophobic chains: a long-chain base (LCB) and a fatty acid (FA). LCBs in the yeast *Saccharomyces cerevisiae* are dihydrosphingosine (DHS) and phytosphingosine (PHS), the latter of which is 4-hydroxylated. In addition to these LCBs, mammals have another LCB sphingosine (SPH), which contains a 4,5-*trans* double bond.

In addition to being components of sphingolipids, LCBs function as signaling molecules. In yeast, LCBs activate serine/threonine kinases Pkh1 and Pkh2, which regulate various cellular processes including heat stress response, actin organization, and endocytosis via activation of downstream protein kinases (3). The mammalian homolog of Pkh1/2, PDK1, is activated by SPH *in vitro* (4). A fraction of LCBs are phosphorylated by LCB kinases to LCB 1-phosphates (LCBPs). LCBPs also function as intracellular signaling molecules. In yeast, LCBPs are involved in heat stress resistance and Ca²⁺ mobilization (1). In mammals, SPH 1-phosphate functions as an extracellular lipid mediator and regulates various cellular processes, such as proliferation, cell migration, and cell-cell adhesion through G protein-coupled receptors (5). Both in yeast and in mammals, LCBPs are also important as key metabolic intermediates of LCBs (6). LCBPs are metabolized to fatty acyl-CoAs after being irreversibly degraded by LCBP lyases, Dpl1 in yeast and SGPL1/SPL in mammals, and then incorporated into glycerophospholipids. Given these multiple functions, it is reasonable to assume that proper regulation of LCB/LCBP levels and localization are important for normal cellular function. Indeed, yeast mutant cells lacking two LCBP degradation enzymes, the LCBP phosphatase Lcb3 and the LCBP lyase Dpl1, exhibit a severe growth phenotype and hypersensitivity to exogenous LCBs

(7-9). Likewise, *Sgpl1* knockout mice show abnormalities in various organs, including lung, heart, kidney, and liver, and die within several weeks after birth (10, 11).

Compared to the LCB biosynthetic/degradation pathways, our understanding concerning the intracellular distribution and dynamics of LCBs is limited. Since LCBs suffer phosphorylation at the cytosolic leaflets of the membrane lipid bilayers, spatial regulation of LCBs across the lipid bilayer is considered important for proper control of LCB/LCBP levels. Rsb1 is a putative LCB transporter or floppase that mediates energy-dependent transport or transbilayer movement of LCBs from the cytosolic leaflet of the plasma membrane to the extracellular spaces or to the extracellular leaflet (7). *RSB1* was isolated as a multicopy suppressor that rescued the hypersensitivity of *dpl1Δ* cells to exogenous LCBs (7). Although expression levels of Rsb1 are low under normal conditions, its expression is induced by changes in the plasma membrane lipid asymmetry (non-uniform distribution of lipid molecules between inner and outer leaflets) (12). Maintenance of the plasma membrane lipid asymmetry is important for several cellular events, such as intracellular trafficking, maintenance of organelle structure, and polarized growth (13, 14). The induction of Rsb1 may be one of the cellular responses compensating for functional lipid asymmetry and is mediated by the Rim101 signaling pathway (15-18).

Rsb1 is predicted to span the membrane seven times with the N- and C-terminus oriented to the extracellular space and to the cytosol, respectively. Rsb1 belongs to the Rta1-like family [pf04479 in the pfam database (<http://xfam.org/>)], the members of which may function in lipid transport (19). To date, 1849 sequences are registered to this family. In addition to Rsb1, *S. cerevisiae* contains the Rta1-like family members Rta1, Pug1, and Ylr046c. Rta1 was previously reported to confer resistance to 7-aminocholesterol (20), while Pug1 is implicated in the uptake of protoporphyrin IX and in efflux of heme (21). The function of Ylr046c is still unclear.

To date, a comprehensive understanding of the Rta1-like family concerning lipid dynamics is missing. Furthermore, it remains unclear how Rsb1 mediates transport or the transbilayer movement of LCBs. In the present study, we reveal that only Rsb1 in the Rta1-like family can transport/flop LCBs. The loop 5 is unique to Rsb1 and is required for Rsb1 function. Of the charged amino acid residues in loop 5, E223, D225, and R236 are important for Rsb1 activity. We also suggest that Rsb1 recognizes the C1 hydroxyl group of LCBs.

Materials and methods

Yeast strains and media

We used yeast *Saccharomyces cerevisiae* strains SEY6210 (*MAT α his3 leu2 ura3 trp1 lys2 suc2*) (22), KHY21 (SEY6210, *dpl1 Δ ::TRP1 lcb3 Δ ::LEU2*) (7), and YOK2264 (SEY6210, *rsb1 Δ ::KanMX4*). Cells were grown at 30°C in YPD (1% yeast extract, 2% bactopecton, and 2% D-glucose) or synthetic complete (SC) medium (2% D-glucose and 0.67% yeast nitrogen base without amino acids with appropriate supplements), but lacking uracil (SC-Ura). In LCB sensitivity assays, cells were spotted on YPD plates with or without LCB [10 μ M PHS (Enzo Life Sciences, Farmingdale, USA) or 5 μ M DHS (Avanti Polar Lipids, Alabaster, USA)] and 0.0015% Nonidet P-40 as a dispersant, followed by incubation at 30°C.

Plasmids

Plasmids for the expression of triple HA (3xHA)-tagged Rsb1 (pHM22), Rta1 (pHM25), Pug1 (pHM26), and Ylr046C (pHM27) were constructed by cloning their coding regions with 3'-UTRs into the pAK162 vector, which is designed to express N-terminally 3xHA-tagged protein under the control of the *GAPDH* promoter (7).

The pHM23 plasmid, which encodes HA-Rsb1 lacking the central part of loop 5 (amino acid residues 199-228), was constructed by amplifying the 5' region (1-594) and the 3' region (685-1065) of *RSB1* separately by PCR, followed by connecting them in the pAK162 vector. The pHM24 plasmid, which encodes HA-Rsb1 lacking the C-terminal region (amino acid residues 320-382), was similarly constructed by amplifying 5' region (1-957) of *RSB1* with an artificial stop codon (TAG) and 3'-UTR of *RSB1*. The pHM40 plasmid, which expresses Rsb1-GFP from the *RSB1* promoter, was constructed as follows. First, chromosomal fusion of the *GFP* sequence cassette (containing *GFP*, the *ADHI*

terminator, and *KANMX6* gene) with *RSB1* was performed by PCR-based gene modification method as described elsewhere (23), generating the yeast strain TFY37. The resulting *RSB1-GFP* region was then amplified from the TFY37 genomic DNA by PCR and cloned into the pRS316 vector (24), resulting in pHM40. Introductions of point mutations were performed using the QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, USA) and appropriate primer sets.

Immunoblot analysis

Immunoblot analyses were performed as described previously (25), using anti-HA (TANA2; 0.5 μ g/ml; Medical & Biological Laboratories, Nagoya, Japan) or anti-Pgk1 (0.5 μ g/ml; Thermo Fisher Scientific, Waltham, USA) antibody as a primary antibody. Prior to detection by immunoblotting, we deglycosylated wild type and mutant Rsb1, as well as the other members of the Rta1-like family, as describe previously (18).

Lipid release assay

Cells were grown to ~ 1 A_{600} unit/ml in SC-Ura medium at 30°C and labeled with 0.2 μ Ci/ A_{600} [3 H]DHS (50 Ci/mmol; American Radiolabeled Chemicals, St. Louis, USA), 0.2 μ Ci/ A_{600} [3 H]palmitic acid (60 Ci/mmol; American Radiolabeled Chemicals), or 0.2 μ Ci/ A_{600} [14 C]1-hexadecanol (52 mCi/mmol; Moravek Biochemicals, Brea, USA) and incubated for 5 min at 30°C. Cells were then washed twice with SC-Ura medium containing 1 mg/ml BSA, suspended in SC-Ura medium containing 1 mg/ml BSA, and incubated for 5 or 10 min at 30°C. Cells and medium were separated by centrifugation and their radioactivities were measured by a liquid scintillation counter. The percentages of released lipids were calculated as $100 \times$ radioactivity in the medium/(radioactivity in cells + radioactivity in the medium).

Microscopy

Fluorescence of GFP was visualized under a fluorescence microscope (DM5000B, Leica Microsystems, Wetzlar, Germany) equipped with a cooled CCD camera (DFC365FX, Leica Microsystems). The microscope was operated by LAS AF software (version 2.60, Leica Microsystems). The images were archived using Photoshop CS3 (Adobe, San Jose, USA).

Results

In the Rta1-like family, only Rsb1 can export LCBs

To date, whether members of the Rta1-like family generally have the ability to export LCBs has not been examined. To test this, we overexpressed Rsb1 or one of the other members of the Rta1-like family (Rta1, Pug1, or Ylr046c) in cells lacking LCBP-degrading enzymes (*dpl1Δ lcb3Δ* cells), as well as monitoring their sensitivities to exogenously added PHS. Since highly accumulated LCBPs are toxic to cells, *dpl1Δ lcb3Δ* cells exhibit sensitivity to exogenously added LCBs, the substrates of LCBPs (26). As reported previously (7), overexpression of Rsb1 rescued the sensitivity to exogenous PHS (Fig. 1A). In contrast, *dpl1Δ lcb3Δ* cells overexpressing Rta1, Pug1, or Ylr046c were still sensitive to PHS. We confirmed that 3xHA-tagged Rta1, Pug1, and Ylr046c were expressed (Fig. 1B). These results suggest that only Rsb1 in the Rta1-like family is capable of exporting LCBs.

Loop 5 is important for the function of Rsb1

One unique feature of Rsb1 among the Rta1-like family is the presence of a long loop 5 and C-terminal tail (Fig. S1). The C-terminal tail includes the Walker A motif GXXXXG(K/R/H)(T/S), which is involved in ATP-binding (27), at amino acid residues 305–312. We previously reported that introducing a mutation in the Walker A motif impaired Rsb1 activity (7). In the present study, we investigated the significance of these unique regions of Rsb1 by deletion analyses. Regarding the C-terminal tail, another group has already reported that deletion of C-terminal 22 amino acid residues (361–382) or 47 amino acid residues (336–382) had little effect on Rsb1 function (28). Therefore, we created a mutant protein (Δ C; 320–382) with longer (63 amino acid) deletion at the C-terminal tail, but containing the Walker A motif. We overexpressed Rsb1 Δ C or Rsb1 lacking the loop 5 (Δ loop 5; 199–228) in *dpl1Δ lcb3Δ* cells and examined its sensitivity to PHS. We found that the cells expressing

Rsb1 Δ C were not sensitive to PHS (Fig. 2A). In contrast, Rsb1 Δ loop 5 did not confer PHS resistance to *dpl1 Δ lcb3 Δ* cells. We confirmed that both mutant proteins were expressed (Fig. 2B). These results indicate that the loop 5, but not the C-terminal region after the Walker A motif, is essential for Rsb1 function.

E223, D225, and R236 residues in loop 5 are important for Rsb1 function

The loop 5 appears to have no known motif, but is rich in charged amino acid residues. Therefore, we focused on these charged amino acid residues and substituted them with Ala (Fig. 3A). Among 11 created mutants, Rsb1 R203A, Rsb1 E223A/E225A, and Rsb1 R236A could not rescue the sensitivity of *dpl1 Δ lcb3 Δ* cells to PHS, although the effect of the R203A mutation was mild (Fig. 3B). A similar result was obtained for another LCB, DHS (Fig. 3B). The mutant proteins were expressed normally as revealed by immunoblot analysis (Fig. 3C). Next, we investigated the DHS export activities of the mutant proteins. Deletion of the loop 5 caused a large decrease in the DHS export activity (Fig. 3D). Rsb1 E223A/E225A and Rsb1 R236A mutants also exhibited reduced DHS export activities, but their effects were milder than the Δ loop 5 mutant (Fig. 3D). Conversely, the R203A mutation, which showed a weak effect on LCB sensitivity (Fig. 3B), did not significantly reduce DHS release, although we observed a slight reduction tendency (Fig. 3D). These results suggest that the E223, D225, and R236 residues located in loop 5 are required for the full activity of Rsb1. We then examined the intracellular localization of these mutants. Expression of wild type Rsb1 fused with GFP was mainly localized in the plasma membrane as described previously (29), but also partly in the vacuole (Fig. 3E). Rsb1 R203A, Rsb1 E223A/E225A, and Rsb1 R236A showed similar localization patterns, indicating these charged amino acid residues are not essential for plasma membrane localization of Rsb1. While Rsb1 Δ loop 5 also localized to the plasma membrane in some cells, it

mainly localized to the vacuole (Fig. 3E). Therefore, the decreased DHS release and LCB resistance observed for Rsb1 Δ loop 5 (Fig. 3B and D) may be due to the combined effect of reduced transport activity and partial mislocalization.

C1 hydroxyl group of LCBs is important for recognition by Rsb1

To gain an insight into the mechanism of substrate recognition by Rsb1, we investigated the ability of Rsb1 to export palmitic acid and 1-hexadecanol, which resemble the overall structure of LCBs, *i.e.*, containing one long-chain hydrophobic chain and small functional group(s) at the end. 1-Hexadecanol possesses a C1 hydroxyl group like LCBs, while palmitic acid instead has a carboxyl group. Rsb1 facilitated export of DHS and 1-hexadecanol, but not palmitic acid (Fig. 4A), suggesting the C1 hydroxyl group is important for Rsb1-mediated export. Rsb1-independent DHS export activity was lower than those of palmitic acid and 1-hexadecanol (Fig. 4A). The Δ loop 5 and *E223A/D225A* mutations significantly reduced 1-hexadecanol export activity, while the *R203A* and *R236A* mutations did not (Fig. 4B). Therefore, E223 and D225 residues in loop 5 may be involved in the recognition of the C1 hydroxyl group of LCBs and 1-hexadecanol.

Discussion

In the present study, we revealed that only Rsb1 in the Rta1-like family could confer resistance to exogenous PHS to *dpl1Δ lcb3Δ* cells (Fig. 1). This is the first analysis of the Rta1-like family other than Rsb1 concerning an ability to export LCBs. It is known that Rsb1-mediated export of LCBs requires ATP and the Walker A motif, generally known to mediate ATP-binding (27), at the C-terminal tail of Rsb1 (7). The Walker A motif is not present in other members of the Rta1-like family, which may explain the reason for their inability to export LCBs. Another unique feature of Rsb1 is the presence of an extended cytosolic loop (loop 5) between the fifth and sixth transmembrane segments. We found that loop 5 was essential for Rsb1 function (Fig. 2A). Within loop 5, E223, D225, and R236 residues were important for Rsb1 activity (Fig. 3B and D).

We recently revealed the FA transporter family (also known as acyl-CoA synthetase family) members Faa1 and Faa4 are involved in importing both LCBs and long-chain FAs (30). We found here that Rsb1 facilitated export of DHS and 1-hexadecanol, but not palmitic acid (Fig. 4A). Thus, Rsb1 exhibits more strict substrate specificity than Faa1 and Faa4. Considering that LCBs and 1-hexadecanol, but not palmitic acid, have a C1 hydroxyl group, Rsb1 may recognize the C1 hydroxyl group. The *E223A/D225A* mutations decreased export of both DHS and 1-hexadecanol (Figs. 3D and 4B). One possible explanation may be that E223 and D225 participate, directly or indirectly, in recognition of the C1 hydroxyl group.

Rsb1-independent export of DHS occurred at a much lower level than those of palmitic acid and 1-hexadecanol (Fig. 4A). It is possible that, due to its polarity, the amino group prevents spontaneous transbilayer movement of LCBs across the hydrophobic environment in the membrane. Alternatively, the amino group may inhibit the export of LCBs by unknown transporters/floppases. In either case, the amino group of LCBs is likely important in reducing nonspecific export of LCBs and to guarantee

regulated LCB export.

The E223A/D225A and R236A mutations caused strong effects on the growth-rescuing activity of Rsb1 in *dpl1Δ lcb3Δ* cells, *i.e.*, almost complete loss of the activity (Fig. 3B). However, they only weakly affected DHS release activity of Rsb1 (Fig. 3D). This seemingly inconsistent result may have originated from the differences in the basal LCB/LCBPs levels between the two assays. It is known that the *dpl1Δ lcb3Δ* cells used in the PHS-sensitivity assay accumulate LCBs and exhibit a slow growth phenotype even without exogenous LCBs (8). Therefore, even a mild reduction in LCB export activity due to *E223A/D225A* or *R236A* mutation may be enough to cause severe growth defects.

We previously identified *RSB1* as a multi-copy suppressor against the LCB sensitive phenotype of *dpl1Δ* cells (7). Overproduction of Rsb1 suppressed cell death by enhancing LCB release from the cells (7). From these observations, we speculate that Rsb1 acts as the LCB transporter/floppase. In contrast, Moye-Rowley and his colleagues later proposed Rsb1 functions as a receptor/signaling transducer and regulates an unidentified LCB transporter (28). The basis of their notion are 1) Rsb1 is predicted to have seven transmembrane domains, a transmembrane structure resembling G protein-coupled receptors and 2) deletion of *RSB1* does not cause changes in intracellular LCB levels. However, we cannot agree with their notion and for the following reasons still suggest it is highly likely Rsb1 is the LCB transporter/floppase. First, only having seven transmembrane domains cannot be indicative of receptors. Indeed, Rsb1 shares no sequence similarity with G protein-coupled receptors or other receptors. Rather, multi-membrane spanning structures are often seen in transporters. Furthermore, Rta1-like family members other than Rsb1 are also implicated as transporters (19-21). Second, it is quite reasonable that disruption of *RSB1* does not induce changes in intracellular LCB levels. Sphingolipid synthesis begins in the ER and proceeds at the Golgi apparatus, and the major site of sphingolipid degradation in yeast is the vacuole (lysosome in mammals). Thus,

LCBs in the plasma membrane are expected to compose only a minor fraction of the total amount of LCB or sphingolipid, therefore the loss of Rsb1-mediated LCB export only marginally affects LCB/sphingolipid levels. Finally, our screening was conducted using non-biased methods. Therefore, if it had existed, our screening should have been able to identify the other LCB transporter suggested by Moye-Rowley and his colleagues. Generally, it is difficult to verify transporter activities due to technical difficulties. However, *in vitro* studies must be conducted to establish the transport/floppase activity of Rsb1 in future.

In conclusion, we identified amino acid residues important for Rsb1-mediated LCB export. In addition, we offered insight into substrate recognition by Rsb1. These advances may serve as a solid basis for future studies to understand the mechanism of Rsb1-mediated export of LCBs.

Conflict of interest

The authors declare no conflicts of interest.

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Figure legends

Fig. 1 Only Rsb1 in the Rta1-like family confers resistance to exogenous PHS on *dpl1Δ lcb3Δ* cells. (A) SEY6210 (wild type; WT) cells harboring the pAK162 vector (vec) and KHY21 (*dpl1Δ lcb3Δ*) cells harboring pAK162 or a plasmid encoding the indicated genes tagged with *3xHA* were grown to a stationary phase in SC-Ura medium, serially diluted at 1:10, spotted on YPD plates with or without 10 μM PHS, and grown at 30°C for 35 h. (B) The above described KHY21 cells expressing the indicated genes were grown to an early log-phase. Total cell lysates were prepared, deglycosylated, and subjected to immunoblotting with an anti-HA or, to demonstrate uniform loading, anti-Pgk1 antibody.

Fig. 2 Loop 5 is essential for Rsb1 function. (A) SEY6210 (wild type; WT) bearing the pAK162 vector (vec) and KHY21 (*dpl1Δ lcb3Δ*) cells harboring pAK162 or a plasmid encoding wild type (pHM22) or mutants (Δ loop 5, pHM23; Δ C, pHM24) of 3xHA-tagged Rsb1 were grown to a stationary phase in SC-Ura medium, serially diluted at 1:10, spotted on YPD plates with or without 10 μM PHS, and grown at 30°C for 35 h. (B) Total cell lysates were prepared from the above described KHY21 cells expressing the indicated genes, treated with endoglycosidase H, and subjected to immunoblotting with an anti-HA or anti-Pgk1 antibody.

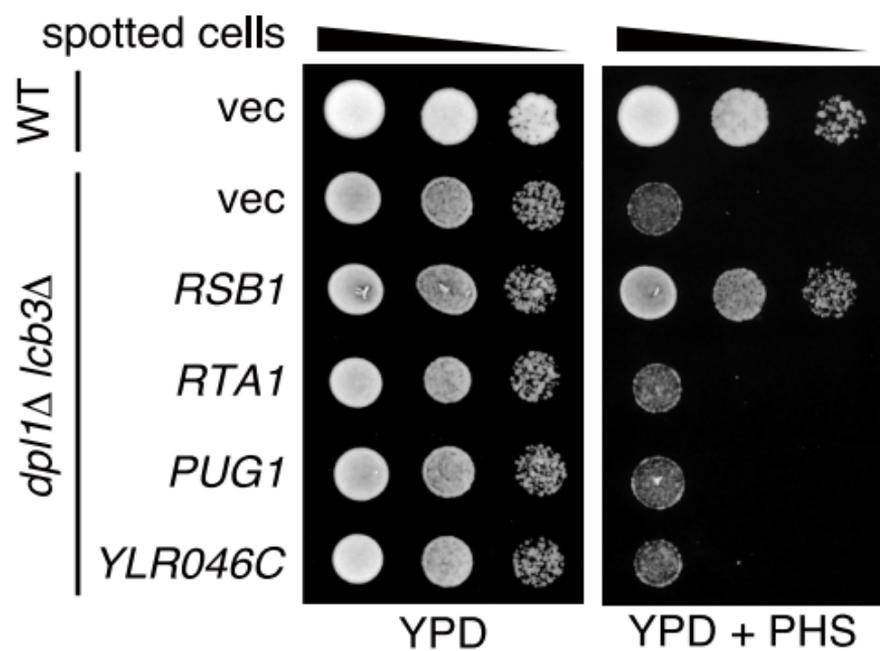
Fig. 3 E223, D225, and R236 are important for Rsb1 activity. (A) Structure of Rsb1 and amino acid sequence of loop 5 are illustrated. Acidic and basic residues are shown in red and blue, respectively. Charged amino acid residues substituted with Ala in this study are boxed. The region deleted in the Rsb1 Δ loop 5 mutant is underlined. (B and C) KHY21 (*dpl1Δ lcb3Δ*) cells bearing the pAK162 vector (vec) or a plasmid encoding the indicated Rsb1 mutants were grown to a stationary

phase in SC-Ura medium. Cells were serially diluted at 1:10, spotted on YPD plates with or without 10 μ M PHS (**B**, upper panel) or 5 μ M DHS (**B**, lower panel), and grown at 30°C for 40 h (upper panel) or 48 h (lower panel). Total lysates were deglycosylated and subjected to immunoblotting with an anti-HA or anti-Pgk1 antibody (**C**). (**D**) YOK2264 (*rsb1* Δ) cells bearing pAK162 vector or a plasmid encoding wild type (WT) or an indicated mutant of 3xHA-tagged Rsb1 were labeled with [³H]DHS for 5 min, washed twice, suspended in medium, and further incubated for 5 min. Values represented are percentages of radioactivities associated with medium to those of total radioactivities and represent the means \pm SD from three independent experiments (**, $P < 0.01$; Student's t test). (**E**) YOK2264 (*rsb1* Δ) cells expressing Rsb1-GFP or its mutants were grown to a log phase in SC-Ura medium at 30°C and subjected to fluorescent microscopy. Bar, 5 μ m.

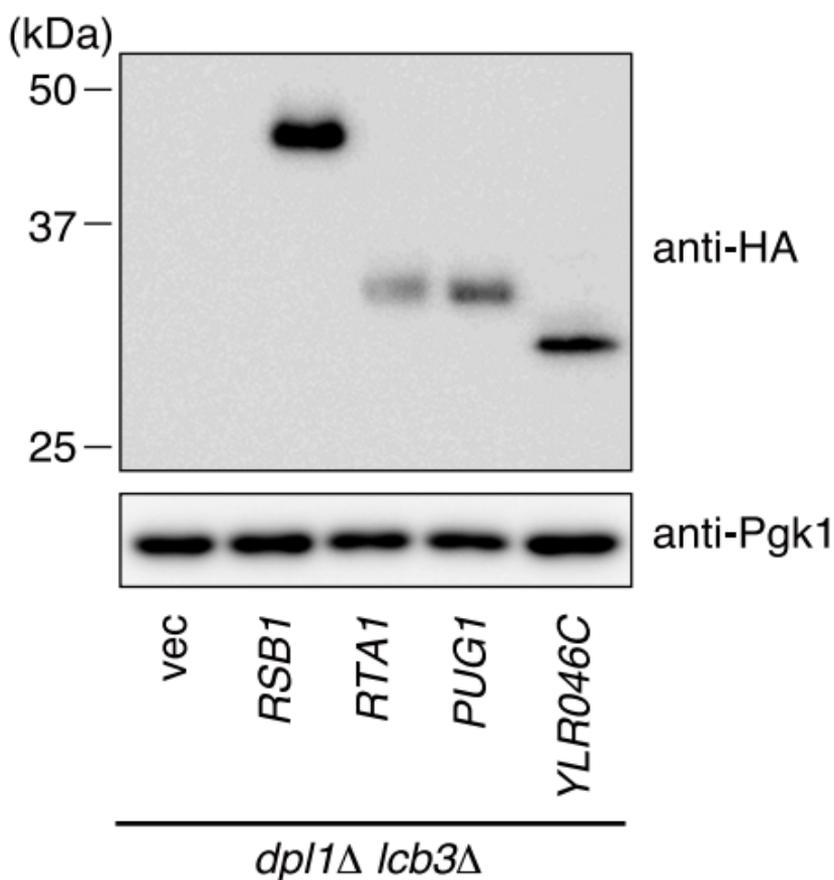
Fig. 4 The C1 hydroxyl group is important for the Rsb1-mediated release of LCBs. (**A**) YOK2264 (*rsb1* Δ) cells bearing a pAK162 vector (vec) or a plasmid encoding 3xHA-RSB1 (pHM22; WT) were labeled with [³H]DHS, [³H]palmitic acid, or [¹⁴C]1-hexadecanol for 5 min, washed twice, suspended in medium, and further incubated for 10 min. Values represented are percentages of radioactivities associated with medium to those of total radioactivities and represent the means \pm SD from three independent experiments (**, $P < 0.01$; Student's t test). (**B**) YOK2264 (*rsb1* Δ) cells bearing pAK162 vector or a plasmid encoding wild type or a mutant of 3xHA-tagged Rsb1 were subjected to [¹⁴C]1-hexadecanol-release assay as in (**A**) except that incubation time after the wash was 5 min. *, $P < 0.05$; **, $P < 0.01$; Student's t test.

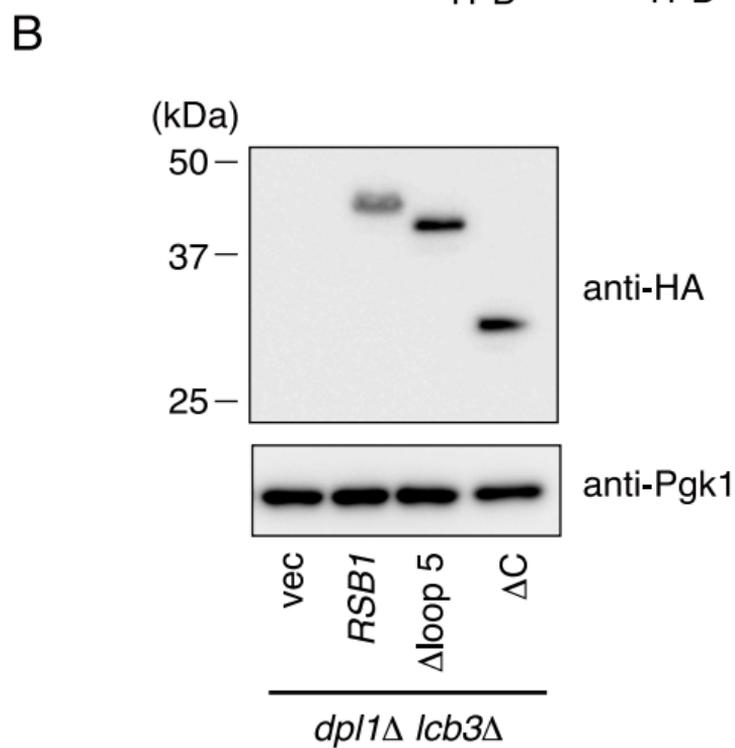
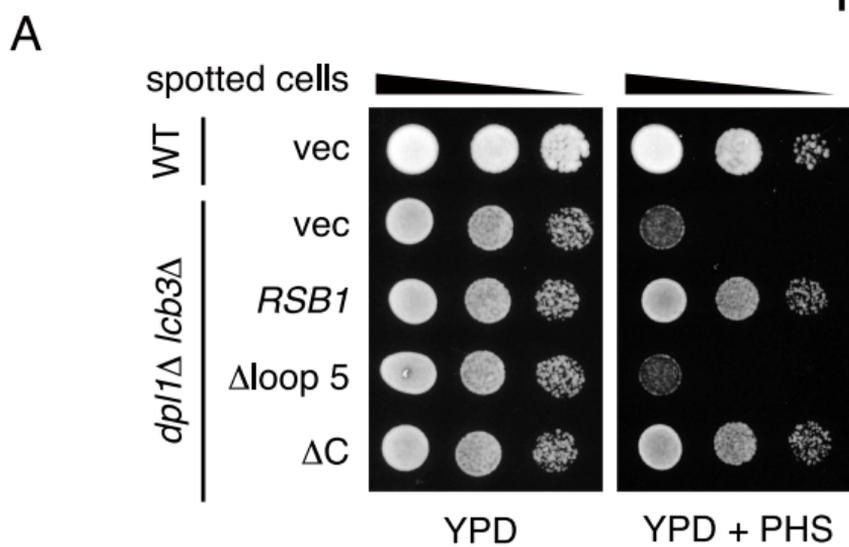
Fig. 1

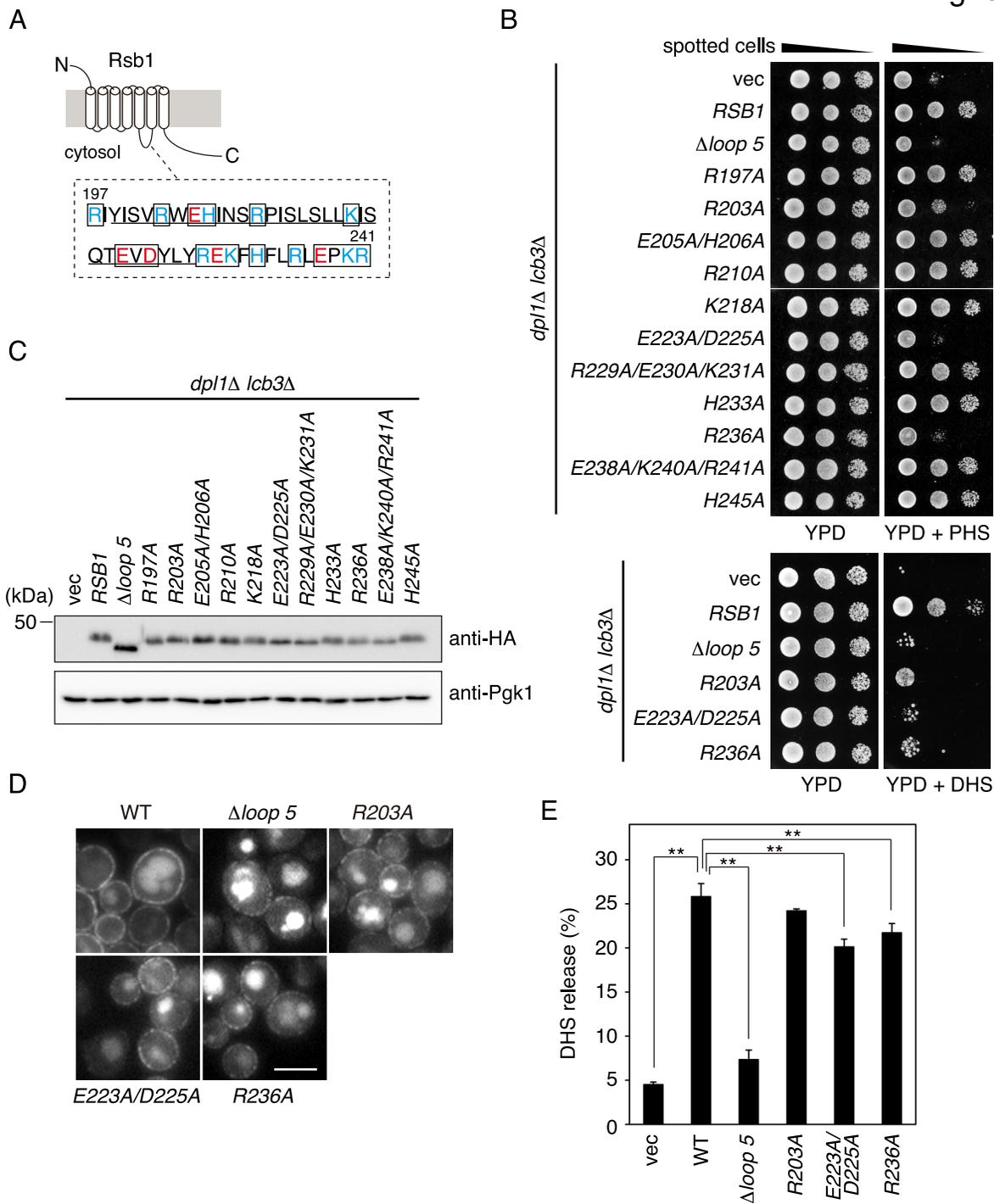
A



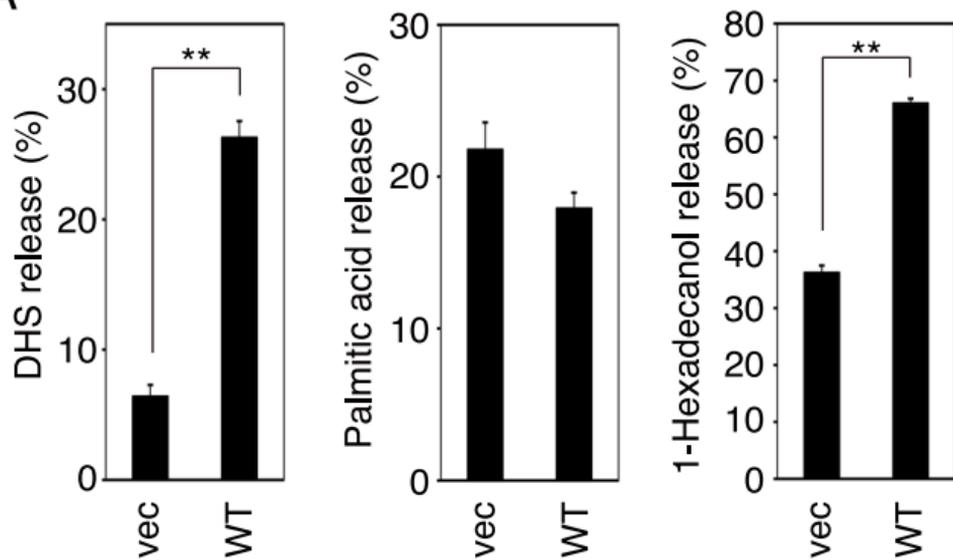
B







A



B

