The C-terminal Cytosolic Region of Rim21 Senses Alterations in Plasma Membrane Lipid Composition

INSIGHTS INTO SENSING MECHANISMS FOR PLASMA MEMBRANE LIPID ASYMMETRY

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Yeast responds to alterations in plasma membrane lipid asymmetry and external alkalization via the sensor protein Rim21 in the Rim101 pathway. However, the sensing mechanism used by Rim21 remains unclear. Here, we found that the C-terminal cytosolic domain of Rim21 (Rim21C) fused with GFP was associated with the plasma membrane under normal conditions but dissociated upon alterations in lipid asymmetry or external alkalization. This indicates that Rim21C contains a sensor motif. Rim21C contains multiple clusters of charged residues. Among them, three consecutive Glu residues (EEE motif) were essential for Rim21 function and dissociation of Rim21C from the plasma membrane in response to changes in lipid asymmetry. In contrast, positively charged residues adjacent to the EEE motif were required for Rim21C to associate with the membrane. We therefore propose an “antenna hypothesis,” in which Rim21C moves to or from the plasma membrane and functions as the sensing mechanism of Rim21.

In the plasma membrane lipid bilayer, lipid molecules are distributed unevenly between the inner (cytoplasmic) and outer (extracellular) leaflets. Phosphatidylserine (PtdSer) and phosphatidylethanolamine are mostly confined to the inner leaflet, whereas complex sphingolipids are enriched in the outer leaflet (1, 2). This phenomenon is called lipid asymmetry and is a common feature of the plasma membrane of eukaryotic cells. Lipid asymmetry does not come about spontaneously but is generated and regulated by ATP-dependent inward (flip) and outward (flop) trans-bilayer movements of lipid molecules, which are catalyzed by flippases and floppases, respectively (3–7).

Regulation of lipid asymmetry plays a central role in a great variety of cellular events, such as generation of membrane potential, vesicular transport, polarized cell growth, cell migration, blood coagulation, and removal of apoptotic cells (8–14). Yeast cells have five flippases in the P4-ATPase family (Dnf1, Dnf2, Dnf3, Dnf4, and Dnf5), and simultaneous deletion of the NEO1 gene has been deleted are inviable (4), and simultaneous deletion of the other four flippase genes is also lethal (13). These results highlight the essential role of lipid asymmetry in cell viability. In humans, impairment of the capacity to properly regulate lipid asymmetry is implicated in several diseases, such as intrahepatic cholestasis, Stargardt macular dystrophy, and Scott syndrome (9, 15–17).

In the yeast Saccharomyces cerevisiae, alterations in lipid asymmetry caused by defective flip and/or flop functioning in phospholipids led to activation of the Rim101 pathway (18), a signal transduction pathway that was first identified as an alkaline-responsive pathway (19). In the Rim101 pathway, a plasma membrane protein, Rim21, acts as the sensor protein for both altered lipid asymmetry and external alkalization (20). However, how this single sensor can sense both altered lipid asymmetry and external alkalization remains an enigma. Upon activation of the Rim101 pathway, expression of Rim8 and Rim21, which are involved in the flip/transport of sphingolipid-long chain bases and phospholipids, respectively, is induced (18, 21–23).

Rim21 transduces the signal to downstream proteins in the Rim101 pathway, such as an arrestin-related protein Rim8, ESCRT proteins, a Bro1 domain-containing protein Rim20, and a calpain-like protein Rim13, at the plasma membrane (24–28). Rim13 then causes proteolytic activation of the transcription factor Rim101.

Rim21 is thought to span the membrane seven times, exposing its N and C termini to the extracellular and cytosolic space, respectively (20) (see Fig. 1A). Rim21 does not have any regions that exhibit sequence similarity with known domains. The C-terminal cytosolic region of Rim21 (Rim21C) is unique in that charged amino acid residues are highly enriched (see Fig. 1A). In the present study, we demonstrate that Rim21C senses altered lipid asymmetry and external alkalization. In addition, we identify motifs important for this sensing function using a systematic mutational analysis. From these results, we proposed an “antenna hypothesis” for the sensing mechanism employed by Rim21.

Experimental Procedures

Yeast Strains and Media—The S. cerevisiae strains used in this study are listed in Table 1. Yeast cells were grown at 30°C to log phase in YPD (1% yeast extract, 2% bactopeptone, 2% d-glucose).
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| Yeast strains used in this study | Genotype | Source |
|---------------------------------|----------|--------|
| **Strain**                      | **Genotype** | **Source** |
| SEY6210                         | MATα his3 leu2 ara3 trp1 lys2 susC | Ref. 42 |
| YOK2027                         | SEY6210, rim21Δ::KanMX4             | Ref. 20 |
| YOK2543                         | SEY6210, pepΔ LEU12 prb1Δ NatNT2 DFG16-FLAG::KanMX6 | This study |
| YOK3073                         | SEY6210, Ppox1-1 Orf11-1-Myc::URA3 | Ref. 24 |
| YOK3208                         | SEY6210, RIM21-2×GFP::KanMX4        | Ref. 20 |
| YOK3249                         | SEY6210, RIM20-2GFP::TRP1 rim21Δ::KanMX4 | This study |
| YOK3395                         | SEY6210, Ppox1-1 GEP::RIM21C::URA3  | This study |
| YOK3396                         | SEY6210, Ppox1-1 GEP::RIM21C::URA3 lem3Δ::KanMX4 | This study |
| YOK3397                         | SEY6210, Ppox1-1 GEP::RIM21C::URA3 pepΔ::KanMX4 | This study |
| YOK3398                         | SEY6210, Ppox1-1 GEP::RIM21C::URA3 choΔ::KanMX4 RSB1-HA::TRP1 | This study |
| YOK3399                         | SEY6210, Ppox1-1 GEP::RIM21C::E335A E335A::URA3 | This study |
| YOK3402                         | SEY6210, Ppox1-1 GEP::RIM21C::E335A E335A::URA3 choΔ::KanMX4 RSB1-HA::TRP1 | This study |
| YOK3405                         | SEY6210, Ppox1-1 GEP::RIM21C::E335A E335A::URA3 lem3Δ::NeoNT2 | This study |
| YOK3437                         | SEY6210, Ppox1-1 GEP::RIM21C::E338A R339A K340A E341A E342A E353A E354A E355A::URA3 | This study |
| YOK3441                         | SEY6210, Ppox1-1 GEP::RIM21C::URA3 HSP104-RedStar2::NatNT2 lem3Δ::KanMX4 | This study |
| YOK3684                         | SEY6210, Ppox1-1 Orf11-1-Myc::URA3 NEO1-FLAG-AID::KanMX6 | This study |
| YOK3689                         | SEY6210, Ppox1-1 Orf11-1-Myc::URA3 NEO1-FLAG-AID::KanMX6 | This study |
| YOK3700                         | SEY6210, Ppox1-1 GEP::RIM21C::URA3 dnf3Δ::KanMX4 | This study |
| YOK3706                         | SEY6210, pepΔ LEU12 prb1Δ NatNT2 DFG16-FLAG::KanMX4rim21Δ::TRP1 rim21Δ::URA3 | This study |
| YOK3762                         | SEY6210, pepΔ LEU12 prb1Δ NatNT2 DFG16-FLAG::KanMX4rim21Δ::TRP1 rim21Δ::URA3 | This study |
| YOK3764                         | SEY6210, pepΔ LEU12 prb1Δ NatNT2 rim21Δ::TRP1 rim21Δ::URA3 | This study |
| YOK3791                         | SEY6210, Ppox1-1 GEP::RIM21C::E338A R339A K340A E341A E342A E343A E344A E345A::URA3 | This study |
| YOK3797                         | SEY6210, Ppox1-1 GEP::RIM21C::E338A R339A K340A E341A E342A E343A E344A E345A::URA3 lem3Δ::KanMX4 | This study |
| YOK3842                         | SEY6210, rim21Δ::KanMX4 RIM21-C3-2×GFP::URA3 | This study |
| YKN58                           | SEY6210, Ppox1-1 GEP::RIM21C::URA3 dnr2Δ::KanMX4 | This study |
| YKN150                          | SEY6210, Ppox1-1 GEP::RIM21C::E338A R339A K340A E341A E342A E343A E344A E345A::URA3 | This study |
| YKN152                          | SEY6210, pepΔ LEU12 prb1Δ NatNT2 rim21Δ::TRP1 rim21Δ::URA3 | This study |

 cose) or synthetic complete (SC) medium (2% D-glucose and 0.67% yeast nitrogen base without amino acids, and appropriate supplements). Alkaline treatment was performed by adding 1 M Tris-HCl (pH 8.0) to the culture medium at a final concentration of 100 mM.

Genetic Manipulation and Plasmid Construction—Gene disruption was performed by replacing the entire coding region of the gene with a marker gene. Integration of the P<sub>ADH</sub>-GFP-RIM21C to the yeast chromosome was performed as follows. The P<sub>ADH</sub> yEGFP sequence was amplified by PCR from pYM-N9 (29) such that the SacI and EcoRI sites were at the 5’ and 3’ ends, respectively, and a guanine was inserted after the yEGFP coding sequence to adjust the coding frame. The RIM21C sequence was amplified using PCR such that the EcoRI and Xhol sites were at the 5’ and 3’ ends, respectively. After digestion of their 5’ and 3’ ends with the respective restriction enzymes, these fragments were cloned together into the SacI-XhoI site of p416CYC1 (30), generating pOK553. The SacI-KpnI fragment of pOK553 was excised and cloned into the SacI-KpnI site of pRS306 and pRS304 (31), creating pOK577 and pOK599, respectively. Introduction of point mutations was performed using the QuickChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). We then linearized pOK577 and pOK599 via digestion by Stul and HindIII, respectively, and inserted them into the URA3 and TRP1 loci, respectively.

Chromosomal fusion of the AID tag to the C terminus of Neo1 was performed using PCR-based gene disruption and modification (32). The FLAG-AID, the ADH1 terminator, and a marker sequence were amplified by PCR from the pFA6a vector series (32) with a primer set specific to NEO1. The amplified fragment was inserted into the chromosome by means of homologous recombination.

We constructed a RIM21-C3-2×GFP strain as follows. The RIM21-2×GFP sequence, including the RIM21 promoter and ADH termination, was amplified using PCR from the genomic DNA of YOK3208 (RIM21-2×GFP) (20). The Xhol sites were at both the 5’ and the 3’ ends, and the PCR product was cloned into the Xhol site of pRS306, generating pOK692. The C3 mutation was introduced into pOK692 using the QuickChange site-directed mutagenesis kit, creating pOK693. We then linearized pOK693 via digestion by Stul and inserted it into the URA3 locus of YOK2027 (rim21Δ) (20). Successful construction of the plasmids was confirmed by sequencing. Successful deletion of the genes and tagging were confirmed by genomic PCR, immunoblot analysis and/or fluorescence microscopy. The plasmid for the expression of HA-Rim101 (pFI1) was given to us by Dr. T. Maeda (University of Tokyo, Tokyo, Japan).

Co-immunoprecipitation—Cells were cultured in YPD medium to log phase, harvested, suspended in lysis buffer (50 mM HEPES-NaOH, pH 8.0, 150 mM NaCl, 5 mM MgCl₂, 1 mM dithiothreitol, 1 mM PMSF, and EDTA-free protease inhibitor mixture (Complete; Roche Diagnostics, Indianapolis, IN)), and broken by mixing vigorously with glass beads at 4 °C for 10 min. The cell lysates were sonicated and centrifuged at 5,000 g for 3 min to remove cell debris. After treatment with 1% Triton X-100 at 4 °C for 1 h, samples were centrifuged at 100,000 × g for 30 min. The supernatant was then incubated with anti-FLAG M2 agarose beads (Sigma) while rotating, and maintained at 4°C for 90 min. The beads were washed three times with lysis buffer containing 0.1% Triton X-100. The bound proteins were eluted with SDS sample buffer and subjected to immunoblot analysis.

Immunoblot Analysis—Proteins were separated by SDS-PAGE and transferred to Immobilon™ polyvinylidene difluoride membrane (Millipore, Billerica, MA), as described previously (33). The membrane was incubated with anti-HA (TANA2; Medical & Biological Laboratories, Nagoya, Japan), anti-FLAG (M2; Stratagene), or anti-Pgk1 (Life Technologies, Inc.) antibody. Signal detection was performed using Western Lightning ECL Pro system (PerkinElmer Life Sciences) with a bioimaging analyzer (LAS4000; Fuji Photo Film) or x-ray film.
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To detect Rim21-HA, proteins were deglycosylated as follows. The prepared total cell lysates were diluted with four volumes of endoglycosidase H buffer (62.5 mM sodium citrate, pH 5.5, and 1.25 mM PMSF) and incubated with 20 units/μl endoglycosidase H (Endo Hf; New England Biolabs, Beverly, MA) at 37 °C for 10 min. then treated with an appropriate volume of 4 M guanidine-HCl and 1.25 mM PMSF and incubated with 20 units/μl endoglycosidase H (Endo Hf; New England Biolabs, Beverly, MA) at 37 °C for 1 h with occasional mixing by tapping. The samples were stored at −80 °C for at least 30 days before use.

Microscopic Observation—Cells were grown to mid-log phase (A600nm = 1.2–2.0) and subjected to microscopic observation before and after alkaline treatment. Fluorescence of GFP was visualized under a fluorescence microscope (DM5000B; Leica Microsystems, Wetzlar, Germany) equipped with a cooled CCD camera (DFC365FX; Leica Microsystems) controlled with LAS AF software (version 2.60; Leica Microsystems). The images were archived using Photoshop CS3 (Adobe, San Jose, CA). Rim21–2×GFP and Rim21–C3–2×GFP cells were observed under a fluorescence microscope (IX-81; Olympus, Tokyo, Japan) equipped with an electron-multiplying CCD camera (ImageEM, C9100-13; Hamamatsu Photonics, Hamamatsu, Japan), controlled with MetaMorph software (Molecular Devices, Sunnyvale, CA). In some cases, a linear adjustment was applied to enhance the image contrast using the level adjustment function of Photoshop.

Quantification of Fluorescence—All images were taken with the same exposure time, gain, and excitation intensity. The fluorescence of GFP-Rim21C was quantified using ImageJ software (National Institutes of Health) as follows. The bud neck and cytosol and the nucleus. Localization of GFP-Rim21C in the cytosol and the nucleus may be the result of the tendency of GFP to enter the nucleus.

Results

Rim21C Dissociates from the Plasma Membrane in Response to Altered Lipid Asymmetry—To gain insight into the molecular mechanism by which Rim21 senses alterations in lipid asymmetry, we first focused on the C-terminal cytosolic region of Rim21 (Rim21C), where charged amino acid residues are highly enriched (Fig. 1A). GFP fusion was fused to Rim21C (GFP-Rim21C), and its localization was monitored. Although Rim21C does not apparently contain transmembrane segments, GFP-Rim21C was primarily detected at the plasma membrane in WT cells (Fig. 1B). Interestingly, a strong signal was often detected at the bud neck, where lipid asymmetry is known to be altered locally, e.g. where phosphatidylethanolamine is exposed to the outer leaflet (34). Some of the GFP-Rim21C was dispersed in the cytosol, and its localization was monitored. Although Rim21C does not apparently contain transmembrane segments, GFP-Rim21C was primarily detected at the plasma membrane in WT cells (Fig. 1B). Interestingly, a strong signal was often detected at the bud neck, where lipid asymmetry is known to be altered locally, e.g. where phosphatidylethanolamine is exposed to the outer leaflet (34). Some of the GFP-Rim21C was dispersed in the cytosol and the nucleus. Localization of GFP-Rim21C in the nucleus may be the result of the tendency of GFP to enter the nucleus.

Because Lem3 is a regulatory subunit of the flippases Dfn1 and Dnf2, plasma membrane lipid asymmetry is altered in the nucleus. These observations suggest that Rim21 may sense altered lipid asymmetry, and that direct interaction of Rim21 with the plasma membrane may be involved in regulating plasma membrane lipid asymmetry in yeast. Further studies are needed to determine whether Rim21 interacts directly with the plasma membrane or whether it senses membrane lipid asymmetry through another mechanism.
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lem3Δ cells as a result of inactivation of Dnf1 and Dnf2 (35, 36). In lem3Δ cells, GFP-Rim21C completely dissociated from the plasma membrane and dispersed in the cytoplasm (Fig. 1B). A portion of GFP-Rim21C was detected as intense punctate signals, some of which were co-localized with Hsp104, a chaperone protein that refolds aggregated proteins, suggesting that excess GFP-Rim21C in the cytoplasm might sometimes form aggregates (Fig. 1C). Dissociation of GFP-Rim21 from the plasma membrane was also observed in deletion mutants for other flippase genes DRS2 and DNF3 (Fig. 1B).

For analysis of NEO1, which encodes another flippase essential for viability, we employed the auxin-inducible degron (AID) system in which proteins tagged with the AID tag can be degraded by the addition of a phytohormone auxin (37). We found that tagging Neo1 with the FLAG-AID tag itself caused a reduction in Neo1 function, as was evident from the observation that the NEO1-FLAG-AID strain was hypersensitive to neomycin (Fig. 1D). In this strain, GFP-Rim21C dissociated from the plasma membrane even without auxin treatment (Fig. 1B).

Lipid asymmetry is the result not only of flippases, but also of floppases. As for flippase gene disruption, deletion of the floppase PDR5 gene caused dissociation of GFP-Rim21C from the plasma membrane (Fig. 1B). These observations indicate that Rim21C alone senses the status of lipid asymmetry and responds to alterations in lipid asymmetry. We evaluated the extent of its membrane binding by quantifying the ratio of the fluorescence intensity of the bud neck to that of the cytosol of the mother cell (Fig. 1E). Again, GFP-Rim21C clearly dissociated from the plasma membrane in mutants deficient in phospholipid flip/flop, although the degree of dissociation varied. Because Rim21C does not contain any transmembrane segments, it must be associated with the cytosolic surface of the plasma membrane. It is therefore highly likely that Rim21C recognizes changes in the membrane surface state on the basis of altered lipid asymmetry. We speculated that Rim21C senses the lipid composition of the inner leaflet of the plasma membrane. Indeed, a considerable fraction of GFP-Rim21C dissociated from the plasma membrane when PtdSer (enriched in the inner leaflet) was eliminated by deleting the sole PtdSer synthase gene CHO1 (Fig. 1, B and E).

We next investigated the dynamics of GFP-Rim21C after external alkalization and reacidification, because Rim21C also senses external alkalization in addition to altered lipid asymmetry (20). Upon external alkalization, GFP-Rim21C dissociated from the plasma membrane and dispersed in the cytosol and the nucleus (Fig. 2). However, it reattached to the plasma membrane within 5 min of reacidification (which was achieved by transferring the cells to the normal acidic medium, at approximately pH 4.5; Fig. 2). This indicates that Rim21C can also sense changes in external pH.

Our findings show that Rim21C contains regions that can detect altered lipid asymmetry and external alkalization. In other words, a sensor motif capable of detecting lipid asymmetry and external alkalization exists in Rim21C.

**A Cluster of Glu Residues in Rim21C Is Essential for the Sensing Function**—To identify the regions of Rim21C that play an important role in sensing lipid asymmetry and external alkalization, we performed a systematic mutational analysis of full-length Rim21. We created a series of Ala-substituted mutants of charged amino acid residue clusters (C1–C18; Fig. 1A) in Rim21C. Each mutant was expressed in rim21Δ cells from a low copy (CEN) plasmid, and activation of the Rim101 pathway was monitored by detecting the processed form of Rim101. Rim21 contains three consecutive Glu residues (C3; amino acids 353–355; the EEE motif). The C3 mutant did not activate the Rim101 pathway upon external alkalization, indicating that the EEE motif is essential for Rim21 function (Fig. 3A). On the other hand, most Rim21 mutants did activate the Rim101 pathway, although their level of activity varied. For example, the C17 mutant, in which 13 acidic residues near the C terminus were mutated, exhibited weak activity. This might have been the result of the low expression level of this mutant protein (Fig. 3A). However, no mutant exhibited constitutive activation of the Rim101 pathway without external alkalization (Fig. 3B).
We previously reported that Rim21 is modified by phosphorylation and N-glycosylation (20). In an immunoblot analysis of deglycosylated lysates, Rim21 was detected as two bands, the faster and slower migrating bands representing the unphosphorylated and phosphorylated forms, respectively (20). Two phosphorylation sites (Ser-409 and Ser-515; arrowheads in Fig. 1A) within Rim21C have been identified by comprehensive analysis (38, 39). Both C6 and C2 mutant proteins, especially from alkali-treated cells, were detected as sharp single bands (Fig. 3A). The Ser-409 residue is adjacent to the C6 region, so phosphorylation at Ser-409 might be affected by C6 mutation. It is possible that C2 is located near the phosphorylation site in three-dimensional conformation. However, it should be noted that processing of Rim101 in response to external alkalization was normal in C2 and C6 mutants. This indicates that the banding pattern of Rim21, which probably represents its phosphorylation status, does not correlate with its function.

The EEE Motif Is Involved in Sensing Altered Lipid Asymmetry—As described above, the C3 EEE motif was found to be essential for the functioning of Rim21 (Fig. 3A). We next examined which elementary processes the C3 EEE motif is involved in. Rim21 forms a complex with Dfg16, another component of the Rim101 pathway (20). HA-tagged Rim21-C3 mutant was co-immunoprecipitated with FLAG-tagged Dfg16, using anti-FLAG antibody. The efficiency of the immunoprecipitation was similar to that of Rim21 WT (Fig. 4A), indicating that the EEE motif is not essential for binding to Dfg16. Rim21 localizes primarily to the plasma membrane and partly to internal membrane structures (20). The Rim21-C3 mutant also did so (Fig. 4B), indicating that the EEE motif is not required for localization of Rim21 to the plasma membrane. At a later stage in Rim101 signaling, Rim20 is recruited to the plasma membrane, where it forms a protein complex that cleaves Rim101 (24, 26). The accumulation of Rim20 in the plasma membrane did not occur in rim21Δ cells expressing the Rim21-C3 mutant (Fig. 4C), indicating that the EEE motif is a prerequisite for Rim20 recruitment to the plasma membrane.

We next analyzed the dynamics of GFP-Rim21C-C3, the GFP-fused, C-terminal domain of the Rim21-C3 mutant. GFP-Rim21C-C3 was attached to the plasma membrane in WT cells, particularly at the bud neck, as was done with GFP-Rim21C (Fig. 4, D and E). Interestingly, a large proportion of the GFP-Rim21C-C3 was still attached to the plasma membrane in the lipid asymmetry mutant (lemΔ3) and in the PtdSer-deficient mutant (cho1Δ). These observations indicate that Rim21C-C3 does not sense altered lipid asymmetry effectively; in other words, the EEE motif is necessary for sensing lipid asymmetry.

Because Rim21C was able to sense external alkalization as well as altered lipid asymmetry, we examined the localization of GFP-Rim21C-C3 after external alkalization. When the pH of the medium was increased to 8.0 from ~4.5, most of the GFP-Rim21C-C3 dissociated from the plasma membrane, like the GFP-Rim21C did (Fig. 5). In case the sudden increase in ambient pH to 8.0 was too drastic a change to properly test this mechanism, we repeated the test with a smaller increase in external pH, to 6.8. At this pH, we still saw dissociation of GFP-Rim21C from the plasma membrane; however, a substantial fraction of GFP-Rim21C-C3 remained bound to the inner leaflet (Fig. 5). The EEE motif is therefore also at least partly involved in sensing external alkalization.

**Opposing Effects of the EEE Motif and the Adjacent ERKEE Motif Regulates Sensing of Lipid Asymmetry by Rim21**—There is a cluster of charged amino acid residues (ERKEE, C2) adjacent to the EEE motif (C3; Fig. 1A). We examined the potential involvement of this motif in sensing lipid asymmetry and its interaction with the EEE motif. We found that GFP-Rim21C-C2 dissociated from the plasma membrane even in WT cells, in contrast to GFP-Rim21C (Fig. 6, A and C). This effect was opposite to that of the EEE (C3) mutation. GFP-Rim21C-C3 remained attached to the plasma membrane even in the lipid asymmetry mutant (lemΔ3; Figs. 4D and 6B). Introduction of both C2 and C3 mutations into GFP-Rim21C caused detachment of the resulting GFP-Rim21C-C2/C3 mutant from the plasma membrane (Fig. 6, B and C). This suggests that the ERKEE and EEE motifs function in an antagonistic manner with respect to sensing of lipid asymmetry. The EEE motif contains only negatively charged residues, whereas the ERKEE motif has two positively charged residues (RK). Considering that Rim21C mostly dissociated from the plasma membrane in cho1Δ cells in which PtdSer, a negatively charged lipid, is absent, we speculated that positively charged RK sequence is involved in the association of Rim21C to the plasma membrane. Consistent with this assumption, GFP-Rim21C in which the RK sequence was substituted with AA (GFP-Rim21C-C2(AA)) dissociated from the plasma membrane of WT cells in a similar manner to the GFP-Rim21C-C2 mutant (Fig. 6, A and C). Likewise, AA substitution of the RK sequence in the GFP-Rim21C-C3 mutant caused detachment of the resulting GFP-Rim21C-C2(AA)/C3 protein from the plasma membrane in lemΔ3 cells (Fig. 6, B and
These results indicate that the RK sequence within the C2 ERKEE motif is required for association of Rim21C with the plasma membrane. As already noted, the C2 mutation did not affect the ability of Rim21 to activate the Rim101 pathway (Fig. 3A), indicating that this motif is not essential for activation of the Rim101 pathway (see “Discussion”).

**Discussion**

We have demonstrated that Rim21C contains a region that functions to sense lipid asymmetry and external alkalization (Figs. 1 and 2). However, at present, we cannot exclude the possibility that the remainder of Rim21, which includes the N-terminal and transmembrane regions, is also partly involved in sensing lipid asymmetry and external alkalization. Within Rim21C, the C3 EEE and the C2 ERKEE motifs are important for the sensor function of Rim21 (Figs. 4–6). The EEE motif and the ERKEE motif seem to behave in an antagonistic manner: EEE is required for dissociation of Rim21C from the plasma membrane, whereas ERKEE seems to be involved in the association of Rim21C with the plasma membrane. We speculate that the EEE and ERKEE motifs constitute the sensor motif and that altered lipid asymmetry is sensed by the balance between their counteracting effects. Mutation in the C3 EEE motif removed the ability of Rim21 to activate the Rim101 pathway (Fig. 3A), suggesting that dissociation of Rim21C from the plasma membrane is required for signal transduction to the downstream proteins. One may expect that mutation in C2 ERKEE causes the opposite effect to C3 mutation on the Rim101 pathway, i.e., stimulation and/or constitutive activation of the pathway. However, the Rim101 pathway was not constitutively activated under normal conditions in cells expressing the Rim21-C2 mutant, and the degree of activation of the Rim101 pathway upon external alkalization was indistinguishable between the WT and C2 mutants (Fig. 3), suggesting that dissociation of Rim21C from the plasma membrane alone is insufficient for activation of the Rim101 pathway. Alterations in lipid asymmetry and external alkalization may also cause some changes in Rim21 or other proteins of the Rim101 pathway. During preparation of this manuscript, it was reported that the activation threshold of the Rim101 pathway is regulated by phosphorylation of Rim8, a downstream molecule of Rim21 (40). Therefore, it is important that future investigations look at the relationship between Rim8 phosphorylation and how Rim21C associates with and dissociates from the plasma membrane.

The most important unsolved issue is the molecular mechanism of lipid asymmetry sensing. Rim21C is predicted to be broadly disordered (unstructured; Fig. 7A), suggesting that Rim21C is highly flexible. Based on this and the fact that Rim21C dissociates from the plasma membrane in response to altered lipid asymmetry (Fig. 1B), we propose the following working hypothesis. Rim21 uses its flexible C-terminal cytosolic region to sense altered lipid asymmetry.
lic tail (Rim21C) like an insect antenna to monitor the status of lipid asymmetry (Fig. 7B). Rim21C may associate with and dissociate from the plasma membrane using charged amino acid residues, particularly the ERKEE and EEE motifs. In the ERKEE motif, the positively charged amino acid residues, RK, mainly contribute to the association of Rim21C with the plasma membrane (Fig. 6). PtdSer is likely to be the main lipid involved in the interaction between Rim21C and the plasma membrane because Rim21C mostly dissociated from the plasma membrane in cho1/H9004 cells, in which PtdSer is absent (Fig. 1B). However, some of the Rim21C was still detectable on the plasma membrane in cho1Δ cells, suggesting that PtdSer is not the sole factor determining the affinity of Rim21C to the plasma membrane. Under normal conditions, ERKEE-mediated association with the plasma membrane is dominant. Alterations in lipid asymmetry cause changes in lipid composition and local charge on the inner leaflet, which can affect the balance between the ERKEE-mediated association with and EEE-mediated repulsion from the plasma membrane, leading to dissociation of Rim21C from the plasma membrane. After Rim21C dissociates from the plasma membrane, downstream proteins in the Rim101 pathway may be recruited to Rim21.

Rim21 senses external alkalization, as well as altered lipid asymmetry. How a single sensor can sense separate stimuli remains an enigma. We previously reported that the Rim101 pathway is activated by plasma membrane depolarization induced by a proton ionophore (20). The membrane potential of the yeast plasma membrane is mainly the result of differences in proton concentrations inside (pH ~7.4) and outside the cells (pH ~4.5). External alkalization therefore leads to plasma membrane depolarization. Rim21 may not recognize changes in lipid asymmetry or external alkalization through membrane potential, because Rim21C, which lacks transmembrane seg-
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![Diagram](image)

**FIGURE 7. Antenna hypothesis.** A, the disordered state of Rim21C, as predicted by DRIPPred program. Disordered and ordered regions are shown in red and blue, respectively. The ERKEE and EEE motifs are boxed. B, illustration of a working hypothesis concerning the mechanism by which lipid asymmetry is sensed. Rim21C uses its flexible C-terminal cytosolic region (Rim21C) like an insect antenna to monitor lipid asymmetry by sensing the state of the cytotoxic surface of the plasma membrane. The ERKEE motif, especially the positively charged RK residues, mediates association of Rim21C with the plasma membrane, whereas the EEE motif promotes repulsion from the plasma membrane. Usually the ERKEE-mediated association is dominant. When lipid asymmetry is altered, changes occur in the balance between the effects of these two motifs. As a result, the EEE-mediated repulsion of Rim21C becomes dominant. Rim21C dissociates from the plasma membrane, is then recognized by downstream proteins in the Rim101 pathway and activates signal transduction.

Our findings provide a clue to understanding the molecular mechanism by which lipid asymmetry is sensed. However, further research is needed to elucidate the exact recognition target of Rim21C. In addition, further detailed analyses are necessary to confirm the antenna hypothesis.

**Author Contributions**—K. N. and K. O. performed the experiments. K. O. designed the experiments. N. K., K. O., and A. K. analyzed the data. K. O. and A. K. wrote the manuscript.

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