Elucidation of Lipid Asymmetry Signaling for the Prevention of Fungal Infections

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Research objective

Deep-seated mycosis, in which pathogenic fungi penetrate deep into the organs, has a high mortality rate and poses a major threat to human health. Plant fungal infections, such as blast disease and powdery mildew, have long been a threat to crops. Because infectious fungi are eukaryotes, chemical targets without side effects to humans are limited. Recently, the Rim101 pathway, which is activated by altered lipid asymmetry in the plasma membrane for adaptation, has been reported to be required for the proliferation and virulence of fungi infecting their host organisms. We identified Rim21 as a sensor protein for alterations in lipid asymmetry in yeast and we provide an overview of the Rim101 signaling pathway. Rim21 is considered an ideal novel drug target because it regulates the Rim101 pathway at its most upstream position, and is exposed on the cell surface. A large black box in the Rim101 signaling pathway is the molecular mechanism by which Rim21 senses alterations in lipid asymmetry and outputs the signal to downstream molecules. In this study, we aimed to elucidate this black box and form a foundation for the development of drugs targeting Rim21.

Methods

It has been proposed that Rim21 uses its flexible C-terminal cytosolic tail region (Rim21C) as an insect antenna to monitor lipid asymmetry through repetitive interactions with the inner leaflet of the plasma membrane[1]. Given this model, a recombinant protein containing the Rim21C moiety was prepared and subjected to a lipid overlay assay to investigate Rim21C-lipid interactions. We did not succeed in expressing GST-Rim21C in *E. coli* or mammalian cells; thus, we employed a cell-free expression system, that is, a wheat germ extract system.

We have previously reported that the ubiquitin ligase Rsp5 is essential for the Rim101 signaling pathway[2]. Ubiquitination of the sensor protein, Rim21, was examined using a pull-down assay of Rim21-HA from cells expressing His-tagged ubiquitin. Comprehensive analysis showed that the K363 residue of Rim21 was ubiquitinated. Given this information, we constructed a *RIM21(K363R)* mutant strain and monitored the progression of the Rim101 pathway and the ubiquitination of Rim21.

Rsp5 has three substrate-recognition domains, the so-called WW domains. Mutations were

introduced into each WW domain and activation of the Rim101 pathway was examined. Many Rsp5 substrates contain PxxY motifs, to which the WW domain of Rsp5 binds. Rim21 contains a PxxY-like motif in the Rim21C moiety. The Rim101 pathway was monitored in cells harboring mutations in the PxxY-like motif of Rim21.

The Rim21C moiety was cloned from the *C. glabrata* genome and expressed in *S. cerevisiae* cells as a fusion protein with GFP. The response of GFP-CgRim21C to altered lipid asymmetry was also observed.

Results

Recombinant GST-Rim21C protein, prepared using the cell-free wheat germ extract system, was subjected to a lipid overlay assay. GST-Rim21C bound to acidic lipid species, such as phosphatidylserine, phosphatidylinositol, phosphatidic acid, and phosphatidylinositol(4,5)*bis*-phosphate (Fig. 1). This result is consistent with our previous report that basic residues in the Rim21C moiety are required for plasma membrane binding, whereas a cluster of acidic residues is responsible for repulsion from the membrane[1]. Phosphatidylethanolamine does not have a charge, but it mostly localizes to the inner leaflet of the plasma membrane and is, thus, one of the lipids showing an asymmetric distribution. Phosphatidylethanolamine was then mixed with the lipids to which GST-Rim21C bound, and a lipid overlay assay was performed. Of note, binding of GST-Rim21C to acidic lipids was enhanced in the presence of phosphatidylethanolamine (Fig. 1, right panel). Phosphatidylethanolamine has an amino group that accepts protons. We hypothesized that the equilibrium of the acidic lipids is tilted to a negatively charged state in the presence of phosphatidylethanolamine.

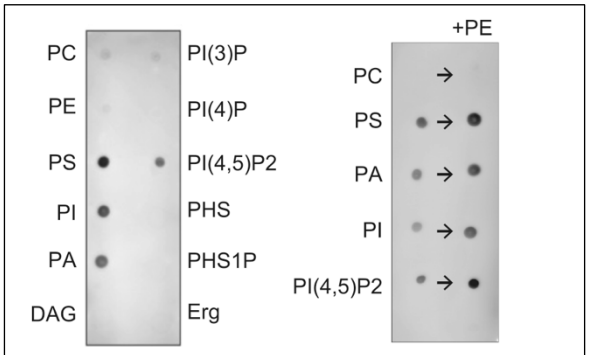

Fig. 1
Rim21C bound to phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidic acid (PA), and phosphatidylinositol (4,5) bisphosphate [PI(4,5)P2] (left panel).
The Interactions between RIm21C and these lipids were enhanced in the presence of phosphatidylethanolamine (PE) (right panel).

Ubiquitinated proteins were pulled down from the membrane fraction of cells expressing His-tagged ubiquitin. Subsequent immunoblotting analysis revealed that Rim21-HA was polyubiquitinated. Ubiquitination of Rim21-HA was facilitated by activation of the Rim101 pathway. Ubiquitination of Rim21-HA in the logarithmic growth phase was mostly abolished by introducing the K363R mutation into Rim21-HA. However, the ubiquitination of Rim21(K363R)-HA was restored upon activation of the Rim101 pathway. Introducing mutations into the PxxY-like motif reduced Rim21-HA ubiquitination. The ubiquitination of Rim21-HA was nearly undetectable when both mutations were introduced (Fig. 2). The Rim101 pathway progressed normally in cells expressing Rim21(K363R)-HA. In contrast,

activation of the Rim101 pathway was completely abolished in cells expressing Rim21-HA harboring mutations in the PxxY-like motif.

Among the three WW domains of Rsp5, the introduction of mutations into the third WW domain strongly inhibited the Rim101 pathway.

We monitored the dynamics of GFP-CgRim21C in *S. cerevisiae* cells. Similar to GFP-ScRim21C, GFP-CgRim21C bound to the plasma membrane when the Rim101 pathway was inactive, but dissociated from the plasma membrane in cells with disturbed lipid asymmetry.

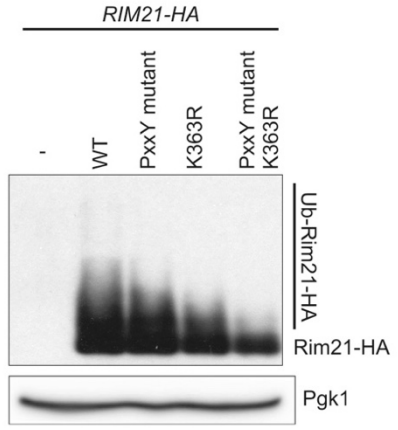

Fig. 2

Ubiquitination of Rim21-HA was observed upon activation of the Rim101 pawthway (WT; Ub-Rim21-HA).
Ubiquitination of Rim21-HA was less evident in the mutants of the PxxY motif and the K363 residue. In the mutants harboring the both mutations, Ub-Rim21-HA was nearly undetectable.

Conclusion

Rim21C bound to negatively charged lipid species, and this binding was enhanced by phosphatidylethanolamine. All bound lipids and phosphatidylethanolamine are mostly localized to the inner leaflet of the plasma membrane; thus, such interactions may occur in living cells as well. Rim21 can detect alterations in the negative charge on the inner leaflet surface and can be activated *in vivo*.

Ubiquitination of Rim21 is facilitated by the Rim101 pathway. Rim21 undergoes ubiquitination at an unknown position in addition to the known K363 residue. Ubiquitination at an unknown residue, but not at K363, is likely important for the activation of the Rim101 pathway. The PxxY-like motif in Rim21 is involved in Rim21 ubiquitination and Rim101 pathway progression. The third WW domain of Rsp5 is involved in Rim101 signaling.

CgRim21C behaves in a manner similar to ScRim21C.

References

1) Nishino, K., Obara, K., and Kihara, A. (2015) The C-terminal cytosolic region of Rim21 senses alterations in plasma membrane lipid composition: Insights into sensing mechanisms for plasma membrane lipid asymmetry. *J. Biol. Chem.* **290:** 30797-30805.
2) Obara, K., and Kihara, A. (2014) Signaling events of the Rim101 pathway occur at the plasma membrane in a ubiquitination-dependent manner. *Mol. Cell. Biol.* **34:** 3525-3534.