Kei1: A Novel Subunit of Inositolphosphorylceramide Synthase, Essential for Its Enzyme Activity and Golgi Localization

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Submitted March 24, 2009; Revised August 14, 2009; Accepted August 20, 2009
Monitoring Editor: Sean Munro

Fungal sphingolipids have inositol-phosphate head groups, which are essential for the viability of cells. These head groups are added by inositol phosphorylceramide (IPC) synthase, and *AUR1* has been thought to encode this enzyme. Here, we show that an essential protein encoded by *KEI1* is a novel subunit of IPC synthase of *Saccharomyces cerevisiae*. We find that Kei1 is localized in the medial-Golgi and that Kei1 is cleaved by Kex2, a late Golgi processing endopeptidase; therefore, it recycles between the medial- and late Golgi compartments. The growth defect of *kei1-1*, a temperature-sensitive mutant, is effectively suppressed by the overexpression of *AUR1*, and Aur1 and Kei1 proteins form a complex in vivo. The *kei1-1* mutant is hypersensitive to aureobasidin A, a specific inhibitor of IPC synthesis, and the IPC synthase activity in the mutant membranes is thermolabile. A part of Aur1 is missorted to the vacuole in *kei1-1* cells. We show that the amino acid substitution in *kei1-1* causes release of Kei1 during immunoprecipitation of Aur1 and that Aur1 without Kei1 has hardly detectable IPC synthase activity. From these results, we conclude that Kei1 is essential for both the activity and the Golgi localization of IPC synthase.

INTRODUCTION

The budding yeast *Saccharomyces cerevisiae*, a unicellular eukaryote widely used as a model organism in cell biology, has three major types of membrane lipids. The most abundant are glycerophospholipids that have glycerol backbones. The others are sterols and sphingolipids. Ergosterol is specific for fungi and corresponds to cholesterol in mammals (Holthuis *et al.*, 2001). Sphingolipids are phospholipids derived from ceramides, and the yeast sphingolipids are characterized by modification with inositol-phosphate (IP). The composition of membrane lipids changes along with the secretory pathway in *S. cerevisiae*; e.g., sphingolipids and ergosterol are more abundant in the plasma membrane than in other membranes (Hechtberger *et al.*, 1994). The deficiency of sphingolipid biosynthesis results in the instability of a subset of proteins localized to the plasma membrane (Lauwers *et al.*, 2007). It is proposed that sphingolipids and ergosterols form specific domains called “lipid rafts” that are distinct from glycerophospholipid-rich regions both structurally and functionally in the plasma membrane (Edidin, 2003). It is

suggested that certain proteins are assembled in the lipid rafts and fulfill specific and essential functions such as signal transduction (Simons and Toomre, 2000; Allen *et al.*, 2007). The existence of lipid raft is still controversial (Munro, 2003), but sphingolipids are important and interesting irrespective of whether or not lipid rafts really exist.

In *S. cerevisiae*, sphingolipids are composed of inositol-phosphorylceramide (IPC) and its mannosylated derivatives, namely, mannose inositolphosphorylceramide (MIPC) and mannose di(inositolphosphoryl)ceramide [M(IP)₂C] (Figure 1). There are five types of ceramide backbones with different positions and number of hydroxide groups in these IP-containing sphingolipids (Funato *et al.*, 2002). De novo synthesis of ceramide is carried out by ceramide synthase composed of Lag1, Lac1, and Lip1 (Guillas *et al.*, 2001; Schorling *et al.*, 2001; Vallee and Riezman, 2005). The conversion of ceramide to IPC is catalyzed by IPC synthase that transfers IP from phosphatidylinositol to ceramide. *AUR1* is the essential gene responsible for this enzymatic activity (Nagiec *et al.*, 1997; Dickson and Lester, 1999; Levine *et al.*, 2000; Funato *et al.*, 2002). Aur1 is localized to the medial-Golgi compartment, indicating that modifications of ceramide occur after it is transported to the Golgi (Levine *et al.*, 2000). Conversion of IPC to MIPC is suggested to be executed by the Csg1–Csg2 or Csg1–Csh1 complex (Uemura *et al.*, 2003), and subsequent conversion of MIPC to M(IP)₂C is catalyzed by Ipt1 (Dickson *et al.*, 1997; Uemura *et al.*, 2003). In this pathway in which ceramides change to complex sphingolipids, only the first step, i.e., conversion of ceramide to IPC, is essential for the viability of the cell. Because it is thought that both the accumulation of ceramides and the loss of sphingolipids exert fatal effects on yeast cells, it seems obvious that the conversion of ceramide into IPC is stringently regulated. To understand the regulation of IPC synthesis, detailed characterization of the enzyme responsible is crucial.

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E09-03-0235>) on September 2, 2009.

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Abbreviations used: CHAPS, 3-[3-(cholamidopropyl)dimethylammonio]propanesulfonate; ER, endoplasmic reticulum; GDP, guanosine diphosphate; GFP, green fluorescent protein; GST, glutathione transferase; HA, hemagglutinin; IPC, inositolphosphorylceramide; MIPC, mannose inositolphosphorylceramide; M(IP)₂C, mannose di(inositolphosphoryl)ceramide; TLC, thin layer chromatography.

Figure 1. Biosynthetic pathway of sphingolipids in *S. cerevisiae*. Precursors, products, and proteins required for the individual enzymatic steps are shown. DHS, dihydrosphingosine; PHS, phytosphingosine; IPC, inositolphosphorylceramide.

Sphingolipids have attracted the attention of many researchers, and striking advances in this field have been made in the past couple of decades. Initially, genetic approaches such as isolating mutants, screening their suppressors, and searching for homologues to known genes related to sphingolipids have mainly contributed the advances; but more recently, different approaches have discovered novel proteins involved in the sphingolipid biosynthetic pathway. Riezman's group found Lip1 through the purification of the ceramide synthase complex (Vallee and Riezman, 2005), and Weissman's group uncovered a previously unidentified 3-hydroxyacyl-CoA dehydratase Phs1 through a large-scale analysis of essential genes whose products are located in the early secretory pathway (Denic and Weissman, 2007). These findings indicate the possibility that any essential genes involved in sphingolipid biosynthesis remain unidentified. It is probably difficult to find them by conventional approaches.

In the *S. cerevisiae* genome, >200 genes encode membrane proteins essential for cell viability. In spite of enormous efforts in genome-wide analyses, several essential membrane proteins remain uncharacterized. We have tried to assign functions to such essential proteins that are integrated into membranes by our strategy of using temperature-sensitive mutants. To date, we have reported the characterization of two previously uncharacterized endoplasmic reticulum (ER) proteins Pga1 and Keg1 (Nakamata *et al.*, 2007; Sato *et al.*, 2007). *YDR367w* is one of such uncharacterized essential gene, encoding a 221-amino acid polypeptide with four predicted transmembrane domains. Here, we

show that the product of *YDR367w* is a novel component of IPC synthase. It interacts with Aur1 *in vivo*, is essential for the enzymatic activity that transfers IP to ceramide, and it is also involved in the localization of IPC synthase to the medial-Golgi. We also found that it undergoes cleavage by Kex2, a late Golgi endoprotease. Therefore, we have designated *YDR367w* as Kex2-cleavable protein Essential for IPC synthesis 1 (*KEI1*).

MATERIALS AND METHODS

Strains, Plasmids, Media, and Reagents

S. cerevisiae and plasmids used in this study are listed in Table 1 and 2, respectively. The construction of the strains and plasmids are described in Supplemental Data.

Yeast cells were grown in YPD medium (1% Bacto yeast extract [BD Biosciences, Franklin Lakes, NJ], 2% Bacto peptone [BD Biosciences], and 2% glucose) or SD medium [0.17% yeast nitrogen base without amino acids [BD Biosciences], 0.5% ammonium sulfate, 2% glucose, and appropriate supplements] at 30°C unless other temperatures were indicated. YPD was used as a growth media unless otherwise specified. Solid media were made with 1.5% agar. Twenty micrograms per milliliter each of histidine, tryptophan, and uracil, and 0.1% 5-fluoroorotic acid (Sigma-Aldrich, St. Louis, MO) were added in SD to make 5-fluoroorotic acid (5-FOA) plates for the Ura3⁺ counterselection. Twenty micrograms per milliliter phloxine B (Sigma-Aldrich, St. Louis, MO) was added in YPD to make phloxine plates (Tsukada and Ohsumi, 1993) for the selection of temperature-sensitive mutants. Aureobasidin A was purchased from Takara Bio (Shiga, Japan). *Escherichia coli* DH5 α (F⁻, supE44 Δ lacU169 ϕ 80lacZ Δ M15 hsdR17 recA1 endA1 gyrA96 thi-1 relA1) was used in plasmid propagation. *E. coli* was grown in a Luria-Bertani (LB) medium (1% Bacto tryptone [BD Biosciences], 0.5% Bacto yeast extract [BD Biosciences], and 0.5% NaCl).

Antibodies

Antisera against Scs2, Ret1, and Sec21 were kindly provided by Dr. Satoshi Kagiwada (Nara Women's University, Nara, Japan), Dr. Pierre Cosson (Basel Institute for Immunology, Basel, Switzerland), and Dr. Rainer Duden (University of Cambridge, Cambridge, United Kingdom), respectively. Anti-3-phosphoglycerate kinase mouse (anti-PGK, 22C5; Invitrogen, Carlsbad, CA), anti-myc mouse (9E10; Berkeley Antibody, Richmond, CA), anti-hemagglutinin (HA) mouse (12CA5; Roche Diagnostics, Indianapolis, IN), and anti-carboxypeptidase Y (CPY) rabbit (Rockland Immunochemicals, Gilbertsville, PA) polyclonal antibody were purchased. The anti-green fluorescent protein (GFP), anti-Sed5, anti-Van1, anti-Kex2, and anti-Gas1 antisera were prepared by immunizing rabbits with the glutathione transferase (GST) fusion protein as the antigen. For Western blotting, these antisera were diluted at 1:1000.

Construction of Temperature-sensitive Mutant Allele of *KEI1*

The DNA fragment containing the whole *KEI1* gene with the authentic promoter and terminator was amplified under an error-prone polymerase chain reaction (PCR) condition (Muhlrad *et al.*, 1992). KSY66 was transformed with the mixture of the amplified fragment and the SmaI-cleaved pKS26 to integrate fragments into the plasmid by gap repair. The His⁺ transformants were replicated to 5-FOA plates to remove pKS23 at 26.5°C and then replicated to phloxine B plates and incubated at 37°C for 4–12 h. Cells with reduced viability become unable to protect the entry of this dye. The cells that formed pink colonies were tested for single colony formation at 26.5 and 37°C. After confirming that the temperature sensitivity was due to the plasmid, the nucleotide sequences of the mutant *kei1* were determined. The allele *kei1-1* was integrated into the chromosome of KSY66 at the Δ *kei1::kanMX4* region by recombination with *kei1-1::LEU2* fragment by double crossing-over at the surrounding homologous sequences. The Leu⁺ cells were streaked on a 5-FOA plate to remove pKS23, grown cells were tested for genotype, and a correct strain was stocked as KSY271.

Subcellular Fractionation

Cells were converted to spheroplasts and burst in B88 (20 mM HEPES, pH 6.8, 150 mM potassium acetate, 5 mM magnesium acetate, and 200 mM sorbitol) containing protease inhibitors (1 μ g/ml each of chymostatin, aprotinin, leupeptin, pepstatin A, antipain, and 1 mM phenylmethylsulfonyl fluoride). Unbroken cells were removed by centrifugation at 1000 $\times$ g for 5 min. For differential fractionation, the cleared lysate was sequentially centrifuged to generate 10,000 $\times$ g $\times$ 10-min pellet (P10), 100,000 $\times$ g $\times$ 60-min pellet (P100), and 100,000 $\times$ g $\times$ 60-min supernatant (S100). Each fraction was adjusted to the original volume of the lysate, and the same amount was applied for SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting.

Table 1. Strains used in this study

Strain name	Description	Source
KA31a	<i>MATa, Δhis3Δ leu2Δ trp1Δ ura3</i>	EUROSCARF ^a
BY4741	<i>MATa, his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	EUROSCARF
BY4742	<i>MATa, his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	EUROSCARF
BY4743	<i>MATa/MATα, his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ0</i>	EUROSCARF
KSY66	As BY4741, <i>Δkei1::kanMX4</i> , pKS23 (CEN, URA3 <i>KEI1</i>)	This study
KSY271	As BY4741, <i>Δkei1::kei1-1 LEU2</i>	This study
KSY431	As BY4741, <i>Δkei1::kanMX4</i> , pKS243 (CEN, <i>HIS3 KEI1-GFP</i>)	This study
KSY506	As BY4743, <i>Δkei1::kanMX4/KEI1</i>	This study
KSY507	As BY4741, <i>Δkei1::kanMX4</i> , pKS301 (CEN, <i>HIS3 KEI1^{-intron}-GFP</i>)	This study
KSY512	As BY4741, <i>Δaur1::AUR1-GFP HIS3</i>	This study
KSY513	As KSY271, <i>Δaur1::AUR1-GFP HIS3</i>	This study
KSY525	As BY4741, <i>Δaur1::AUR1-3HA HIS3</i>	This study
KSY527	As BY4741, <i>Δkei1::KEI1-GFP URA3</i>	This study
KSY530	As KSY271, <i>Δaur1::AUR1-3HA HIS3</i>	This study
KSY531	As BY4741, <i>Δaur1::AUR1-3HA HIS3, Δkei1::KEI1-GFP URA3</i>	This study
KSY548	As BY4741, <i>Δkex2::kanMX4</i>	EUROSCARF
KSY552	As KSY271, <i>Δubc7::kanMX4</i>	This study
KSY553	As KSY271, <i>Δubc7::kanMX4</i>	This study
KSY554	As KSY271, <i>Δdoa10::kanMX4</i>	This study
KSY559	As KSY271, <i>Δhrd1::kanMX4</i>	This study
KSY560	As KSY271, <i>Δhrd1::kanMX4</i>	This study
KSY561	As KSY271, <i>Δdoa10::kanMX4</i>	This study
KSY562	As KSY271, <i>Δubx2::kanMX4</i>	This study
KSY563	As KSY271, <i>Δubx2::kanMX4</i>	This study
KSY571	As KSY512, <i>Δpep4::LEU2</i>	This study
KSY572	As KSY513, <i>Δpep4::LEU2</i>	This study
KSY607	As BY4741, pKS342 (CEN, URA3 <i>kei1^{K141S}-GFP</i>)	This study
KSY609	As BY4741, pKS344 (CEN, URA3 <i>kei1^{R135S}-GFP</i>)	This study
KSY611	As BY4741, pKS346 (CEN, URA3 <i>kei1^{K131S}-GFP</i>)	This study
KSY614	As KSY548, pKS342 (CEN, URA3 <i>kei1^{K141S}-GFP</i>)	This study
KSY616	As KSY548, pKS344 (CEN, URA3 <i>kei1^{R135S}-GFP</i>)	This study
KSY618	As KSY548, pKS346 (CEN, URA3 <i>kei1^{K131S}-GFP</i>)	This study
KSY625	As BY4741, pKS353 (CEN, URA3 <i>KEI1-GFP</i>)	This study
KSY627	As KSY548, pKS353 (CEN, URA3 <i>KEI1-GFP</i>)	This study
KSY686	As BY4741, <i>Δkei1::kanMX4, ura3-52:: KEI1-GFP, URA3</i>	This study
KSY692	As BY4741, <i>Δkei1::kanMX4, ura3-52:: kei1-1-GFP, URA3</i>	This study
KSY694	As BY4741, <i>Δkei1::kanMX4, ura3-52:: kei1^{ΔC}-GFP, URA3</i>	This study
KSY696	As BY4741, <i>Δkei1::kanMX4, ura3-52:: KEI1-GFP, URA3</i>	This study
KSY698	As BY4741, <i>Δkei1::kanMX4, ura3-52:: kei1^{F103I}-GFP, URA3</i>	This study
KSY714	As KSY527, <i>Δkre2::KRE2-3HA LEU2</i>	This study
KSY721	As KSY525, <i>ura3-52::GFP-KEI1, URA3</i>	This study
KSY723	As BY4741, <i>ura3-52::GFP-KEI1, URA3</i>	This study
KSY725	As KSY723, <i>Δkre2::KRE2-3HA LEU2</i>	This study
KSY728	As KSY692, <i>Δpep4::LEU2</i>	This study
KSY730	As KSY694, <i>Δpep4::LEU2</i>	This study
KSY732	As KSY696, <i>Δpep4::LEU2</i>	This study
KSY753	As BY4741, <i>Δkei1::kanMX4, ura3-52:: GFP-kei1^{F103I}, URA3</i>	This study
KSY765	As KSY723, <i>Δpep4::LEU2</i>	This study
KSY767	As KSY753, <i>Δpep4::LEU2</i>	This study
KSY771	As BY4741, <i>Δkei1::kanMX4</i> , pKS380 (CEN, <i>LEU2 KEI1-GFP</i>), <i>Δaur1::AUR1-3HA HIS3</i>	This study
KSY773	As BY4741, <i>Δkei1::kanMX4</i> , pKS381 (CEN, <i>LEU2 kei1^{F103I}-GFP</i>), <i>Δaur1::AUR1-3HA HIS3</i>	This study
KSY775	As BY4741, <i>Δkei1::kanMX4</i> , pKS382 (CEN, <i>LEU2 kei1^{ΔC}-GFP</i>), <i>Δaur1::AUR1-3HA HIS3</i>	This study
KSY780	As KSY698, <i>Δpep4::LEU2</i>	This study
KSY784	As KSY271, <i>Δkre2::KRE2-3HA HIS3</i>	This study
KSY790	As BY4741, <i>Δkre2::KRE2-3HA HIS3</i>	This study
KSY792	As KSY696, <i>Δaur1::AUR1-3HA HIS3</i>	This study
KSY794	As KSY698, <i>Δaur1::AUR1-3HA HIS3</i>	This study
KSY799	As BY4741, <i>Δkei1, ura3-52:: GFP-kei1^{F103I}, URA3</i>	This study
KSY801	As KSY753, <i>Δaur1::AUR1-3HA HIS3</i>	This study
KSY805	As BY4741, <i>Δkei1::kei1^{F103I} LEU2, Δaur1::AUR1-3HA HIS3</i>	This study
THY4-2	As KA31a, <i>Δkre2::KRE2-3HA LEU2</i>	This study
YNY309	<i>sec12-4</i>	Randy Schekman
YNY401	As KA31a, <i>Δemp24</i>	This study

^a EUROpean *Saccharomyces Cerevisiae* ARchive for Functional Analysis, Frankfurt, Germany.

Fractionations using 22–60% sucrose density gradient centrifugation were performed basically as described previously (Becherer *et al.*, 1996; Conchon *et*

al., 1999). In brief, spheroplasts were lysed in 1 ml of ice-cold B88 containing protease inhibitors by incubation for 30 min at 4°C and sequential 20 strokes

Table 2. Plasmids used in this study

Plasmid name	Description	Source
pKS23	<i>CEN-URA3-KEI1</i> , own promoter	This study
pKS26	Gap-repair for <i>KEI1</i> , <i>CEN</i> , <i>HIS3</i>	This study
pKS181	<i>kei1-1</i> integration (<i>LEU2</i>)	This study
pKS243	<i>CEN-HIS3-KEI1-GFP</i> , own promoter	This study
pKS262	<i>2μURA3-AUR1</i> , own promoter	This study
pKS290	<i>AUR1-GFP</i> integration (<i>HIS3</i>)	This study
pKS293	<i>AUR1-3HA</i> integration (<i>HIS3</i>)	This study
pKS301	<i>CEN</i> , <i>HIS3 KEI1^{-intron}-GFP</i> , own promoter	This study
pKS307	<i>KEI1-GFP</i> integration (<i>URA3</i>)	This study
pKS342	<i>CEN</i> , <i>URA3 kei1^{K141S}-GFP</i> , own promoter	This study
pKS344	<i>CEN</i> , <i>URA3 kei1^{R135S}-GFP</i> , own promoter	This study
pKS346	<i>CEN</i> , <i>URA3 kei1^{K131S}-GFP</i> , own promoter	This study
pKS349	<i>KEX2</i> disruption (<i>LEU2</i>)	This study
pKS353	<i>CEN</i> , <i>URA3 KEI1-GFP</i> , own promoter	This study
pKS360	<i>PEP4</i> disruption (<i>LEU2</i>)	This study
pKS361	<i>URA3-kei1-1-GFP</i>	This study
pKS362	<i>URA3-kei1^{ΔC}-GFP</i>	This study
pKS363	<i>URA3-KEI1-GFP</i>	This study
pKS364	<i>URA3-kei1^{F103I}-GFP</i>	This study
pKS378	<i>URA3-GFP-KEI1</i> , <i>YPT1</i> promoter	This study
pKS380	<i>CEN-LEU2-KEI1</i> , own promoter	This study
pKS381	<i>CEN-LEU2-kei1^{F103I}</i> , own promoter	This study
pKS382	<i>CEN-LEU2-kei1^{ΔC}</i> , own promoter	This study
pKS384	<i>URA3-GFP-KEI1^{F103I}</i> , <i>YPT1</i> promoter	This study
pKS393	<i>KRE2-3HAs</i> integration (<i>HIS3</i>)	This study
pKS394	<i>kei1^{F103I}</i> integration (<i>LEU2</i>)	This study
pHL63	<i>KEX2</i> disruption (<i>URA3</i>)	Tomishige <i>et al.</i> (2003)
pTH6-3	<i>KRE2-3HA</i> integration	This study

in a Dounce homogenizer. The lysate was centrifuged at $1000 \times g$ for 5 min to remove unlysed cells. Then, 0.65 ml of the supernatant was recovered, and the pellet was extracted again with 0.45 ml of ice-cold B88 containing protease inhibitors and centrifuged at $1000 \times g$ for 5 min. The combined supernatant was centrifuged at $10,000 \times g$ for 10 min, and 1 ml of the resultant supernatant was loaded on a sucrose step gradient, which was generated using the following steps (all sucrose solutions were made [wt/wt, %], in 10 mM HEPES-KOH, pH 7.6, 1 mM $MgCl_2$): 1 ml 60%, 1 ml 40%, 1.2 ml 37%, 1.8 ml 34%, 2 ml 32%, 1.8 ml 29%, 1.2 ml 27%, and 1 ml 22%. After 16-h centrifugation in an SW40Ti rotor (Beckman Coulter, Fullerton, CA) at $170,000 \times g$, 12 fractions of 1 ml were sequentially collected from the top of the gradient. Aliquots of each fraction were mixed with the SDS sample buffer, and proteins were resolved by SDS-PAGE and detected by immunoblotting using anti-Kex2, anti-Sed5, anti-GFP, and anti-HA antisera. Enhanced chemiluminescence signals were captured by an image analyzer equipped with a cooled charge-coupled-device camera (LAS-1000plus; Fuji Film, Tokyo, Japan), and digital images were quantified using ImageJ software (National Institutes of Health, Bethesda, MD) and graphed on Excel (Microsoft, Redmond, WA).

For fractionation using 18–60% sucrose density gradient centrifugation, spheroplasts were lysed as described above, and 1 ml of the supernatant of $1000 \times g$ was loaded on a sucrose step gradient that was made as described previously (Inadome *et al.*, 2005). The quantification of the digital images and creation of the graph were carried out as described above.

Immunoprecipitation

Cells were grown at 30°C in the YPD medium to an $OD_{600\text{ nm}} = 1.0$, and 50 OD units of cells were converted to spheroplasts and suspended in 1 ml of B88 containing 1% Triton X-100 and protease inhibitors. After rotating the mixture for 30 min at 4°C, unbroken cells were removed by centrifugation at $1000 \times g$ for 5 min at 4°C and then centrifuged at $16,100 \times g$ for 10 min. The supernatant was centrifuged at $100,000 \times g$ for 60 min. A part of the supernatant was recovered as the fraction “supernatant.” The rest of the supernatant was mixed with anti-HA antibody and kept gently rotating at 4°C for 1 h. Protein A-Sepharose beads (Amersham Biosciences, Uppsala, Sweden) washed with B88 containing 1% Triton X-100 were added, and incubation was continued at 4°C overnight. The mixture was centrifuged at $500 \times g$ for 1 min, and the supernatant was recovered as the fraction “unbound.” The beads were washed five times with 500 μ l of B88 containing 1% Triton X-100. The bound proteins were eluted twice by incubating at 4°C for 10 min in 50 μ l of SDS sample buffer and recovered as the fraction “bound.” Material derived from 10-fold more cells was loaded for sample bound than

for others in SDS-PAGE. Indicated proteins were detected by Western blotting.

In Vitro Assay for IPC Synthesis

For membrane fractions, cells were grown at 26.5°C to an $OD_{600\text{ nm}} = 1.0$. Half of the cells were grown at 26.5°C, and the other half were grown at 37°C for 2 h. Cells were collected, washed with water and B88, and suspended in ice-cold B88 containing protease inhibitors. The mixture was added with 1 g of glass beads and broken with Multi-Beads Shocker (Yasui Kikai, Osaka, Japan). Unbroken cells were removed by centrifugation at $1000 \times g$ for 5 min at 4°C, and the supernatant was centrifuged on 60% sucrose cushion at $100,000 \times g$ for 30 min (TLS-55 rotor; Beckman Coulter). The membrane fraction was suspended in ice-cold B88 containing protease inhibitors, dispensed to 60 μ l each, frozen with liquid N_2 , and preserved at -80°C until being used. The membrane fraction was mixed with 60 μ l of the reaction mix (10 mM HEPES-KOH, pH 7.2, 1 mM EDTA, 250 mM sucrose, 10 mg/ml bovine serum albumin BSA [fatty acid-free; Sigma-Aldrich], 2 mM 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate [CHAPS, Dojindo, Kumamoto, Japan], 2 mM $MnCl_2$, and 2 mM $MgCl_2$) with or without 10 μ g/ml aureobasidin A (Takara Bio), and incubated at the indicated temperature for 30 min (preincubation). Then, 0.6 μ l of 1 M 6-[(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoyl]sphingosine (C_6 -NBD-ceramide; Invitrogen) dissolved in dimethyl sulfoxide was added to the mixture and incubated at the indicated temperature for 1 h. The reaction was stopped by adding 400 μ l of 4:10:1 mixture of chloroform:methanol:1 M HCl. Lipids were extracted by sequential additions of 50 μ l of 1 M HCl and 100 μ l of chloroform and vigorously vortexing, after which phase separation was achieved by centrifugation for 5 min at $16,100 \times g$. The chloroform layer was collected and dried by evaporation with Speed-Vac, and the resulting pellet was resuspended in 15 μ l of chloroform. Recovered lipids were separated on a thin layer chromatography (TLC) plate (Silica gel 60; Merck, White Station, NJ) in an 11:9:2 mixture of chloroform:methanol:30 mM KCl. Fluorescent bands on the TLC were detected using LAS-1000plus.

For the assay of immunologically purified IPC synthase, cells were grown at 26.5°C to an $OD_{600\text{ nm}} = 1.0$, and 50 OD units of cells were converted to spheroplasts and suspended in 1 ml of B88 containing 1% Triton X-100 and protease inhibitors. After rotating the mixture for 30 min at 4°C, unbroken cells were removed by centrifugation at $1000 \times g$ for 5 min at 4°C and then centrifuged at $16,100 \times g$ for 10 min at 4°C. The supernatant was centrifuged at $100,000 \times g$ for 60 min at 4°C. The supernatant was mixed with washed protein A-Sepharose beads for 30 min at 4°C, and then centrifuged at $500 \times g$ for 1 min at 4°C. The supernatant was mixed with anti-HA antibody and

kept gently rotating at 4°C for 1 h. Washed protein A-Sepharose beads were added, and incubation was continued at 4°C overnight. The mixture was centrifuged at $500 \times g$ for 1 min, and the beads were washed twice with 1 ml of B88 containing 1% Triton X-100. The beads were further washed three times with 1 ml of B88 containing 1 mM CHAPS. The supernatant was removed and resuspended in 1 ml of B88 containing 1 mM CHAPS and dispensed into four tubes (500, 250, $2 \times 125 \mu\text{l}$). Each tube was centrifuged at $500 \times g$ for 1 min, and the supernatant was completely removed. Bound proteins on beads of one 125- μl dispensed tube were eluted with $1 \times$ SDS-PAGE sample buffer to monitor the amount of bound proteins. Beads in other tubes were resuspended in 120 μl of the reaction mix (10 mM HEPES-KOH, pH 7.2, 1 mM EDTA, 250 mM sucrose, 5 mg/ml fatty acid-free BSA, 1 mM CHAPS, 2 mM MnCl_2 , 2 mM MgCl_2 , 0.15 mg/ml L- α -phosphatidylinositol ammonium salt from bovine liver [Sigma-Aldrich], and 0.05% Triton X-100). After preincubation at 26.5°C for 30 min, the reaction was started by adding 0.6 μl of 1 M C_6 -NBD-ceramide. After incubation at 26.5°C for 1 h, the lipids were extracted and analyzed as explained above.

GST Pull-Down Assay

E. coli BL21 (DE3) cells transformed with the plasmid pKSEC7 (GST-Kei1CT) or pGEX4T-3 (GST) were grown at 37°C in the LB medium containing ampicillin (100 $\mu\text{g}/\text{ml}$), and protein production was induced by the addition of isopropyl β -D-thiogalactoside (final concentration, 0.5 mM) when $\text{OD}_{600 \text{ nm}}$ reached 0.5. The cultures were grown at 37°C for an additional 2 h. The cells were then collected by centrifugation, resuspended in the STE buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 1 mM EDTA) containing 100 $\mu\text{g}/\text{ml}$ lysozyme, and kept on ice for 20 min. Protease inhibitors and dithiothreitol (final concentration, 1 mM) were added to the mixture, which was then sonicated for 1 min in an ice bath with a UD-201 Ultrasonic Disruptor (Tomy, Tokyo, Japan) with the appropriate setting (output, 4; duty cycle, 40%). The cell homogenate was centrifuged, and the supernatant was mixed with one-ninth volume of 10% Triton X-100. Glutathione-Sepharose 4B beads (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) were added to the mixture and rotated gently for 2 h at 4°C. Protein-bound beads were washed three times in ice-cold phosphate-buffered saline. The yeast cytosol prepared from BY4741 was mixed with Triton X-100 (final concentration, 1%), added to the beads containing purified GST-Kei1CT, and gently rotated for 2 h at 4°C. After extensive washing, bound proteins were eluted by boiling the beads in the SDS sample buffer and subjected to SDS-PAGE. The released proteins were detected by Western blotting.

Indirect Immunofluorescence

The localization of Aurl-3HA and Kre2-3HA was observed by the indirect immunofluorescence as described previously (Sato *et al.*, 2007) except that anti-HA antibody and Alexa 568-conjugated goat antibody to mouse immunoglobulin G were used as the primary antibody and the secondary antibody, respectively.

Invertase Detection

Secretory invertase was recovered and analyzed as described previously (Sato *et al.*, 2007).

RESULTS

Kei1/Ydr367w Is Conserved among Fungi

According to *Saccharomyces* Genome Database, *KEI1/YDR367w* encodes a 221-amino acid polypeptide of molecular weight 25,484 and *pI* 8.06, the biochemical function of which remains unknown. Homologous sequences were found in several fungal genome databases by FASTAP search (Figure 2A), but no homologues were found in databases of higher eukaryotes. The hydropathy plot indicated that there are four hydrophobic segments, which are predicted as transmembrane helices by the TMHMM program (<http://www.cbs.dtu.dk/services/TMHMM/>) (Figure 2B).

Kei1 Is an Integral Membrane Protein and Undergoes Cleavage by Kex2

We constructed a plasmid that produces Kei1 which is C-terminally fused with green fluorescent protein (Kei1-GFP) and introduced it to a diploid strain in which one of the *KEI1* alleles was disrupted by *kanMX4*. By tetrad analysis, a haploid disruptant strain producing the fusion protein was obtained. Kei1-GFP fully rescued the $\Delta kei1$ disruption because *KEI1-GFP* cells showed no apparent growth defects (data not

shown). Interestingly, Kei1-GFP yielded two major bands when resolved and detected by SDS-PAGE and Western blotting at the estimated molecular masses of 36.5 and 27 kDa (Figure 3A). When fractionated by the differential centrifugation, the 36.5-kDa polypeptide was mainly recovered in the P10 fraction, and 27-kDa was almost evenly found in P10 and P100 fractions (Figure 3A). From this result, it is likely that 36.5-kDa polypeptide is localized to the ER and 27-kDa polypeptide is localized to the Golgi. Thus, there are at least two types of Kei1 polypeptides, which are different not only in molecular mass but also in subcellular localization.

KEI1 contains an intron of 31 nucleotides after the predicted start site of translation. We tested whether the intron would result in two types of Kei1 polypeptides, but deletion of the intron did not affect the pattern of Kei1-GFP on SDS-PAGE (Figure 3B). This result is consistent with the analyses of transcripts (Miura *et al.*, 2006) and transcription start sites (Zhang and Dietrich, 2005) in *S. cerevisiae*, which indicate that there is only one transcript for *KEI1*. Next, we suspected that Kei1 undergoes processing after being transported to the Golgi. Because Kex2 is a major protease in the Golgi (Fuller *et al.*, 1989; Redding *et al.*, 1991), we tested whether Kex2 would process Kei1. Kei1-GFP was expressed in wild-type or $\Delta kex2$ cells, and the molecular mass of Kei1-GFP was analyzed by SDS-PAGE. As a result, the 27-kDa band was diminished when *KEI1-GFP* was expressed in $\Delta kex2$ cells (Figure 3C). Kex2 prefers basic residues, especially dibasic sequences, as its cleavage site (Rockwell and Fuller, 1998). As it is predicted from the apparent molecular masses of the two species of Kei1-GFP that the cleavage occurs between the third and fourth predicted transmembrane segments, we replaced each of the three basic residues in this region (¹³¹Lys, ¹³⁵Arg, and ¹⁴¹Lys) by Ser. The modified Kei1-GFP constructs were produced in wild-type and $\Delta kex2$ cells. As shown in Figure 3C, only the R135S substitution resulted in the disappearance of the 27-kDa Kei1-GFP band in the wild-type cells, indicating that Kex2 recognizes this ¹³⁵Arg residue to cleave Kei1. From these results, we propose that Kei1 is transported to the late Golgi and subsequently processed by Kex2. We will call the full-length Kei1 as Kei1F, and the N- and C-terminal fragments as Kei1N and Kei1C, respectively.

Next, we carried out a solubility test of Kei1-GFP and found that both Kei1F-GFP and Kei1C-GFP were found in the precipitate after $100,000 \times g$ centrifugation even when the cell lysate was treated with 1 M sodium chloride, 0.1 M sodium carbonate, pH 11.0, or 2 M urea. However, upon treatment with 1% Triton X-100, both proteins were found in the supernatant (Supplemental Figure S1A). In addition, GFP-Kei1F and GFP-Kei1N were also found in the supernatant only when treated with 1% Triton X-100 (Supplemental Figure S1B). Therefore, after Kex2 cleavage, both fragments of Kei1 remain integrated in the membrane, as predicted by the hydropathy profile.

Kei1 Localizes to the Medial-Golgi after Kex2 Cleavage

To determine the localization of Kei1 more precisely, we constructed a strain that express *KEI1-GFP* and *KRE2-3HA* from their own loci on the chromosome by their own promoters, and investigated where Kei1 localizes within the cell in several ways.

First, we compared the localization of Kei1C-GFP with the early Golgi-marker Van1, the medial-Golgi-marker Kre2-3HA and the late Golgi-marker Kex2 by subcellular fractionation. The cell lysate was centrifuged at $10,000 \times g$ for 10 min to remove the ER fraction, and the resulting supernatant

Figure 2. Sequence alignment of Kei1 protein and its homologues and the hydropathy profile of Kei1 protein. (A) Amino acid sequence alignment of the Kei1 homologues. Transmembrane domains predicted by the TMHMM program are indicated by bars over the sequences. The C-terminal peptide that fused to GST in Figure 9 (Kei1CT) is indicated by a dotted line. The sequence alterations in *kei1-1* (F103I and ΔC) are indicated by arrows. The Kex2 recognition residue (¹³⁵R) is indicated by an arrowhead. The identical amino acids were highlighted with reverse letters. S.c., *S. cerevisiae*; C.g., *Candida glabrata*; V.p., *Vanderwaltozyma polyspora*; A.g., *Ashbya gossypii*; and K.l., *Kluyveromyces lactis*. (B) The hydropobicity index was calculated according to Kyte and Doolittle (1982), with a window size of 19 amino acids. Bold lines indicate the hydrophobic domains.

was loaded on the 22–60% sucrose density gradient, and then proteins were fractionated by ultracentrifugation. Distribution of the indicated proteins was analyzed by SDS-PAGE and Western blotting. As shown in Figure 4A, the peak of Kei1C-GFP was found between the peaks of Sed5 and Kex2 and exhibited a very similar distribution to Kre2-3HA, indicating that Kei1C-GFP localizes to the medial-Golgi. Next, we examined the localization of Kei1-GFP, GFP-Kei1, and Kre2-3HA by confocal laser microscopy (Figure 4B). Kei1-GFP and GFP-Kei1 signals showed punctate patterns throughout the cytoplasm, most of which overlapped with Kre2-3HA signals. All Kei1-GFP dots were apparently coincident with Kre2-3HA dots, but some GFP-Kei1 signals did not coincide with Kre2-3HA. They probably correspond to GFP-Kei1F in the ER, because a considerable amount of the uncleaved GFP-Kei1F was cofractionated with the ER-marker Scs2 in our sucrose density gradient centrifugation experiments (Supplemental Figure S1C). GFP-Kei1F is more abundant than Kei1F-GFP in *KRE2-3HA* strains (see Figure 6), which also supports this possibility. From these results, we conclude

that Kei1 localizes to the medial-Golgi compartment after Kex2 cleavage.

Isolation of *kei1-1*, a Temperature-sensitive Allele of *KEI1*

To investigate what function Kei1 exerts in the medial-Golgi, we sought temperature-sensitive (ts) alleles of *KEI1* by error-prone PCR mutagenesis. We obtained one ts allele *kei1-1*, in which a base substitution caused replacement of ¹⁰³Phe with Ile (F103I) and a nucleotide deletion caused truncation of the C-terminal region ¹⁹³Gln...²²¹Glu-COOH to ¹⁹³Lys-¹⁹⁴Thr-COOH (ΔC). Both of the mutations are required for temperature sensitivity, because the cells carrying only the F103I or ΔC mutation can form colonies to a similar extent as wild-type cells at 37°C (Supplemental Figure S2A). The chromosomal *KEI1* allele was replaced with the mutant *kei1-1* by homologous recombination for further analysis.

Kei1 Genetically and Physically Interacts with Aur1

To test whether Kei1 is involved in protein transport or modification, the processing of carboxypeptidase Y, glycosylphosphatidylinositol (GPI)-anchored protein Gas1, and

Figure 3. Kei1 is a novel substrate of the late Golgi endoprotease Kex2. (A) *KEI1*-GFP was expressed from *CEN* plasmid. The total cell lysate was subjected to differential centrifugation to obtain indicated fractions. Proteins were resolved and detected by SDS-PAGE and Western blotting. Two bands at the molecular mass of 36.5 kDa and 27 kDa were detected by anti-GFP antibody. The 36.5-kDa band was mainly recovered in the P10 fraction, and the 27-kDa band was recovered evenly in P10 and P100 fractions. The early Golgi membrane protein Van1 was detected by anti-Van1 antibody. (B) Effect of intron on Kei1 protein size. *KEI1* with or without its intron sequence was expressed from the *CEN* plasmid and proteins were detected by the C-terminal GFP tag. (C) Kei1 protein and its derivatives without possible Kex2 cleavage sites. *KEI1*-GFP, *KEI1*^{K131S}-GFP, *KEI1*^{R135S}-GFP, or *KEI1*^{K141S}-GFP was expressed from *CEN* plasmid in wild-type (+) and $\Delta kex2$ (-) cells.

secretory invertase were analyzed, but no apparent defects were observed in any of the analyses (Supplemental Figure S2, B–D).

Next, we screened for multicopy suppressor genes of the temperature sensitivity of the *kei1-1* mutant, because it often becomes an effective clue to elucidate the functional role of uncharacterized gene products. We constructed a 2- μ m genomic library of the *kei1-1* chromosome and introduced it to *kei1-1* cells and screened for colonies whose viability at 37°C depended on the introduced plasmids. Consequently, we identified *AUR1*, *PSD1*, *GDA1*, and *SFH1* (*YKL091c*) as multicopy suppressors of the temperature sensitivity of *kei1-1* (Figure 5A and Supplemental Figure S3). Importantly, *AUR1* could significantly recover the growth defect of the *kei1-1* mutant at 37°C (Figure 5A). *AUR1* is now thought to encode IPC synthase (Funato *et al.*, 2002; Cowart and Obeid, 2007). High copy introduction of *AUR1* gene could not suppress the lethality of the $\Delta kei1$ null mutant (Figure 5B), indicating that Kei1 has an indispensable role related to Aur1.

The strong suppressor activity of *AUR1* suggests that the *kei1-1* mutant has defects in IPC synthesis. We therefore tested whether the *kei1-1* mutant has altered sensitivity to aureobasidin A, a specific inhibitor of IPC synthase (Nagiec *et al.*, 1997). The *kei1-1* mutant showed a strong hypersensitivity to this inhibitor (Figure 5C), which further supports the direct involvement of Kei1 in IPC synthesis.

When the total cell lysate was subjected to sucrose density gradient centrifugation, the distribution of Kei1C-GFP and

Figure 4. Kex2-cleaved Kei1 is localized to the medial-Golgi. (A) The lysate of cells expressing *KEI1*-GFP and *KRE2-3HA* (KSY714) was centrifuged at 10,000 $\times g$ for 10 min, and then the supernatant was subjected to 22–60% sucrose density gradient fractionation (see *Materials and Methods* for details). Each fraction was subjected to SDS-PAGE, and proteins were detected by Western blotting. The signal intensity of indicated proteins was quantified with ImageJ software and graphed with Excel. (B) Localization of Kei1 with C-terminal GFP tag (Kei1-GFP, top; KSY714) or Kei1 with N-terminal GFP tag (GFP-Kei1, bottom; KSY725) was shown with indirect immunofluorescence of the medial-Golgi protein Kre2-3HA (Al-exa568 anti-mouse). Bar, 5 μ m.

Aur1-3HA were almost identical (Supplemental Figure S1C). So, we next investigated the physical interaction of Kei1 with Aur1 *in vivo*. The lysate of *KEI1*-GFP *AUR1*-3HA or *GFP*-*KEI1* *AUR1*-3HA cells were treated with 1% Triton X-100 to solubilize the membrane. Aur1-3HA was immunoprecipitated with anti-HA antibody, and the GFP signal was analyzed. Cells expressing *KRE2-3HA* instead of *AUR1*-3HA were used as negative controls. As shown in Figure 6, A and B, both Kei1C-GFP and GFP-Kei1N were coprecipitated with Aur1-3HA, indicating that Kei1 and Aur1 form a complex *in vivo*. Coprecipitation of Kei1F-GFP or GFP-Kei1F with Aur1-3HA was much less efficient, but we reproducibly observed the coprecipitated band. This may indicate that Aur1 interacts with Kei1F that has not yet arrived in the late Golgi compartment where Kex2 cleaves Kei1.

Activity of the IPC Synthase Significantly Decreases in the *kei1-1* Mutant

The *in vivo* interaction of Kei1 and Aur1, in addition to the hypersensitivity to aureobasidin A of *kei1-1* cells, led us to suppose that Kei1 is essential for IPC synthase activity. Therefore, we tested whether the membrane preparation from the *kei1-1* mutant has any difference in the activity of

Figure 5. Isolation of *kei1-1*, a temperature-sensitive mutant allele of *KEI1* and identification of *AUR1* as a strong multicopy suppressor of *kei1-1* temperature sensitivity. (A) *KEI1* was introduced to *kei1-1* mutant on *CEN* plasmid and *AUR1* was introduced to *kei1-1* mutant on 2μ plasmid. pRS426 is the empty vector. Cells were spotted onto the YPD plates with $10\times$ serial dilution and incubated at 26.5 and 37°C. (B) *KEI1*/ Δ *kei1*:*kanMX4* diploid cells were introduced with empty vector pRS426, *KEI1* on *CEN* plasmid, or *AUR1* on 2μ plasmid, sporulated, and then the viability of the generated tetrads were analyzed. Four spores from a single ascus were vertically aligned. (C) BY4741 and *kei1-1* mutant (KSY271) were streaked onto the SC plates with or without 0.08 μ g/ml aureobasidin A.

IPC synthesis. We used C_6 -NBD-ceramide as a substrate of IPC synthase, because it has been widely used to assay IPC synthesis activity of yeast microsomal membrane in vitro (Zhong *et al.*, 1999; Levine *et al.*, 2000; Aeed *et al.*, 2004). Synthesis of NBD-IPC is completely blocked by aureobasidin A (Figure 7A, lane 1). When cells were grown at 26.5°C, no apparent difference was observed in the IPC synthesis activity at 26.5°C between the wild-type and *kei1-1* mutant membranes (Figure 7A, lanes 2 and 3). However, when the membrane was incubated at 37°C before the reaction, the

Figure 6. Kei1 is a subunit of the IPC synthase complex. (A) The lysate of KRE2-3HA *KEI1*-GFP (KSY714) or AUR1-3HA *KEI1*-GFP (KSY525) cells were treated with 1% Triton X-100 and centrifuged to prepare the supernatant (S) for immunoprecipitation with anti-HA antibody. U, unbound; B, bound materials. Material derived from 10-fold more cells was loaded for sample B than for samples S and U. (B) Immunoprecipitation was performed from the cleared lysate of KRE2-3HA GFP-*KEI1* (KSY725) or AUR1-3HA *KEI1*-GFP (KSY721) cells and analyzed as described in A.

activity of mutant membrane was reduced (lanes 4 and 5). Furthermore, when the reaction was carried out at 37°C, it was greatly reduced (lanes 8 and 9). Pre-incubation of the cells for 2 h at 37°C before collection also reduced the IPC synthesis activity of the mutant membrane at 26.5°C (lanes 6 and 7), and the membrane had no detectable activity at 37°C (lanes 10 and 11). Therefore, the *kei1-1* mutant has heat-labile IPC synthesis activity.

It is yet unclear whether the specific activity of the IPC synthase was actually reduced in the *kei1-1* mutant, because the amount of Aur1-GFP concomitantly decreased in the *kei1-1* membrane, although that of Van1 was not affected (Figure 7A). To avoid this problem and to directly assess the activity of IPC synthase, the enzyme was isolated by immunoprecipitation of Aur1-3HA with anti-HA antibody. As shown in Figure 7B, the immunoprecipitates from the lysate of AUR1-3HA cells converted C_6 -NBD-ceramide to NBD-IPC in a dose-dependent manner (lanes 4–6). However immunoprecipitates from the lysate of KRE2-3HA cells produced no detectable NBD-IPC (lanes 1–3), which confirms the validity of the IPC synthase assay system. Therefore we then assessed the IPC synthase activity isolated from the *kei1-1* mutant cells. As a result, it showed no detectable IPC synthesis activity (lanes 10–12), even though the cultivation of cells and the IPC synthesis reaction were carried out at the permissive temperature, and the amount of Aur1-3HA protein in the *kei1-1* sample was not less than that in the wild-type sample (lanes 7–12, immunoblots). The *kei1-1* IPC synthase may be very labile in the absence of membrane lipids and loses its activity during the solubilization and immunoprecipitation steps.

Figure 7. Kei1 is essential for the activity of IPC synthase. (A) *AUR1-3HA*-expressing cells (KSY512 and KSY513) were grown at 26.5°C and further incubated at indicated temperatures for 2 h. Lysate was prepared from the cells, and membrane fraction was recovered by ultracentrifugation. Membrane fractions were preincubated at the indicated temperatures, after which the reaction was performed at the indicated temperatures for 1 h. (B) Immunoisolation with anti-HA was performed from cells expressing *KRE2-3HA* (THY4-2) or *AUR1-3HA* (KSY525 and KSY530) grown at 26.5°C, and the activity of IPC synthase on the beads at 26.5°C for 1 h was tested. The product NBD-IPC was detected on the TLC by LAS-1000plus. The proteins on the beads were monitored by Western blotting. (C) Immunoisolation with anti-HA was performed from *KEI1 AUR1-3HA* (KSY771), *kei1^{F103I} AUR1-3HA* (KSY773), or *kei1^{ΔC} AUR1-3HA* (KSY775) cell lysate, and the activity of IPC synthase was determined as described in B. (D) Immunoprecipitation of Aur1-3HA was performed from the cleared lysate of *KEI1-GFP AUR1-3HA* (KSY792) or *kei1^{F103I}-GFP AUR1-3HA* (KSY794) cells, and the indicated proteins were monitored by Western blotting. S, supernatant; U, unbound; and B, bound. Material derived from 10-fold more cells was loaded for sample B than for samples S and U. (E) Immunoprecipitation of Aur1-3HA was performed from the cleared lysate of *GFP-KEI1 AUR1-3HA* (KSY799) or *GFP-kei1^{F103I} AUR1-3HA* (KSY801) cells and analyzed as described in D.

We next tested which of the two mutations of *kei1-1* is responsible for the loss of IPC synthase activity. Whereas the IPC synthase from *kei1^{ΔC}* cells showed no significant reduction of the IPC synthase activity than that from *KEI1* cells (Figure 7C, lanes 1–3 and 7–9), the activity of the IPC synthase from *kei1^{F103I}* cells was significantly decreased (lanes 4–6). This result clearly indicates that the F103I replacement is responsible for inactiveness of IPC synthase isolated from *kei1-1* cells.

The inactivation of isolated IPC synthase by the F103I replacement is surprising because *kei1^{F103I}* cells show no

apparent growth defect (Supplemental Figure S2A). To examine whether the activity of the IPC synthase of the *kei1^{F103I}* cells is also significantly affected in vivo, we assayed conversion of C₆-NBD-ceramide to NBD-IPC by the cells as described by Levine *et al.* (2000). As a result, no significant decrease in NBD-IPC production in *kei1^{F103I}* cells was observed (Supplemental Figure S4), suggesting that the inactivation of IPC synthase from *kei1^{F103I}* cells occurs in the process of immunoisolation.

If Kei1 is an essential subunit for the IPC synthase activity, it is possible that Kei1^{F103I} may be released from Aur1-3HA

Figure 8. Aur1-GFP in *kei1-1* cells is delivered to and degraded in the vacuole. (A) The Aur1-GFP levels in wild type (lane 1; KSY512), $\Delta pep4$ (lane 2; KSY571), *kei1-1* (lane 3; KSY513), *kei1-1* $\Delta ubc7$ (lanes 4 and 5; KSY552, KSY553), *kei1-1* $\Delta doa10$ (lanes 6 and 7; KSY554, KSY561), *kei1-1* $\Delta hrd1$ (lanes 8 and 9; KSY559, KSY560), *kei1-1* $\Delta ubx2$ (lanes 10 and 11; KSY562, KSY563), and *kei1-1* $\Delta pep4$ (lane 12; KSY572) cells were compared. Two independent isolates of ERAD mutants were used and cells were incubated at 26.5°C. Similar results were obtained after further incubation at 37°C for 2 h (data not shown). (B) The localization of Aur1-GFP was observed by fluorescence microscopy at the left panels. Nomarski images are shown at the right, and the merge of fluorescence and Nomarski images at the middle. The wild-type (KSY512), $\Delta pep4$ (KSY571), *kei1-1* (KSY513), and *kei1-1* $\Delta pep4$ (KSY572) cells were incubated at 26.5°C. (C) The localization of Kre2-3HA in the *KEI1* (KSY790) or *kei1-1* (KSY784) cells was observed by fluorescence microscopy. Cells were incubated at 26.5°C and then at 26.5 or 37°C for 2 h. Bars, 5 μ m.

during immunoprecipitation, which may result in enzyme inactivation. To test this possibility, we investigated the association of Kei1^{F103I} to Aur1-3HA on the beads. Aur1-3HA was immunoprecipitated from *AUR1-3HA KEI1-GFP* cells or *AUR1-3HA kei1^{F103I}-GFP* cells as in Figure 7C, and the amount of bound proteins were analyzed by Western blotting. As expected, a significant amount of Kei1-GFP was found in the bound fraction (Figure 7D, lane 5), but the signal of Kei1^{F103I}-GFP in bound fraction could be hardly detected (Figure 7D, lane 6). Similar result was obtained in the case of GFP-Kei1^{F103I}N (Figure 7E). These results demonstrate that Aur1 alone does not have IPC synthase activity and that Kei1 is indeed the essential subunit of IPC synthase for its activity.

The Golgi Localization of Aur1 Is Diminished in *kei1-1* Mutant and Aur1 Is Degraded in the Vacuole

As shown in Figure 7A, the amount of Aur1-GFP protein had decreased in *kei1-1* cells, even when they grew at 26.5°C and almost an equal amount of Van1 was found in the membrane (lanes 2 and 3). When cells were grown at 37°C

for 2 h, the amount of Aur1-GFP in *kei1-1* cells decreased further (lanes 6 and 7). These results suggest that degradation of Aur1 occurred in the *kei1-1* mutant. There are two possible explanations for this. One explanation is that the newly produced Aur1 accumulates in the ER and is subsequently degraded by ER-associated degradation (ERAD) pathway. The other explanation is that the Golgi localization of Aur1 is unstabilized, and Aur1 is degraded in the vacuole. To determine which possibility actually occurs in the *kei1-1* mutant, we deleted genes involved in the ERAD pathway or protein degradation in the vacuole. Deletion of genes encoding Ubc7 (ubiquitin-conjugating enzyme E2; Bays *et al.*, 2001), Doa10 (ubiquitin ligase E3; Carvalho *et al.*, 2006), Hrd1 (ubiquitin ligase E3; Bays *et al.*, 2001), or Ubx2 (membrane protein that recruits AAA ATPase Cdc48 to substrate proteins; Neuber *et al.*, 2005; Schubert and Buchberger, 2005) did not result in any stabilization of Aur1-GFP in the *kei1-1* mutant (Figure 8A, lanes 4–11). Aur1-GFP was restored to the wild-type level by deletion of *PEP4*, which encodes a master protease of the vacuole (Van Den Hazel *et al.*, 1996) (Figure 8A, lane 12). This result suggests that Aur1 is unable

Figure 9. The C-terminal region of Kei1 is important for stable Golgi localization of Kei1 and Aur1. (A) *PEP4* (+) or *Δpep4* (−) with *KEI1*-GFP (+: KSY696, −: KSY732), *kei1-1*-GFP (+: KSY698, −: KSY780), *kei1^{ΔC}*-GFP (+: KSY694, −: KSY730), and *kei1^{F103I}*-GFP (+: KSY692, −: KSY728) cells were incubated at 26.5°C. The amounts of Kei1F-GFP and Kei1C-GFP were analyzed by Western blotting. Lanes 1–6 were tested on the same gel and membrane and duplicated lanes were removed. The same applies to lanes 7–10. (B) *PEP4* (+) or *Δpep4* (−) with GFP-*KEI1* (+: KSY723, −: KSY765) and GFP-*KEI1^{F103I}* (+: KSY753, −: KSY767) cells were incubated at 26.5°C. The amounts of GFP-Kei1F and GFP-Kei1N were analyzed by Western blotting. Lanes 1–4 were tested on the same gel and membrane and duplicated lanes were removed. (C) The amounts of Aur1-3HA in the *KEI1*-GFP (*KSY771*), *kei1^{F103I}*-GFP (*KSY773*) and *kei1^{ΔC}*-GFP (*KSY775*) cells were analyzed by Western blotting. Duplicate samples were tested. (D) GST or GST-fusion protein of C terminus of Kei1 (GST-Kei1CT) expressed in *E. coli* was purified and mixed with the yeast cell lysate. Bound proteins were precipitated with glutathione-Sepharose and detected by Western blotting with indicated antibodies to α-COP and γ-COP. Phosphoglycerate kinase (PGK) was also detected as a negative control.

to stably localize to the Golgi in the absence of wild-type Kei1, and consequently it tends to be delivered to and degraded in the vacuole. To verify that Aur1 is transported to the vacuole in *kei1-1* cells, we observed the localization of Aur1-GFP in *kei1-1* cells at the permissive temperature. In the *KEI1* cells, regardless of the presence or absence of *PEP4*, Aur1-GFP displayed the punctate Golgi patterns (Figure 8B), which is consistent with the previous observation that showed Aur1-3HA localizes to the medial-Golgi (Levine *et al.*, 2000). In the *kei1-1 PEP4* cells, the fluorescence of Aur1-GFP was obscure and Golgi pattern could be hardly detected (Figure 8B); in the *kei1-1 Δpep4* cells, Aur1-GFP was accumulated in the vacuole (Figure 8B, bottom). In contrast, Kre2-3HA, a control medial-Golgi protein that shows similar punctate immunofluorescence pattern, was not affected by *kei1-1* at either temperature (Figure 8C). From these results, we concluded that the stable Golgi localization of Aur1 is compromised in *kei1-1* cells and a fraction of Aur1 is delivered to the vacuole and degraded in a *Pep4*-dependent manner. It may be worth noting that, when GFP-fusion proteins are mislocalized and degraded in the vacuole, the fluorescence signal of the GFP remnants remains detectable in many cases (Reggiori and Pelham, 2002; Sato and Nakano, 2002; Valdez-Taubas and Pelham, 2005). However, in this case the GFP remnants also seemed to be digested.

Deletion of the C Terminus of Kei1 Causes Mislocalization of Kei1 and Aur1

How does Kei1 contribute to the stable localization of Aur1 to the Golgi? To address this question, we first tested whether Golgi localization of Kei1 is also unstable due to the *kei1-1* mutant. Kei1-1-GFP was used in this experiment. To make the construct of Kei1-1-GFP, the nucleotide sequence was manipulated so that F103I, Q193K, and K194T replacements were introduced in the Kei1 sequence, and GFP was directly fused to the ¹⁹⁴Thr residue in Kei1. Kei1-1-GFP was produced in cells whose genomic *KEI1* were replaced with

kanMX4, and the amounts of Golgi-localized Kei1-1C-GFP in *PEP4* and *Δpep4* cells were compared. If Kei1-1C-GFP is unable to stay in the Golgi and is delivered to the vacuole like Aur1 in *kei1-1* cells, the amount of Kei1-1C-GFP should be restored by the deletion of *PEP4*. As expected, the amount of Kei1-1C-GFP was significantly increased by *Δpep4* (Figure 9A, lanes 1 and 2), whereas the amount of wild-type Kei1C-GFP was not affected by the deletion of the *PEP4* gene (Figure 9A, lanes 5 and 6). From this result, we conclude that Kei1-1C itself is unable to stably localize in the Golgi and is delivered to the vacuole.

To test whether either the F103I substitution or ΔC truncation causes loss of Golgi localization, we checked the amount of GFP-Kei1^{F103I}N and Kei1^{ΔC}-GFP in *PEP4* and *Δpep4* cells. The amount of GFP-Kei1^{F103I}N in *Δpep4* cells was slightly increased compared with that in *PEP4* cells (Figure 9B, lanes 1 and 2); however, this increase was also observed in GFP-Kei1N (Figure 9B, lanes 3 and 4), which suggests that even the wild-type Kei1 is constitutively degraded in a *Pep4*-dependent manner when GFP is fused to its N terminus. Therefore it is unlikely that F103I substitution affects the Golgi localization. In contrast, the amount of Kei1^{ΔC}-GFP significantly increased when *PEP4* was disrupted (Figure 9A, lanes 3 and 4), although that of Kei1^{F103I}C-GFP or Kei1C-GFP did not (Figure 9A, lanes 5–10). These results indicate that the C-terminal region of Kei1 is important for localization to the Golgi. The observation that the amount of Aur1-3HA in *kei1^{ΔC}* cells was less than that in *KEI1* and *kei1^{F103I}* cells (Figure 9C) also supported the importance of the Kei1 C-terminal region in its Golgi localization.

Cytosolic C-Terminal Peptide of Kei1 Can Interact with Coat Protein (COPI) Coatomer

Kex2 exerts its protease activity in the lumen of the Golgi; therefore, the Kex2 recognition site of Kei1, the ¹³⁵Arg residue, should also locate in the lumen. Because there is only

one hydrophobic segment between ¹³⁵Arg and the C terminus, the C terminus of Kei1 is very likely to be exposed to the cytoplasm. Many cases are known, where the cytoplasmic C-terminal peptide of the ER and Golgi membrane proteins interacts with coatamer, which is responsible for retrograde trafficking in the secretory pathway. For example, the dilysine motif KKXX or KKKXX binds to coatamer directly and supports the incorporation of proteins with this motif into the COPI vesicle (Jackson *et al.*, 1990; Cosson and Letourneur, 1994). Furthermore, although the dilysine motif does not exist in Rer1, Emp24, or Vrg4, their relatively basic C-terminal tails interact with coatamer and are important for their Golgi localization (Belden and Barlowe, 2001; Sato *et al.*, 2001; Abe *et al.*, 2004). We hypothesized that Kei1 also interacts with coatamer through its C-terminal tail like these proteins, and tested this by GST pull-down assay. The peptide from ¹⁷⁶Gln to ²²¹Glu of Kei1 was fused to the C terminus of GST. The fusion protein was produced in *E. coli* and purified with glutathione-Sepharose affinity beads. The beads were mixed with the yeast cell lysate, and proteins that bound to the beads were analyzed. As shown in Figure 9D, we found that the coatamers, α -COP Ret1 and γ -COP Sec21, bound to the C-terminal peptide of Kei1. Phosphoglycerate kinase, a negative control, was not detected. This result suggests that Kei1 helps the medial-Golgi localization of IPC synthase in a COPI vesicle-dependent manner.

DISCUSSION

Genetic and biochemical approaches using temperature-sensitive mutants provide effective clues to elucidate the biological roles of unknown essential proteins, as we reported previously on the study of Pga1, a novel subunit of GPI-mannosyltransferase II (Sato *et al.*, 2007). The *kei1-1* mutant, which was obtained by our screening for temperature-sensitive mutants of *KEI1*, has a F103I substitution and Δ C truncation, and both are required for the temperature sensitivity. The F103I substitution seems to be silent in the cell, and the mutant IPC synthase has enough activity to supply IPC for cell growth. However, Kei1^{F103I} is released from Aur1 during immunoprecipitation and abolishes IPC synthase activity. This clearly shows that Aur1 alone is unable to catalyze IPC synthesis. The Δ C truncation disturbs Golgi localization and causes degradation of the enzyme in the vacuole. Binding of the C-terminal peptide to coatamer suggests that Kei1 may have a role to incorporate IPC synthase in the COPI vesicles for recycling. Thus F103I and Δ C have different effects on Kei1 protein, and when combined, synergistically cause temperature sensitivity. We conclude that Aur1 requires Kei1 both for IPC synthase activity and correct localization to the Golgi.

The finding that Kex2 cleaves Kei1 (Figure 3) led us to hypothesize that IPC synthase activity is regulated through its proteolytic processing. However, contrary to this, no apparent difference has been found in the activity, the subunit interaction, or the localization of IPC synthase (our unpublished data). Therefore, we have found no evidence that the Kex2 cleavage regulates IPC synthesis. Although Kei1 sequence is highly homologous to its fungal counterparts, the hydrophilic region that has the ¹³⁵Arg cleavage site is not conserved (Figure 2A). Arginine is only found in *Candida glabrata*, and no basic residue is found in *Vanderwaltozyma polyspora*. We suggest that generation of Kex2 cleavage site in *S. cerevisiae* Kei1 was an accidental event in its evolution without specific advantages or disadvantages in IPC synthesis.

Although the cleavage of Kei1 by Kex2 does not seem to be biologically important, it has allowed us to investigate the localization of IPC synthase in detail. Most Kei1 in the Golgi fractions has been cleaved by the Kex2 protease that resides in the late Golgi compartment, although Kei1 is mostly found in the medial-Golgi (Figure 4). This suggests that Kei1 is temporarily transported to the late Golgi whereby it is cleaved by Kex2. Kei1 is then efficiently transported retrograde to the medial-Golgi. Because Kei1C-GFP was hardly detected in Sed5-rich fractions when the Golgi compartments were fractionated by sucrose density gradient centrifugation (Figure 4A), Kei1 is likely to recycle between the medial- and late Golgi in a COPI-dependent manner. This idea is consistent with the previous report that Aur1 does not recycle back to the ER in secretion block experiments (Levine *et al.*, 2000). In mammalian cells, sphingomyelin (SM) synthase 1, which produces SM from newly synthesized ceramide, localizes to the *trans*-Golgi network (Huitema *et al.*, 2004). Ceramide transfer protein (CERT) directly transfers newly synthesized ceramide from the ER to the site of SM synthesis in the *trans*-Golgi network (Hanada *et al.*, 2003). As it has been reported that ceramide is transferred from the ER to the Golgi by both vesicular and nonvesicular transport in yeast (Funato and Riezman, 2001), it is possible that an unidentified functional homologue of CERT carries ceramide from the ER to the medial-Golgi. However, no protein homologous to CERT in sequence has been found thus far.

We identified *GDA1*, *PSD1*, and *SFH1* as multicopy suppressors of the *kei1-1* mutant. Their suppressor activities were obviously lower than *AUR1* but were both reproducible and significant. Currently, the mechanism of the suppression is not clear, but sphingolipid metabolism may be somehow involved. *GDA1* encodes a GDPase that converts guanosine diphosphate (GDP) to guanosine monophosphate after mannose is transferred to the substrate of mannosyltransferases in the Golgi, and this conversion is required for the transport of cytosolic GDP-mannose into the Golgi lumen (Abeijon *et al.*, 1993; Berninsone *et al.*, 1994). We suppose that the multicopy expression of *GDA1* may activate the sphingolipid synthetic pathway in the Golgi by increasing the supply of GDP-mannose, the substrate of MIPC synthase. *PSD1* encodes a phosphatidylserine decarboxylase of the mitochondrial inner membrane, which converts phosphatidylserine to phosphatidylethanolamine (Clancey *et al.*, 1993). No relationship between *Psd1* and Kei1 has been reported, but it is possible that the mitochondrial lipid composition affects the lipid homeostasis of the Golgi or that the reduction of IPC impairs the function of mitochondria and *Psd1* repairs it. *Sfh1* is one of five homologues of the lipid transfer protein Sec14 in *S. cerevisiae* and has the highest similarity but the lowest functional relationship with Sec14. Moreover, *Sfh1* lacks the lipid transfer activity in vitro; therefore, the molecular function of *Sfh1* in vivo is unclear (Li *et al.*, 2000). However, the multicopy suppressor activity of *SFH1* may indicate that *Sfh1* is actually involved in lipid metabolism. Previous investigations using DNA microarray analysis revealed that *SFH1* gene expression was significantly enhanced by various stress conditions (Griac, 2007). Considering that a number of studies demonstrated that sphingolipids are important for stress responses, *Sfh1* may regulate genes related to sphingolipid metabolism. Although this hypothesis has not been examined, the observation of *Sfh1* localization to the nucleus and Golgi (Schnabl *et al.*, 2003) suggests a possible role of *Sfh1* that links the lipid composition in the Golgi membrane and the regulation of gene expression.

Concerning the role of sphingolipids in the stress response, interesting findings are reported in experiments using a *Δlcb1 slc1-1* double mutant that has no sphingolipids but produces mannosylated phosphatidylinositol derivatives containing a C26 fatty acid (Dickson *et al.*, 1990; Patton *et al.*, 1992; Lester *et al.*, 1993). This mutant can grow normally in adequate growth conditions, but the growth is significantly inhibited at high temperature or low and high pH, indicating that cells cannot adapt to such immoderate environments without sphingolipids. These results demonstrate that ceramides are a key mediator of signaling in the stress response. IPC synthase activity can define the balance of ceramide and complex sphingolipids; therefore, it should be regulated stringently in response to stimuli caused by intracellular and environmental changes. Kei1 is responsible for both the localization and the activity of IPC synthase, which is an ideal feature to be a regulatory subunit. Interestingly, we observed that the amount of Kei1 was significantly increased in *kei1-1* cells compared with wild-type cells (our unpublished data), suggesting that cells up-regulate *KEI1* in response to the decreasing amounts of IPC or the increase of ceramide. This hypothesis is currently under investigation.

Because tagging 3HA to the C terminus of Aur1 affected the ratio of Kei1C to Kei1F (Figure 6), it is possible that Aur1 is responsible for in the transport of Kei1. Indeed, our preliminary data show that Kei1 accumulates in the ER by reduction of Aur1 in promoter shut-off experiments, suggesting that the exit of IPC synthase from the ER to the Golgi requires Aur1–Kei1 complex formation. It must be interesting to investigate further how cells regulate the production, complex formation, and localization of Aur1 and Kei1.

ACKNOWLEDGMENTS

We thank Drs. Satoshi Kagiwada, Pierre Cosson, and R. Rainer Duden for kindly providing antisera; Dr. Randy Schekman for kindly providing a yeast strain; Kentaro Kajiwaru for valuable advice in the analysis of aureobasidin A sensitivity; Dr. Hiroyuki Adachi for helpful discussions; and Dr. Ricardo Costa and Christopher J. Noakes for critical reading of the manuscript. This work was supported by a grant-in-aid for JSPS Fellows from the Japan Society for the Promotion of Science (to K. S.), grants-in-aid for Scientific Research from the Japan Society for the Promotion of Science (to Y. N. and K. Y.), and a grant from the RIKEN Institute (to K. Y.) and grant from the Noda Institute of Scientific Research (to K. Y.).

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