Mitochondrial membrane biogenesis: phospholipids and proteins go hand in hand

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Mitochondrial membrane biogenesis requires the import and synthesis of proteins as well as phospholipids. How the mitochondrion regulates phospholipid levels and maintains a tight protein-to-phospholipid ratio is not well understood. Two recent papers (Kutik, S., M. Rissler, X.L. Guan, B. Guiard, G. Shui, N. Gebert, P.N. Heacock, P. Rehling, W. Dowhan, M.R. Wenk, et al. 2008. *J. Cell Biol.* 183:1213–1221; Osman, C., M. Haag, C. Potting, J. Rodenfels, P.V. Dip, F.T. Wieland, B. Brügger, B. Westermann, and T. Langer. 2009. *J. Cell Biol.* 184:583–596) identify novel regulators of mitochondrial phospholipid biosynthesis. The biochemical approach of Kutik et al. (2008) uncovered an unexpected role of the mitochondrial translocator assembly and maintenance protein, Tam41, in the biosynthesis of cardiolipin (CL), the signature phospholipid of mitochondria. The genetic analyses of Osman et al. (2009) led to the discovery of a new class of mitochondrial proteins that coordinately regulate CL and phosphatidylethanolamine, another key mitochondrial phospholipid. These elegant studies highlight overlapping functions and interdependent roles of mitochondrial phospholipid biosynthesis and protein import and assembly.

The mitochondrial inner membrane has a unique composition of proteins and phospholipids, whose interdependence is crucial for mitochondrial function. It is highly enriched in proteins specific to this membrane, the majority of which are encoded by the nuclear genome and imported from the cytosol. The lipid component contains the major classes of phospholipids found in all cell membranes, including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidic acid (PA), as well as phosphatidylglycerol (PG) and cardiolipin (CL), which are located predominantly if not exclusively in the mitochondria (Zinser et al., 1991). PE, PG, and CL are synthesized "in house," whereas the others must be imported. Mitochondrial membrane biogenesis, therefore, requires the coordinated import and synthesis of proteins as well as phospholipids. Historically, mitochondrial protein and phospholipid import and biosynthesis have been studied separately. In the last decade, however, it has become apparent that mitochondrial phospholipids play a fundamental role in the function and import of mitochondrial proteins, which in turn regulate the synthesis of these phospholipids.

CL, the signature phospholipid of the mitochondrion, is a dimeric phospholipid with the capacity to form nonbilayer structures in the presence of calcium ions. The CL biosynthetic pathway is well-characterized in *Saccharomyces cerevisiae* (see Fig. 1 A), and the availability of null mutants of the genes encoding the CL biosynthetic enzymes has made in vivo studies of CL function possible. From yeast mutant studies, it is now clear that CL has diverse mitochondrial functions in respiratory chain supercomplex stabilization, mitochondrial protein import, ceramide synthesis, aging and apoptosis, and the translation of electron transport chain components (Joshi et al., 2009). Moreover, CL also affects cellular processes not previously associated with mitochondrial function, including cell wall and vacuolar biogenesis (Zhong et al., 2005; Chen et al., 2008). The significance of CL in human health is apparent from clinical studies showing that perturbation of CL metabolism leads to the life-threatening disorder known as Barth syndrome (Schlame and Ren, 2006).

Although CL plays a key role in many mitochondrial functions, recent evidence suggests that at least some functions overlap with those of the phospholipid PE, which comprises >25% of total mitochondrial phospholipids. Mitochondrial PE is synthesized by the Psd1p-catalyzed decarboxylation of PS. Overlapping functions of CL and PE were suggested by the finding that deletion of *PSD1* is synthetically lethal in CL-deficient yeast cells (Gohil et al., 2005), and may be attributed to the shared biophysical properties of PE and CL, both of which have the ability to form nonbilayer structures in membranes.

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A

Preprotein with Pre-sequence Preprotein with internal signals

H^+ H^+ H^+

+++

Tim23 Tim22 +++

$\Delta\Psi$

Tam41 ---

I III IV

II

V

MATRIX

ΔpH

IMM

H^+

(?)

PA → CDP-DG → PGP → PG → CL PG → CL

Cds1 Pgs1 ? Crd1 Crd1

→ Transport

→ Enzymatic synthesis

↶ ↶ Positive regulation

B

CL ←

UPS1
MDM34
GEP4
MDM32
CRD1

↔ Phb1/2 ↔

MMM1
MDM31
YTA10
YTA12
COX6
GEP3
GEP5
GEP6

→ CL + PE

PSD1
MDM35
GEP1

→ PE

● Phb1

○ Phb2

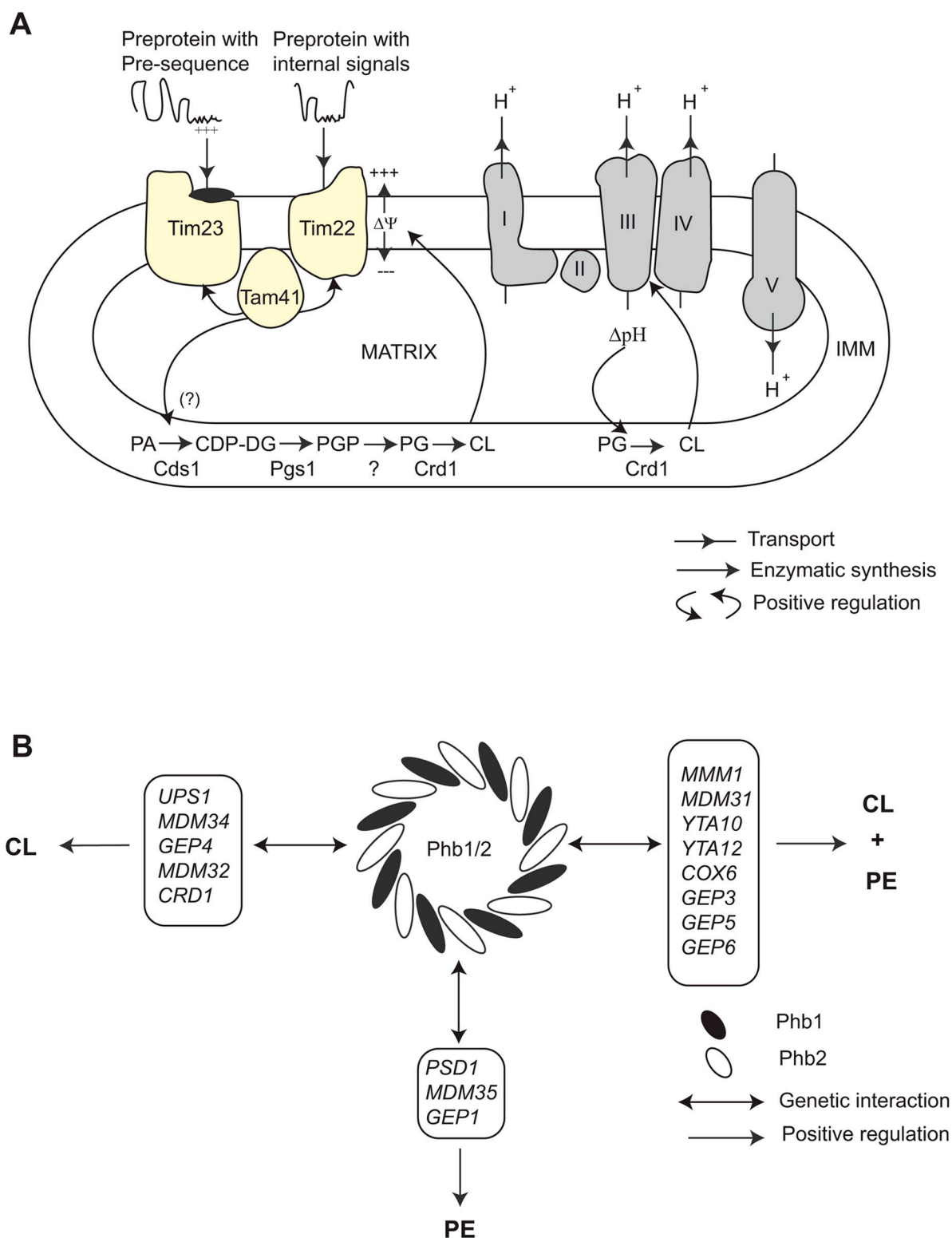↔ Genetic interaction

→ Positive regulation

Figure 1. **Identification of novel regulators of mitochondrial nonbilayer-forming phospholipids.** (A) Interdependence of CL biosynthesis and function. Four enzymatic steps catalyze CL biosynthesis in the inner mitochondrial membrane (IMM). The genes encoding three steps have been identified. Import machinery for proteins with N-terminal cleavable sequences (Tim23) or internal signals (Tim22) is also localized in the IMM. The newly identified Tam41 regulates Tim23- and Tim22-dependent protein import as well as CL biosynthesis, possibly by activating Cds1. CL, in turn, regulates protein import by maintaining the IMM potential by a mechanism not currently understood. CL stabilizes supercomplex assembly of complex III and IV, and stimulates complex IV activity. The pH gradient generated by respiratory chain activity increases CL synthesis by activating Crd1 synthase activity. (B) Genetic interactome of prohibitins regulates PE and/or CL levels. Genes identified in a synthetic genetic screen as genetic interactors of prohibitins (Phb1/2) consist of three classes that regulate (1) CL levels, (2) PE levels, or (3) both CL and PE. CDP-DG, cytidine diphosphodiacylglycerol; CL, cardiolipin; PA, phosphatidic acid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PGP, phosphatidylglycerolphosphate.

The functional importance of CL biosynthesis is underscored by the intricate regulation of CL synthesis via multiple novel mechanisms (Li et al., 2007). Evidence for cross-pathway control is seen in the down-regulation of CL synthesis by the phospholipid precursor inositol, possibly by posttranslational modification of *PGS1* (He and Greenberg, 2004). Additionally, factors affecting mitochondrial biogenesis, such as carbon source and the presence of mitochondrial DNA, regulate the expression of *PGS1* and *CRD1* (Jiang et al., 1999; Zhong and Greenberg, 2003). A novel mechanism of regulation was demonstrated by the finding that the last step in CL biosynthesis, catalyzed by CL synthase, is stimulated by the increase in matrix pH that accompanies respiration (Gohil et al., 2004). This elegant mechanism clearly demonstrates the interdependence of CL biosynthesis and electron transport chain function; CL promotes supercomplex formation and cytochrome oxidase activity, which, in turn, up-regulate CL synthesis (Fig. 1 A). This theme is repeated in the work of Kutik et al. (2008), albeit in a different context.

The work of Kutik et al. (2008) identifies a new function for Tam41 (translocator and maintenance protein 41) in the regulation of CL biosynthesis. Tam41 is a component of the mitochondrial protein translocator system, peripherally attached to the inner mitochondrial membrane from the matrix side (Gallas et al., 2006; Tamura et al., 2006). Although previous studies have identified the role of Tam41 in the import of cleavable presequence-containing proteins via assembly and maintenance of the Tim 23 complex, its role in the import of noncleavable carrier proteins via Tim22 remained controversial (Gallas et al., 2006; Tamura et al., 2006). In seeking to resolve this controversy, Kutik et al. (2008) discovered that the *tam41Δ* mutant exhibited phenotypes that were remarkably similar to those of the CL-deficient *crd1Δ* mutant. As seen with the loss of CL, which is required for the optimal activity and assembly of the ATP-ADP carrier (AAC; Claypool et al., 2008), the *tam41Δ* mutant exhibited a marked absence of AAC oligomers, as visualized by blue native PAGE. Mitochondrial respiratory chain supercomplex assembly was also perturbed in the *tam41Δ* mutant. These shared phenotypes suggested a possible role of Tam41 in CL regulation. Indeed, phospholipid analysis of mitochondria isolated from *tam41Δ* cells showed an almost complete absence of CL and its precursor PG. The expression of Crd1 and Pgs1 proteins was unaffected by the loss of Tam41; however, the accumulation of PA in *tam41Δ* mitochondria suggested that regulation may occur at the level of CDP-DAG synthase (Fig.1 A). These data suggest that Tam41 is primarily required for PG and CL biosynthesis, and that defects in protein import in *tam41Δ* are a secondary consequence of the loss of these lipids. However, it is likely that Tam41 has an additional function, as *tam41Δ*, unlike *pgs1Δ*, exhibits a true petite negative phenotype that cannot be rescued by 1 M sorbitol (Gallas et al., 2006). In light of earlier work showing that CL is required for maintenance of the mitochondrial membrane potential (ψ) and the import of proteins across the inner mitochondrial membrane (Jiang et al., 2000), the current report (Kutik et al., 2008) shows that the function of CL in mitochondrial protein import is interdependent with the regulation of CL biosynthesis by components of the mitochondrial protein import apparatus (Fig. 1 A). Tam41, therefore, has a dual role in maintaining mitochondrial structural integrity by regulating both protein and phospholipid composition. The unique

regulatory function of this protein would allow the coordinated biogenesis of mitochondrial components and prevent the formation of structurally and functionally aberrant mitochondria.

A fascinating new dimension of CL regulation is seen in the study of Osman et al. (see p. 583 of this issue), which shows that the biosynthesis of CL and PE is coordinately regulated and tied to the prohibitins (Phbs). Phbs are multimeric, ringlike structures present in many different cellular membranes, including the mitochondrial inner membrane. The rings, which consist of multiple copies of Phb1 and Phb2 subunits with a molecular mass of >1 MD, play a role in cell proliferation, cristae morphogenesis, and the functional integrity of mitochondria (Merkwirth and Langer, 2009). In *S. cerevisiae*, Phb complexes associate with mitochondrial proteolytic machinery, thereby controlling protein turnover (Steglich et al., 1999). They also bind to mitochondrially translated proteins, raising the possibility that they have a chaperone-like function (Nijtmans et al., 2000). The biochemical activity of Phbs remains unknown.

To gain insight into the function of Phbs, Osman et al. (2009) used the synthetic genetic array strategy to identify genetic interactions with Phb. In this manner, they identified 35 genes that are required for the survival of Phb-deficient cells. These genes encode four classes of proteins: (1) respiratory chain assembly proteins, (2) mitochondrial morphology regulating proteins, (3) uncharacterized proteins that the authors name genetic interactors of prohibitins (GEP), and (4) proteins with diverse functions, including enzymes that synthesize CL and PE. The observed synthetic lethality of Phb with genes encoding PE- and CL-synthesizing enzymes suggested that the other genes identified in the screen may play an important role in phospholipid regulation.

The first indication of linkage between Phb function and phospholipid regulation came from the finding that synthetic lethality of *phb1Δgep1Δ* cells could be rescued by increased expression of *CHO1*, which encodes PS synthase. This enzyme catalyzes the synthesis of PS, the precursor of PE. Consistent with this, the mitochondrial phospholipid profile of *gep1Δ* cells showed increased PE turnover and significantly reduced PE levels, which were restored by the overexpression of *CHO1*. A further link between Phb and phospholipid synthesis was apparent in the observation that loss of the GEP gene family member *UPS1* resulted in a dramatic sevenfold reduction of CL without affecting PE levels. Interestingly, deletion of *GEP1* restored normal CL levels in *ups1Δ* mitochondria, which suggests coordinated regulation of mitochondrial nonbilayer-forming phospholipids by GEP family proteins. Mitochondrial phospholipid profiles in the remaining Phb-interacting genes identified three functional classes of genes that regulate levels of CL, PE, or both (Fig. 1 B). This study, therefore, highlights critical overlapping roles of Phbs and the phospholipids CL and PE in maintaining mitochondrial inner membrane integrity. These findings are completely consistent with earlier studies showing synthetic lethal interactions of Phb with the PE-synthesizing enzyme Psd1p (Birner et al., 2003), and between mitochondrial PE and CL biosynthetic pathways (Gohil et al., 2005). Based on these data, the authors proposed the presence of defined lipid clusters in the mitochondrial membrane facilitated by the ringlike Phbs. The demonstration of such structures and the function of these PE-CL–rich domains remains an open question.

Other plausible explanations for the synthetic lethal interactions between PE, CL, and Phbs are certainly possible. For example, all three may act as chaperones for a common set of proteins. This would be consistent with previous reports demonstrating the capability of Phbs (Nijtmans et al., 2000), PE (Bogdanov and Dowhan, 1998), and CL (Zardeneta and Horowitz, 1992) to act as molecular chaperones. Another possibility is that Phbs, PE, and CL have a partially redundant role in mitochondrial or cellular division. Consistent with this, Phbs are known to regulate the replicative life span of *S. cerevisiae* (Coates et al., 1997), and the redistribution of PE at the cleavage furrow is essential for cytokinesis (Emoto and Umeda, 2000). Furthermore, the fact that synthetic interactions were observed with genes involved in the maintenance of mitochondrial morphology lends credence to a possible role of Phbs, PE, and CL in mitochondrial fusion and fission.

In summary, the identification of a link between mitochondrial import proteins and CL, and the identification of a conserved family of GEP-like proteins that function as novel regulators of CL and PE have substantially enhanced our understanding of mitochondrial biogenesis. The studies described here also raise many important questions, the answers to which will further our understanding of the interdependent role of CL function and its biosynthesis, and of the coordinate regulation of CL and PE in the mitochondria. First, although many proteins were identified in these reports, it is possible, and even likely, that many key players have yet to be identified. For example, the full spectrum of proteins required for the interorganelle and intramitochondrial transport of PE and CL has yet to be characterized. To this end, a genome-wide lipidomic approach involving quantitation of phospholipid levels in the yeast deletion collection may identify novel regulators of phospholipid transport, turnover, and synthesis. Second, what proteins mediate CL localization? Although it is clear that CL is synthesized in the inner mitochondrial membrane by a matrix-facing CL synthase, what proteins are required to flip the lipid to the outer leaflet of the inner membrane, and to transport CL to the outer mitochondrial membrane? How does CL become enriched in outer and inner membrane contact sites? Third, not all of the biosynthetic enzymes and regulators of CL metabolism have been identified. Mechanisms underlying the turnover and remodeling of CL have not yet been characterized. On a more basic level, the phosphatase that dephosphorylates phosphatidylglycerolphosphate (Fig. 1 A) has not been identified. Finally, many of the questions pertaining to CL synthesis and localization are also relevant to PE. For example, how is PE exported from the mitochondrial inner membrane to the ER? Does CL regulate the synthesis of PE by controlling expression and/or activity of Psd1? We may now begin to answer these questions in the context of the GEP-like and other regulatory proteins and their lipid interactors.

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