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Accessibility
Genetic interactions between a phospholipase A2 and the Rim101 pathway components in S. cerevisiae reveal a role for this pathway in response to changes in membrane composition and shape

M. Mattiazzi · A. Jambhekar · P. Kaferle · J. L. DeRisi · I. Križaj · U. Petrovič

Abstract Modulating composition and shape of biological membranes is an emerging mode of regulation of cellular processes. We investigated the global effects that such perturbations have on a model eukaryotic cell. Phospholipases A2 (PLA2s), enzymes that cleave one fatty acid molecule from membrane phospholipids, exert their biological activities through affecting both membrane composition and shape. We have conducted a genome-wide analysis of cellular effects of a PLA2 in the yeast Saccharomyces cerevisiae as a model system. We demonstrate functional genetic and biochemical interactions between PLA2 activity and the Rim101 signaling pathway in S. cerevisiae. Our results suggest that the composition and/or the shape of the endosomal membrane affect the Rim101 pathway. We describe a genetically and functionally related network, consisting of components of the Rim101 pathway and the prefoldin, retromer and SWR1 complexes, and predict its functional relation to PLA2 activity in a model eukaryotic cell. This study provides a list of the players involved in the global response to changes in membrane composition and shape in a model eukaryotic cell, and further studies are needed to understand the precise molecular mechanisms connecting them.

Keywords Rim101 pathway · Phospholipase A2 · Endosomal membrane · Genetic interactions

Introduction

Membrane curvature is emerging as an active means to create membrane domains and to organize membrane trafficking and signaling (McMahon and Gallop 2005). The aim of this study was to identify genes and biological processes that are involved in the adaptation of a model eukaryotic cell to perturbed membrane shape, since a global insight into such adaptation has not been obtained yet.

Phospholipases A2 (PLA2s) cleave fatty acids from the sn-2 position of the phospholipid molecules in biological membranes, resulting in changes of both composition and shape of the membranes. PLA2s induce membrane curvature because of the different shape of their substrates (cylindrically shaped phospholipids) and products that
remain inserted in the membrane (inverted cone-shaped lysophospholipids). In addition to this, fatty acids generated by PLA₂ enzymatic activity can be biologically active (Farooqui and Horrocks 2006). PLA₂s are involved in many physiological and pathological processes, such as inflammation, cell injury, tumor resistance and neurotoxicity (Kini 1997; Kudo and Murakami 2002). Probably the best studied example of the biological activity of these enzymes is that of the so-called cytosolic PLA₂s, which have long been known as inducers of the inflammatory reaction by releasing arachidonic acid (Kramer and Sharp 1997; Kita et al. 2006). This example has focused the attention mainly on fatty acids and lysophospholipids as the mediators of the biological effects of PLA₂s. However, it is clear that the increased positive membrane curvature caused by the PLA₂s’ enzymatic activity could represent a further important consequence of the action of these enzymes, especially in endocytosis and other vesicular transport-related cellular processes (McMahon and Gallop 2005). Unbiased analyses that take into account all the potential modes of action of PLA₂s are therefore needed to understand the biology of this group of enzymes. For this reason, in the current study ammodytoxin (Atx) was selected, which is a PLA₂ with a high degree of phospholipolytic efficiency on phospholipid membranes of different chemical compositions (Singer et al. 2002; Petan et al. 2005).

Genome-wide analyses have recently attracted much attention because of their ability to provide a global insight into the cellular events following a perturbation, allowing identification of all the biological processes involved in these events, and generating productive hypotheses on the molecular mechanism of the perturbation. *Saccharomyces cerevisiae* is an excellent model organism in which by genome-wide screens, rational hypotheses can be generated that are subsequently tested in more physiologically relevant experimental systems. Atx, a neurotoxic PLA₂ from the long-nosed viper and a model PLA₂, is the only specific PLA₂ (i.e., without any phospholipase B activity, which results in cleavage of both fatty acids from a phospholipid molecule) that has been studied in *S. cerevisiae* cells (Petrovic et al. 2005). When expressed in yeast, Atx was active in the cytosol (Petrovic et al. 2005), and its cytosolic, rather than extracellular, activity is thought to be important for its neurotoxic effects in its physiological target, neuronal cells (Petrovic et al. 2004; Praznikar et al. 2008). In yeast, Atx expression was shown to affect control of the cell cycle, but this effect was most probably dependent on its ability to bind to 14-3-3 proteins (Petrovic et al. 2005). To obtain a broader and more complete picture of the cellular effects of PLA₂s in eukaryotic cells, we performed a genome-wide analysis to identify yeast single-gene deletion strains that are sensitive to PLA₂ activity. We report the identification of a genetic and functional interaction between PLA₂ activity and the Rim101 pathway components.

*Saccharomyces cerevisiae* Rim101p is a zinc finger containing transcription factor (Lamb and Mitchell 2003) that is activated by removal of its C-terminal region by a cysteine protease Rim13p (Futai et al. 1999; Penalva and Arst 2002). The signaling cascade leading to its activation, in which numerous other proteins are involved, is required for the expression of several alkaline pH-induced genes and for alkaline pH-stimulated haploid invasive growth (Su and Mitchell 1993; Lamb et al. 2001). The same pathway is also involved in processes of cell wall construction (Castrejon et al. 2006) and meiosis (Su and Mitchell 1993). A signal transduction pathway relaying information on environmental alkaline pH, leading via the endosomal sorting complex required for transport (ESCRT) and endosomal multivesicular body (MVB) formation to the Rim101 pathway, has recently been proposed (Boysen and Mitchell 2006). However, a complete picture of the functional interactions between the Rim101 pathway components and proteins involved in vesicular transport-related processes is still lacking.

In the absence of known direct molecular targets of a perturbation, analysis of genome-wide experimental data by searching for characteristics linking the identified genes is helpful. Because of the extensively annotated genome, yeast *S. cerevisiae* is an ideal model organism for such an approach (Sturgeon et al. 2006). In this study, we used BioPixie (Myers et al. 2005), a biological data integration and visualization tool that enables, based on a probabilistic system and integration of diverse genome-wide data, discovery of interaction networks in which the genes of interest participate. Annotations of the network’s central genes/proteins and analysis of the actual interactions between the genes/proteins in the discovered networks then represent a basis for the generation of accurate and reliable hypotheses on the molecular mechanistic properties of the perturbation.

Using the combination of genome-wide experimental approach and bioinformatics analysis, we identified a functional interaction between the Rim101 pathway and changes in the shape and composition of cellular membranes. We were able to localize the underlying molecular events to the endosomal membrane and implicated the role of prefoldin, retromer and SWR1 complexes in a functional network including the Rim101 pathway component Rim13p.
Materials and methods

Strains and plasmids

AJY217 (S288c-derived, MATa, can1Δ::MFAIpr-HIS3 his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 lyp1Δ) was used to construct the starting strain for synthetic dosage lethality (SDL) analysis, MMY1001 (MATa, can1Δ::MFAIpr-HIS3 mfa1Δ::GALIpr-ATXA natMX his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 lyp1Δ).

MMY1001 was constructed in three steps. First, the gene coding for mature AtxA was cloned into pRD53 plasmid (pRS316 derivative containing GALIpr) between BamHI and ClaI sites. Secondly, GALIpr-ATXA was inserted between SalI and HindIII sites into pAG25 plasmid, which carries the nat1 gene (natMX resistance cassette) from Streptomyces noursei encoding nourseothricin N-acetyl-transferase and confers resistance to the antibiotic nourseothricin (cNAT). Thirdly, the MFA1 open reading frame (mfa1Δ::GALIpr-ATXA natMX) of strain AJY217 was replaced by the GALIpr-ATXA natMX cassette by integration of a PCR product generated with primers (5′-ATCTGTAACTGTTCGATAAAAACGAAAATA AGTACAAAAGCCATCGAATAGCATTTCCT CGACTCTCCT-3′) and (5′-GGTGTAACGGAAAAAGGA AGATAAAGGAGGGAGAAACGTTTTGTACGC AGAACAACATGGATCGATCTATCA-3′) that contain a 55-bp sequence identical to sequences upstream and downstream of MFA1 (bold). Transformants were selected on YPD plates containing nourseothricin (cNAT; Werner BioAgents).

For SDL, the collection of all the viable MATa single-gene deletion haploid strains (BY4741 derivatives) (Winzeler et al. 1999) and, for additional experiments, individual strains from the same collection were used. BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) was used as wild-type reference strain.

To monitor Rim101p proteolytic activation, RIM101 allele in BY4742 strain (MATa, his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0) was tagged with 3xHA inserted at codon 312 as described (Li and Mitchell 1997).

Plasmid pBJ1.0 was derived from pCM190 (tetO7-CYC1pr, URA3) by cloning ATXA between BamHI and NotI sites. Plasmids pBJ1.0 and pCM190 were transformed into RIM101-ΔHA-tagged BY4742 using the lithium acetate method (Geitz and Woods 1994).

Media

Yeast deletion strains and the corresponding wild-type reference strain were propagated in YPD medium or on YPD plates [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose, 2% (w/v) agar] containing G418 (200 mg/L, Invitrogen). Deletion strains containing the GALIpr-ATXA natMX cassette were grown in YPD medium or on YPD plates containing both G418 and cNAT (100 mg/L, Werner BioAgents). For ATXA expression, galactose [2% (w/v)] was used as carbon source (YPgal).

Growth media and conditions for SGA analysis were as described (Tong et al. 2001). Strains transformed with plasmids were grown in synthetic minimal uracil dropout medium, YNBglc + CSM-Ura (6.7 g yeast nitrogen base, 0.67 g complete supplement mixture without uracil and 2% (w/v) glucose per 1L).

For growth curve analysis, filter-sterilized YPD and YPgal containing 100 mM HEPES at pH 3.5, 4.5 or 7.5 were used. Overnight cultures were grown in unbuffered YPraf [2% (w/v) raffinose as carbon source].

Synthetic dosage lethality (SDL) screening

The selection of haploid double-mutant strains (i.e., single-gene deletion mutants with GALIpr-ATXA cassette) was performed as described in (Tong et al. 2001), with an additional step in the pinning procedure. In the last step, the expression of ATXA was induced by pinning the obtained double mutants on double-mutant selection plates containing galactose as sole carbon source. In parallel, as a control, double mutants were pinned on glucose-containing double-mutant selection plates. To minimize the number of false-positive results, the screen was repeated twice. For both screens, robotic manipulators were used: Virtek colony arraying and picking system (CAPS) was used for the first screen, while for the second screen a custom-made robotic manipulator with 384 floating pin replicator and with 0.787 mm diameter pins (V&P Scientific) was used. The strains that showed significantly decreased growth rate only because of ATXA expression were identified by comparing the growth of the double, as well as single mutant collections, both on glucose and galactose. In the first screen (CAPS pinned), the results were analyzed by visual inspection of the plates. For the analysis of the second screen, colony volume determination, normalization and growth fitness calculation were performed as described in Online Resource 1.

Growth assay

Growth curves were obtained as described in (St Onge et al. 2007) with some modifications. Deletion strains were grown overnight in YPraf. Cell cultures were then diluted to an initial OD600 of ~0.05 in buffered YPD and YPgal and grown in 300 μL volumes in 96-well plates in a Tecan Sunrise microplate reader to stationary phase. The growth of each culture was monitored by measuring the OD595 every 2 min, with constant shaking between measurements.
The growth rate ($K$) was determined as the slope of the linear part of the growth curve during exponential phase. The growth fitness ($R$) of a strain was calculated as:

$$R = (K_{gal}K_{galWT})/(K_{glc}K_{glcWT}),$$

where $K_{gal}$ and $K_{glc}$ are the growth rates of the deletion strain on galactose or glucose, and $K_{galWT}$ and $K_{glcWT}$ are the growth rates of the wild-type strain on galactose or glucose.

Rim101p processing assay

To monitor Rim101p proteolytic activation, BY4742 strain with HA-tagged RIM101, harboring either pBJ1.0 plasmid for ATXA expression or pCM190 plasmid as control, was grown overnight in YNBglc + CSM-Ura. Cells were then resuspended to an OD$_{600}$ of 0.2 in YNBglc + CSM-Ura containing 100 mM HEPES at pH 3.5, 4.5 or 7.5 and harvested in mid-logarithmic phase.

HA-tagged Rim101p was detected as described previously (Li and Mitchell 1997). Pgk1 was used as a loading control.

Endocytosis assay

The quantification of cell-associated Lucifer Yellow (LY; Lucifer Yellow CH dilithium salt, Sigma) was performed as described by Riezman (1985), with minor modifications. Cells were grown to mid-logarithmic phase in unbuffered or alkaline buffered (pH 7.4) medium, and resuspended in fresh medium containing 4 mg/mL LY and 20 mM NaN$_3$. After 1 h incubation at 30 or 0°C, cells were washed eight times with ice-cold buffer A (50 mM Na-succinate, pH 5.0, 100 mM NaCl, 10 mM MgCl$_2$, 20 mM NaN$_3$) containing 10% sorbitol to prevent lysis of more fragile cells. The cells were collected by centrifugation. They were then resuspended in 1 mL of buffer B (50 mM Tris–HCl, pH 7.5, 10 mM 2-mercaptoethanol) and 100 U lyticase was added. After 1 h of incubation at 37°C, 50 µL of 10% SDS was added to the lysed cells. LY fluorescence was excited at 426 nm and emission measured at 550 nm, both with 15 nm slit widths, using a Perkin Elmer LS50B spectrofluorimeter.

**Results**

Genetic interactions of PLA$_2$

with the Rim101-signaling pathway components

In the wild-type *S. cerevisiae* cells under standard growth conditions, Atx does not affect the growth rate (Petrovic et al. 2005). Morphologically, PLA$_2$-expressing cells are indistinguishable from control cells and PLA$_2$ expression does not confer an easily detectable phenotype, such as temperature or hypo-/hyper-osmotic shock resistance (data not shown). To obtain a genome-wide picture of the effects of a PLA$_2$ activity on yeast cells, ATXA gene under the control of inducible GAL1/10 promoter was integrated into the MFA1 locus of all the *S. cerevisiae* strains with single deletion of non-essential genes following the method of Tong et al. (see “Materials and methods”). Two independent replicates of the experiment were performed and in the first one by visual inspection 152 mutants were identified as lethal, very sick or sick (Online Resource 2), whereas in the second screen automated analysis identified 42 mutants as having a significantly decreased growth rate, compared to the wild-type strain (Online Resource 2). In the analysis of the two gene sets, gene ontology enrichment analysis identified only one term with the P value <0.01, namely ‘regulation of transcription’ (P value 0.0057) from the first screen (Online Resource 2). Overlap of the two screens identified eight genes, LGE1, NHP10, QCR6, RIM13, RIM101, VPS1, VPS5 and YTA7 (Table 1), the deletion of which was found to increase sensitivity to induced PLA$_2$ expression. Six of them are not obviously related: NHP10 codes for a component of an ATP-dependent chromatin-remodeling complex (Shen et al. 2003), QCR6 for a subunit of the ubiquinol cytochrome c reductase complex (Yang and Trumpower 1994), VPS1 for a dynamin-like GTPase (Ekena et al. 1993), VPS5 for a nexin-1 homolog (Nothwehr and Hindes 1997), YTA7 for an ATPase that regulates histone gene expression (Gradolatto et al. 2008) and LGE1 for a protein of unknown function involved in histone monoubiquitination (Hwang et al. 2003). Products of two of the identified genes, however, have closely related functions: Rim13p proteolytically activates Rim101p (Futai et al. 1999), thus placing both genes in the same pathway. We therefore looked more closely at other members of this pathway, i.e., genes coding for proteins involved in activation or processing of Rim101p; (Li and Mitchell 1997; Treton et al. 2000; Xu et al. 2004; Rothfels et al. 2005), and found that nine of them (*RIM20*, *RIM8*, *RIM9*, *RIM21*, *VPS23*, *VPS25*, *VPS28*, *VPS37* and *YGR122W*) have been identified in one of our two screenings (Table S1 in Online Resource 1). This indicates that the Rim101 pathway as a whole plays a role in the sensitivity to PLA$_2$ expression, and we therefore focused our subsequent experiments on the effects of intracellular PLA$_2$ activity on this pathway.

Inhibition of growth at alkaline pH by PLA$_2$ activity is functionally linked to RIM101

The Rim101 pathway is required for alkaline pH-stimulated cellular responses (Lamb et al. 2001). To determine whether PLA$_2$ activity affects this function of the Rim101 pathway, we measured the growth rate of PLA$_2$-expressing
strains in media with different pH values. Since the PLA2 acts in the cytosol that contains stable pH environment (Orij et al. 2009) even in the absence of the RIM101 gene (Mira et al. 2009), the activity of the PLA2, featuring enzymatic activity within a broad pH range (Beiboer et al. 1996), should not be affected under these conditions. While PLA2 activity did not affect the growth of the wild-type strain at acidic pH (3.5 and 4.5), the growth rate of the PLA2-expressing strain at pH 7.5 was 83% of that of the wild-type strain without the PLA2 gene. Also at pH 7.5, the growth rate of the rim101Δ strain without the PLA2 gene was 74% of that of the wild-type strain without the PLA2 gene, and when PLA2 was expressed in the rim101Δ strain, the growth rate was only 40% of that of the wild-type strain without the PLA2 gene. This is significantly less than the 61% (=0.83 * 0.74), which would be expected if PLA2 activity and deletion of RIM101 had independent effects on the cellular response to alkaline pH. This result is in agreement with the aggravating synthetic (phenotypic enhancement) effect between PLA2 expression and the Rim101 pathway determined in our screen.

PLA2 expression inhibits endocytosis in S. cerevisiae

It has recently been proposed that endocytosis of plasma membrane pH sensors is an upstream event leading to

Table 1 Genes whose mutation conferred increased sensitivity to induced PLA2 activity in both experiment replicates

| Gene    | Summary of protein function                                                                 | SDL screen |
|---------|------------------------------------------------------------------------------------------------|------------|
| LGE1    | Protein of unknown molecular function, involved in histone methylation and regulation of cell size; homozygous diploid null mutant has delayed premeiotic DNA synthesis and its efficiency of meiotic nuclear division is reduced | s 0.13     |
| NHP10   | Probable component of the INO80 ATP-dependent chromatin-remodeling complex, related to mammalian high-mobility group proteins | s 0.09     |
| QCR6    | Subunit 6 of the ubiquinol cytochrome c reductase complex, a component of the mitochondrial electron transport chain, required for maturation of cytochrome c1 | s 0.37     |
| RIM13   | Calpain-like cysteine protease that proteolytically activates Rim101p in response to alkaline pH | l 0.11     |
| RIM101l | Transcriptional repressor involved in the response to pH and in cell wall construction and required for alkaline pH-stimulated haploid invasive growth and sporulation; activated by proteolytic processing by Rim13p | l 0.46     |
| VPS1    | Dynamin-like GTPase required for vacuolar sorting, which is involved also in actin cytoskeleton organization, late Golgi retention of some proteins and regulation of peroxisome proliferation | s 0.30     |
| VPS5    | Nexin-1 homolog required for localizing membrane proteins from a prevacuolar/late endosomal compartment back to the late Golgi apparatus; structural component of the retromer complex | s 0.29     |
| YTA7    | Protein that localizes to chromatin and has a role in regulation of histone gene expression, has a bromodomain-like region that interacts with the N-terminal tail of histone H3 and an ATPase domain, and is potentially phosphorylated by Cdc28p | vs 0.24    |

In the two right columns are given the growth fitness values from both SDL screens, either determined by visual inspection (1) or calculated as described in Online Resource 1 (2) (relative growth fitness; R) for each of the eight genes

s Synthetic sick phenotype, l synthetic lethal phenotype, vs very synthetic sick phenotype
The WT measurement at acidic pH (endocytosis rates are independent of pH with empty vector) and RIM101 endocytosis in the PLA2-expressing strain (WT end3)

As a control, we measured the rate of endocytosis in the

Fig. 2 PLA2 expression inhibits endocytosis in yeast. The rate of endocytosis in the PLA2-expressing strain (WT + PLA2) is reduced to 45% (±10%) as compared to the control non-PLA2-expressing wild-type strain (WT). As a positive control, we measured the rate of endocytosis in the end3A strain. Lucifer Yellow dye uptake measurement was done as described in the "Materials and methods". Represented are the relative endocytosis rates for the WT + PLA2 and end3A strains that were calculated by normalizing to the WT measurement. The rate of endocytosis was measured for the same three strains also at alkaline pH (3 right columns) and normalized to the WT measurement at acidic pH (left column). The ratios of relative endocytosis rates are independent of pH

The molecular targets of PLA2 activity in yeast cells are not known, thus it is not possible to obtain a deeper insight into the molecular effects of PLA2s via a candidate approach. However, a global picture can be obtained, although at a lower resolution, by genome-wide analysis, relying on previously reported interactions between the yeast genes for the interpretation of results.

According to published data (Futai et al. 1999; Bensen et al. 2000; Kaeberlein and Guarente 2002; Krogan et al. 2003; Tong et al. 2004; Rothfels et al. 2005), the set of eight genes identified above can be regarded as two groups of genetically inter-connected genes (VPS1, VPS5, LGE1, and RIM101, RIM13, YTA7, NHP10; no interactions of QCR6 with the other seven genes have been reported) that are connected to each other by the physical interaction between Vps1p and Nhp10p (Gavin et al. 2002, 2006), (Fig. 3). These seven genes thus constitute a network that represents a context for investigating functional connection between PLA2 activity and the Rim101 pathway.

To define indirectly related genes and proteins with PLA2 genetic interactions, i.e., its process-specific network, we used the bioPIXIE bioinformatics tool (Myers et al. 2005) (Fig. 4). The RIM101 and RIM13 components of the Rim101 pathway are connected to this network primarily via interactions of RIM13, which has the highest confidence interactions with components of the prefoldin complex (GIM3/4/5 and YKE2). Indeed, gene set enrichment analysis of the network genes revealed the highest confidence level for three molecular complexes, SWR1 (P value 1.03e-10), retromer (P value 1.12e-6) and prefoldin (P value 3.35e-6), which, together with VPS1 and LGE1 genes, are central to the gene network thus obtained (Fig. 4). SWR1 complex is involved in chromatin remodeling, specifically it is required for the incorporation of the histone variant H2AZ into chromatin (Korber and Horz 2004). Retromer complex is an evolutionarily conserved protein complex involved in retrograde transport from endosomes to the Golgi apparatus (Verges 2008). Prefoldin complex is a heterohexameric cochaperone complex that delivers unfolded proteins, such as tubulin, to cytosolic chaperonin (Lopez-Fanarraga et al. 2001). These genes/complexes share many genetic interactions (Fig. 5), strongly indicating functional relations.

Rim101p activation (Boysen and Mitchell 2006). We therefore measured LY uptake to determine the effect of PLA2 expression on the rate of endocytosis in yeast cells. As a control, we measured the rate of endocytosis in the end3A strain (Fig. 2). The rate of endocytosis in the wild-type strain expressing PLA2 was reduced to ~45% of the control (wild-type, non-PLA2-expressing) strain. The effect of PLA2 on the rate of endocytosis was also observed under alkaline conditions, since also at pH 7.4 relative differences in the rate of LY uptake remained unchanged, while the absolute values were lower (Fig. 2).

PLA2 and Rim101 pathway components are genetically linked with SWR1, retromer and prefoldin complexes

Fig. 1 Western blot showing the abundance of Rim101p and its proteolytic cleavage products in the presence and absence of PLA2 at different pH values. The RIM101-HA-tagged WT (wild-type strain with empty vector) and RIM101-HA-tagged PLA2-expressing strain (wild-type strain with PLA2-expressing vector) were grown overnight in YNBglc + CSM-Ura medium and then diluted in identical medium buffered to pH 3.5, 4.5 or 7.5. The cells were collected in mid-log phase and the assay was carried out as described in “Materials and methods”. Pgk1 was used as a loading control. Full-length and cleaved Rim101p are indicated. In the PLA2-expressing strain, the ratio of cleaved to non-cleaved Rim101p under all experimental conditions is normal. The abundance of Rim101p at alkaline pH is unchanged. At acidic pH, on the other hand, significantly more Rim101p is present in PLA2-expressing cells.
Fig. 3  Diagram representing known interactions between seven of the eight genes identified as the highest confidence PLA₂ genetic interactors (a) and their overall confidence of the interaction (b) that was obtained with the bioPIXIE tool (Myers et al. 2005, http://pixie.princeton.edu/pixie).

Fig. 4  Functional genetic network as defined by the bioPIXIE tool (Myers et al. 2005, http://pixie.princeton.edu/pixie) with genes in genetic interaction with PLA₂ expression as input. Genes in genetic interaction with PLA₂ expression are marked in gray. Components of the prefoldin, SWR1 and retromer complexes are circled with dashed, full and dotted lines, respectively.
Relationship between PLA2 activity and the Rim101 pathway

In the set of eight genes identified in the whole genome screen for strain sensitive to heterologous expression of the PLA2 gene, two central genes of the Rim101 pathway were identified (Table 1). Moreover, all the strains containing mutations in the genes known to be required for activation or processing of Rim101p that were included in our analysis were identified as being at least somewhat sensitive to induced PLA2 intracellular activity in yeast cells (Table S1 in Online Resource 1), making the prediction that PLA2 activity and the Rim101 pathway are functionally related very likely. Western blot analysis revealed increased Rim101p expression at acidic pH in PLA2-expressing cells, where the ratio of cleaved (active) to full-length Rim101 protein resembled that in control cells (Fig. 1). Constitutive activation of Rim101p has previously been reported on mutation of ESCRT-III complex components Did4p/Vps2p, Vps4p and Vps24p, which are required for sorting of proteins into lumenal vesicles of MVBs. However, the PLA2-mediated phenotype appears to be different, since, in the case of the vps mutants, increased processing of Rim101p has been observed at acidic pH, without overall increase in its amount (Hayashi et al. 2005), whereas we observed an increase in total Rim101 protein in PLA2-expressing strains, with unaltered processing of Rim101p. Interestingly, the promoter region of the RIM101 gene contains the oleate response element (ORE) (Karpichev and Small 1998). This DNA sequence is bound by the transcription-activating heterodimer Oaf1p/Pip2p, which modulates fatty acid-induced activation of gene expression (Luo et al. 1996). Although fatty acids generated by PLA2 activity could explain the increased amount of Rim101p following PLA2 expression in the assay at pH 3.5 and 4.5, this fact alone does not explain the synthetic lethal interaction between the two. Rather, the presence of ORE in the RIM101 promoter could indicate a functional interaction between the Rim101 pathway and endogenous fatty acid-related cellular processes, in agreement with two recent reports (Ikeda et al. 2008; Yazawa et al. 2009).

It has recently been proposed that endocytosis of plasma membrane pH sensors is an upstream event leading to Rim101p activation (Boysen and Mitchell 2006). Atx, the PLA2 used in this study, is known to inhibit endocytosis in mammalian cells when applied extracellularly (Pungercar and Krizaj 2007) and in yeast cells when expressed intracellularly (Fig. 2). Our data show that receptor-mediated endocytosis is inhibited by the PLA2 activity in yeast cells, exemplified by the increased lifetime of Sla1-GFP-marked endocytotic patches (Mattiazzi and Drubin, manuscript in preparation). Since Rim101p activation is not inhibited in PLA2-expressing cells (Fig. 1), inhibition of endocytosis does not explain the functional link between PLA2 activity

![Diagram](image-url)
and the Rim101 pathway, but from the genome-wide data hypotheses on the mechanistic link could be drawn (see below).

Genome-wide view of the gene network including PLA2 genetic interactors

Our genome-wide analysis indicated that the major effect of PLA2 is alteration of membrane chemistry and/or trafficking. Two of the eight genes identified in our SDL screening, VPS1 and VPS5, encode membrane-bound proteins directly involved in vesicle sorting. Vps1p is a dynamin-like GTPase localized to peroxisomal and outer mitochondrial membranes, and is involved in various cellular processes such as vacuolar sorting and late Golgi retention of proteins, as well as actin and peroxisome organization and biogenesis (Hoepfner et al. 2001; Zahedi et al. 2006). Vps5p, the nexin-1 homolog, localizes to the endosome membrane and is a component of the outer shell of the retromer complex that is required for late endosome to late Golgi protein transport (Nothwehr and Hindes 1997; Seaman et al. 1998). Consistent with the finding that Vps1p and Vps5p are required for cells to counteract the effects of PLA2, we found that PLA2 expression reduced endocytosis rates in yeast (Fig. 2), similarly to neurotoxic PLA2 effects in mammals (Dixon and Harris 1999; Harris et al. 2000). Membrane remodeling during fission makes lipids more accessible to PLA2, which further alters membrane curvature and thus likely leads to the observed endocytosis defects. Our results thus indicate that additional defects in vesicle sorting induced by VPS1 or VPS5 mutations exacerbate the endocytosis defect caused by PLA2 expression. Besides Vps5p, other components of the retromer complex (Vps17p, Vps35p, Vps29p and Vps26p), which consists of membrane-associated proteins involved in vesicle trafficking, were also predicted to functionally interact with PLA2 (Fig. 4). Our genetic screen showed a consistent slight synthetic sick phenotype for all four strains (the average relative growth fitness measures (R) were: 0.77 for vps17A, 0.81 for vps35A, 0.81 for vps29A, and 0.82 for vps26A), which confirms this prediction.

Additionally, the retromer complex along with Ypt6p, which mediates fusion of ER-derived vesicles with Golgi and is predicted to interact with PLA2 expression (Fig. 4), could indirectly counteract PLA2 toxicity by activating the Rim101 pathway. MVBs are formed by invagination of the endosomal membrane and, in A. thaliana and in human cells, retromer complex components have been localized to MVBs (Seaman 2004; Oliviusson et al. 2006). Proteins residing in the membranes of MVBs have been shown to play a crucial role in the Rim101 pathway activation (Xu et al. 2004). Accordingly, RIM13 was predicted to interact with the retromer complex (Fig. 4). These facts combined suggest a hypothesis that the endosomal membrane is a direct target for PLA2 activity in yeast cells and that cleavage of endosomal membrane phospholipids, and/or modulation of the shape of this membrane, interferes with the Rim101 pathway. This hypothesis is further reinforced by the fact that two genes identified in our screen, VPS1 and VPS5, are functionally linked to phosphatidylinositol 3-phosphate (Burda et al. 2002; Efe et al. 2005), which is the key endosomal signaling lipid (Schu et al. 1993).

Three out of the eight genetic interactors of PLA2 (LGE1, NHP10 and YTA7) are involved in chromatin modification: LGE1 is in direct genetic interaction with SWR1 complex components (Fig. 5) and involved in histone H2B monoubiquitination (Hwang et al. 2003). Nhp10p is a component of the INO80 complex that shares components with the SWR1 complex (van Vught et al. 2007), whereas Yta7p is involved in maintaining the barrier between heterochromatin and euchromatin and in the regulation of histone gene expression (Gradolatto et al. 2008). Interestingly, it has recently been shown that Htz1p, the Z variant of H2A histone, which is introduced by the activity of the SWR1 complex, and its chemical modification are crucially involved in fatty acid-induced transcriptional changes (Wan et al. 2009). Moreover, strains mutated in HTZ1, SWR1, VPS71, ARP6 and YAF9 genes were shown to be sensitive to the presence of free oleic acid in the medium (Lockshon et al. 2007). A hypothesis can therefore be proposed that fatty acids generated by the PLA2 activity have similar effects on the cells, and further work will be required to test it. Congruent genetic interactions between SWR1 complex components and RIM13 indicate a potential functional interaction also between SWR1 complex and the Rim101 pathway.

In addition to the Rim101 pathway and retromer complex, the members of which were identified by SDL screening, our computational analysis predicted that the SWR1 and prefoldin complexes also functionally interact with PLA2 expression. These complexes are strongly connected to VPS1 and LGE1, and to both the retromer complex and Rim101 pathway via RIM13 (Figs. 4, 5). The fact that SWR1 and prefoldin complexes genes were not identified in our screen indicates a more indirect relation with PLA2 activity, or a relation in the context of a non-essential cellular process.

What could be a physiological counterpart of Atx, i.e., an endogenous PLA2, in yeast cells? Recently, a spore membrane bending pathway (SpoMBe), involved in spore formation/meiosis and in cell wall function, was described (Maier et al. 2008). One of SpoMBe components is also Spo1p, a structural homolog of phospholipases B and A2. Also, the Rim101 pathway has been reported to be involved in meiosis/sporulation (Su and Mitchell 1993; Castrejon et al. 2006). Moreover, it has been proposed that
membrane remodeling/curvature could be a trigger for Rim101p processing and activation (Rothfels et al. 2005). Since the promoter of the RIM101 gene also contains ORE and given the data presented in this study, a possibility exists that the Rim101 pathway, besides its other known functions, is involved in membrane-related cellular processes, such as membrane rearrangements during sporulation.

In this study, we identified the Rim101 pathway and the prefoldin, retromer and SWR1 complexes as players in the response of yeast cells to changes in membrane composition and shape caused by PLA2 activity. We activity described a network connecting the genes involved, and further studies are needed to understand the precise molecular mechanisms connecting them.

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