The double lives of phosphatases of regenerating liver: A structural view of their catalytic and noncatalytic activities

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Phosphatases of regenerating liver (PRLs) are protein phosphatases involved in the control of cell growth and migration. They are known to promote cancer metastasis but, despite over 20 years of study, there is still no consensus about their mechanism of action. Recent work has revealed that PRLs lead double lives, acting both as catalytically active enzymes and as pseudophosphatases. The three known PRLs belong to the large family of cysteine phosphatases that form a phosphocysteine intermediate during catalysis. Uniquely to PRLs, this intermediate is stable, with a lifetime measured in hours. As a consequence, PRLs have very little phosphatase activity. Independently, PRLs also act as pseudophosphatases by binding CNNM membrane proteins to regulate magnesium homeostasis. In this function, an aspartic acid from CNNM inserts into the phosphatase catalytic site of PRLs, mimicking a substrate–enzyme interaction. The delineation of PRL pseudophosphatase and phosphatase activities *in vivo* was impossible until the recent identification of PRL mutants defective in one activity or the other. These mutants showed that CNNM binding was sufficient for PRL oncogenicity in one model of metastasis, but left unresolved its role in other contexts. As the presence of phosphocysteine prevents CNNM binding and CNNM-binding blocks catalytic activity, these two activities are inherently linked. Additional studies are needed to untangle the intertwined catalytic and noncatalytic functions of PRLs. Here, we review the current understanding of the structure and biophysical properties of PRL phosphatases.

Phosphatases and kinases regulate the phosphorylation state of molecules involved in cell proliferation, adhesion, migration, differentiation, survival, and apoptosis, etc (1). Cysteine-based phosphatases (CBPs) constitute the largest family of phosphatases and include protein tyrosine phosphatases (PTPs), dual-specificity phosphatases (DUSPs) as well as lipid phosphatases such as PTEN (2, 3). Structurally, the catalytic portion of CBPs consists of a domain of roughly 150 residues present either alone or with other domains. The first CBPs identified were in the PTP family, which has led to the incongruous use of the term to refer to all members despite widely differing substrate specificities. The roughly 100 human CBPs also include a small number of pseudophosphatases, which have lost their catalytic activity through evolution and act through the direct binding of proteins.

Here, we review structural aspects of a unique family of protein phosphatases that have both catalytic and noncatalytic functions. Termed phosphatases of regenerating liver (PRLs) due to their initial discovery in the liver (4), their biological function has remained incompletely understood for over 25 years. Biochemically, PRLs exhibit very unusual enzyme kinetics and tightly bind to a family of membrane ion transporters. The interpretation of the catalytic and noncatalytic functions has divided the research literature on PRLs. We present different hypotheses to bridge the gap with perspectives for future studies.

PRLs are members of CBP superfamily

The three mammalian PRLs are highly related in their amino acid sequences. PRL-1 and PRL-2 are the most similar with 88% identity while PRL-1 and PRL-3 share 79% identity. The proteins are classified as class IVa protein tyrosine phosphatases, leading to their gene designations: *PTP4A1*, *PTP4A2*, and *PTP4A3*. The phosphatases are often considered to be DUSPs, which are phosphatases that act on both phosphotyrosine and phosphothreonine/serine. Among CBPs, the PRL phosphatases are most similar to cell division cycle 14 (CDC14) phosphatases and share approximately 30% sequence identity. Structurally, they consist of a single catalytic domain followed by an unfolded C-terminal tail of ∼20-residues capped with a CAAX farnesylation motif (Fig. 1*A*). Among protein phosphatases, the C-terminal farnesylation is unique to PRLs and serves to localize them to cellular membranes including the plasma membrane (5–9).

PRL function

PRLs have been implicated in a wide variety of cellular functions. In mammals, PRLs appear to be primarily involved in regulation of cell proliferation and migration. PRL-1 and -2 have broad tissue distributions while PRL3 is normally largely restricted to muscle (10). Much of the interest in PRLs arises from their overexpression in cancer. PRL-3 is highly upregulated in metastatic colon cancer and is associated with poor prognosis in many types of cancer (11). PRL-1 and PRL-2 overexpression is less prevalent in human cancers, but they

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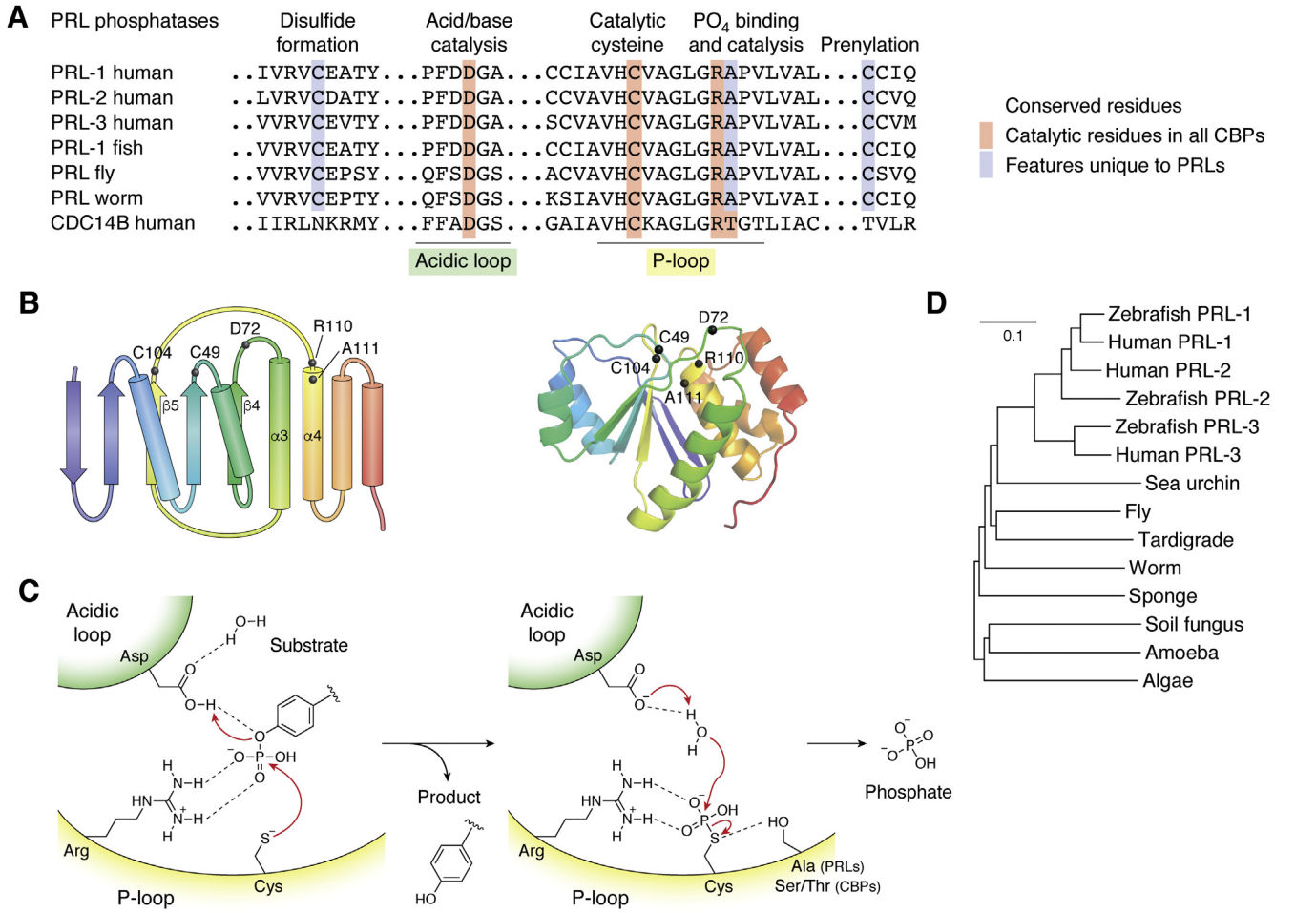

Figure 1. Structure and sequence of PRL phosphatases. *A*, conserved elements include catalytic residues present in all CBPs (*yellow*) and features unique to PRLs (*cyan*). *B*, secondary structure of PRLs and key residues in the catalytic site, numbered according to PRL-1/3. *C*, catalytic mechanism of CBPs. Cysteine in its ionized form attacks the phosphorus atom to form a phosphocysteine intermediate, which is hydrolyzed to regenerate the active enzyme (91, 92). The serine/threonine residue present in other CBPs is replaced by an alanine in PRLs (25). *D*, phylogenetic tree of PRLs.

have been shown to be oncogenic in cell and animal model systems (reviewed in (10, 12)).

The genetic knockout of the PRLs is viable in mice (13–17). This is likely due to complementation between the highly related genes as the double knockout of PRL-1 and PRL-2 is embryonic lethal (18). PRL-2$^{-/-}$ mice show defects in placental development and spermatogenesis, which are accentuated with partial (heterozygotic) loss of PRL-1. In zebrafish, PRL-3 expression affects melanocyte stem cell differentiation (19).

PRLs have been implicated in many signaling pathways, reviewed in (10, 12, 20–22). The PTEN/PI3K/Akt cancer pathway is the most frequently mentioned although there are papers suggesting the involvement of the Rho-family GTPase, Src, p53, STAT3/5, cKit/Notch, and Ras/MAPK pathways, among others. In the majority of studies, PRLs were proposed to act *via* dephosphorylation with the substrates identified from coimmunoprecipitation experiments and changes in protein phosphorylation in cells. Confirmation of the substrates has been difficult due to the low activity of PRLs *in vitro*. As an example, Li *et al.* (23) identified phosphorylated PTEN as a substrate of PRL-2 based on coimmunoprecipitation assays and decreased phosphorylation in cells when PRL-2 is overexpressed. However, direct measurements of phosphatase activity with purified components measured very low turnover numbers, suggesting that additional factors, such as membrane localization or unidentified cofactors, are required for full phosphatase activity (23). PRLs have also been suggested to be lipid phosphatases. Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) was dephosphorylated by PRL-3 in two different biochemical assays (24). Given the membrane localization and involvement of PRLs in the PTEN/PI3K/Akt pathway, PTEN and PI(4,5)P2 are compelling substrates from a biological standpoint, but their identification requires further confirmation.

PRL structure

The first atomic structure of a PRL phosphatase (PRL-3) was solved by solution NMR in 2004 (25, 26) followed by X-ray crystal structures of PRL-1 in 2005 (27, 28). At present, almost 20 structures are known, either alone or in complexes

Table 1
Structures of PRL phosphatases

Protein/complex	Species	PDB code	Resolution (Å)	Comments
PRL-1				
PRL-1 (1–173)	*H. sapiens*	1XM2	2.7	C104S mutant; trimeric
PRL-1 (7–160)	*R. norvegicus*	1ZCK	1.9	oxidized form; trimeric
PRL-1 (1–160)	*R. norvegicus*	1ZCL	2.9	C104S mutant; trimeric
PRL-1 (1–160)	*R. norvegicus*	1X24	3.2	oxidized form; trimeric
PRL-1 (1–160)/peptide	*R. norvegicus*	3RZ2	2.8	oxidized form; trimeric
PRL-1 (8–160)/compound 3	*H. sapiens*	5BX1	1.9	oxidized form
PRL-1 (1–173)/CBS CNNM2	*M. musculus*	5LXQ	3.33	oxidized form
PRL-1 (1–173)/CBS CNNM2	*M. musculus*	5MMZ	2.4	oxidized form
PRL-1 (7–160)/CBS CNNM2	*H. sapiens*	6WUS	2.76	C104D mutant
PRL-2				
PRL-2 (1–163)/CBS CNNM3	*H. sapiens*	5K22	3.0	reduced form
PRL-2 (1–167)/CBS CNNM3	*H. sapiens*	5K23	2.96	oxidized form
PRL-2 (1–163)/CBS CNNM3	*M. musculus*	5K24	3.1	oxidized form
PRL-2 (1–167)/CBS CNNM3/ADP	*H. sapiens*	5K25	3.05	oxidized form
PRL-2 (1–167)/CBS CNNM3	*H. sapiens*	6WUR	2.88	C101D mutant
PRL-3				
PRL-3 (1–169)	*H. sapiens*	1R6H	n/a	NMR structure
PRL-3 (1–173)	*H. sapiens*	1V3A	n/a	NMR structure
PRL-3 (1–162)	*H. sapiens*	2MBC	n/a	NMR structure; vanadate complex
PRL-3 (1–169)/CBS CNNM3	*H. sapiens*	5TSR	3.19	C104A mutant
Nonmammalian PRL				
PRL (1–163)	*Leishmania*	3S4O	2.3	oxidized form

(Table 1). PRL phosphatases have the typical CBP-fold consisting of a five-stranded central β-sheet covered by two α-helices on one side and a four-helical bundle on the other (Fig. 1*B*). The β-sheet is mostly parallel with only one antiparallel strand (β1).

The first structures of PRL-1 showed the protein crystallized as a trimer (27, 28). This led to the hypothesis that PRLs exist as trimers *in vivo*. Although oligomers have been detected in cells by chemical crosslinking, their physiological relevance remains unclear. Trimers have not been observed in structures of other PRLs (Table 1).

The PRL catalytic site is highly conserved and formed by residues from two loops. The phosphate-binding loop (P-loop) connecting strand β5 and helix α4 contains an arginine that coordinates phosphate binding and a cysteine (C104 in PRL-3) that forms a phosphocysteine intermediate as part of the catalytic cycle. In the acidic loop between strand β4 and helix α3, an aspartic acid acts both as a general acid and a base during the two-step catalytic cycle (Fig. 1*C*). At physiological pH, the catalytic cysteine is deprotonated due to the spatial alignment of the sulfur with the dipole of the following α4 helix. Ionization of the cysteine is required for catalysis but also increases the propensity of the cysteine to form a disulfide with an adjacent cysteine (C49 in PRL-3). PRLs readily crystallize as disulfides and, in fact, only one PRL crystal structure has the catalytic cysteine in its reduced form.

To initiate catalysis, the aspartic acid acts as a general acid to prime the phosphorus atom for nucleophilic attack by the cysteine and release of the dephosphorylated product. In the second step, the aspartic acid, now acting as a general base, activates a water molecule for hydrolysis of the phosphocysteine intermediate. In CBPs other than PRLs, attack of the water is facilitated by a serine or threonine residue from the P-loop. In PRLs, this residue is always alanine (A111 in PRL-3). This serves to stabilize the phosphocysteine intermediate and is a unique feature of the PRL family. The phosphocysteine intermediate has been visualized by X-ray crystallography in the CBP family phosphatase PTP1B (29).

Defining features and phylogeny

Within the CBP family of phosphatases, PRLs are distinguished by their farnesylation site and two features of the catalytic site: the alanine in the P-loop and the second cysteine residue (also present in DUSPs). PRLs from different species show the greatest divergence in the length of the loop between helix α3 and strand β5, which is far from the active site and unlikely to affect the distinctive catalytic properties of PRLs.

PRL phosphatases are absent in prokaryotes but occur widely in eukaryotes from unicellular organisms to mammals (30) (Fig. 1*D*). While many fungi species (such as *S. cerevisiae* and *C. albicans*) do not contain PRLs, their presence in algae, unicellular parasites, and some fungi points to their development early in evolution. Most vertebrates contain three PRLs. Phylogenetic analysis suggests that gene duplication occurred early as the isoforms from different vertebrates are evolutionarily more related to each other than to isoforms in the same species. The proliferation of forms also occurred independently in Leishmania and Trypanosoma protozoan parasites, which contain two or three PRL proteins, respectively.

Invertebrates generally contain only a single PRL gene. The PRL paralog in flies has received attention with recent reports that it plays developmental and protective roles in the nervous system (31–33). In worms, PRL was shown to signal through a lysosomal Ca^{2+} channel, TRPML (34). Leishmania and trypanosoma have multiple PRL genes, which have been suggested to contribute to the intracellular survival of the parasites (35, 36).

Kinetics of phosphatase activity

CBPs, including PRLs, catalyze the dephosphorylation of substrates through a two-step reaction: (i) formation of a

phosphoenzyme intermediate and release of the dephosphorylated substrate, and (ii) hydrolysis of phosphocysteine and release of free phosphate (Fig. 2*A*) (37). The two-step reaction leads to generation of a phosphoenzyme intermediate and a phenomenon known as burst kinetics. The existence of two steps has been observed in multiple CBPs but, in general, it is not detectable due to the rapid breakdown of the phosphoenzyme intermediate relative to formation ($k_3 >> k_2$). When the opposite occurs ($k_2 >> k_3$), there is an accumulation of the phosphoenzyme intermediate, which gives rise to burst kinetics: an initial burst of activity followed by a slow steady-state rate (38). The size of the burst step is stoichiometric with the amount of enzyme while the steady-state rate depends on the rate of the second step in the catalytic cycle (Fig. 2*C*).

PRLs are unusual in that the rate of hydrolysis of the phosphoenzyme intermediate (k_3) is several orders of magnitude slower than for other CBPs. Typical values for k_3 of protein phosphatases are in the range of 10 to 0.1 s^{-1} while, for PRLs, the rate is <1 h^{-1} (39–44). The ratio of k_2 divided by k_3 provides a measure of the burst-like character. For PRL-2, the ratio is roughly 400 compared with values of 10 or less for other CBPs. The stability of the PRL phosphoenzyme intermediate is, in part, due to the absence of serine or threonine residue in the P-loop (Fig. 1*C*). Replacement of the alanine (A111 in PRL-3) with serine increases the rate of steady-state catalysis, eliminating much of the burst effect (25). Conversely, when Denu *et al.* replaced the naturally occurring serine with alanine in VHR (vaccinia H1-related) phosphatase, the mutant enzyme showed slowed hydrolysis, which allowed the detection of phosphocysteine by NMR spectroscopy (45, 46). The strict conservation of alanine in PRLs in different species suggests that the stability of the phosphocysteine intermediate is a general feature of PRLs that has been maintained during evolution.

Although the existence of PRL burst kinetics has been known for many years (25, 27), it is often overlooked. Typical phosphatase assays use a small amount of enzyme compared with substrate, so the initial burst of phosphatase activity is small and transient. However, the existence of the burst has major consequences for the interpretation of the kinetic constants. During the steady-state condition, PRLs exhibit classical Michaelis–Menten kinetics with the extra reaction rate (k_3) folded into the values for the Michaelis constant (K_M) and k_{cat} (Fig. 2*C*) (38). This means that it is not possible to detect the two-step kinetic cycle from simply measuring the

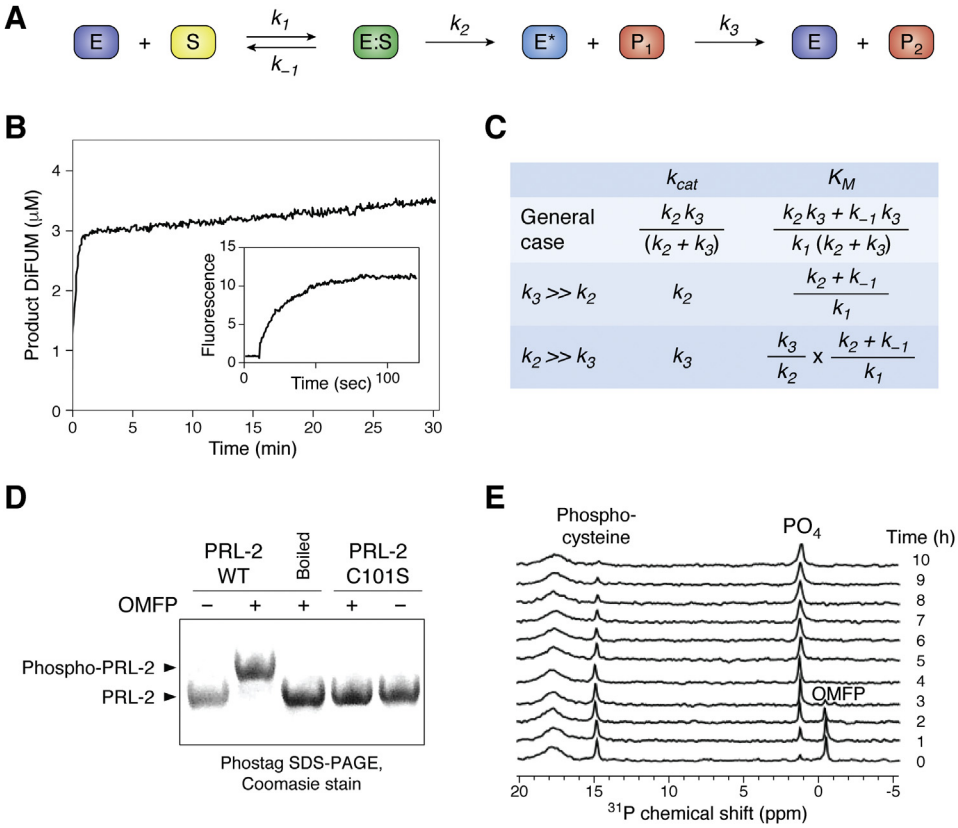

Figure 2. Enzyme kinetics of PRLs. *A*, phosphatase activity proceeds in two steps with a phospho-enzyme intermediate (E*). *B*, burst kinetics visualized at two different timescales. Stopped-flow traces of phosphatase activity of 3 μM PRL-2 measured with a fluorogenic substrate. Comparison of the slope of initial (*inset*) and steady-state curves reveals a roughly 400-fold difference in reaction rates. *C*, kinetic terms for a two-step enzyme mechanism (38). When the k_3 is larger than k_2, the equations simplify to the usual Michael–Menton kinetics. When k_3 is smaller, the turnover rate (k_{cat}) reduces to k_3 and the K_M is decreased by the burst ratio (k_2/k_3). Paradoxically, the enzyme appears to have higher affinity for substrates. *D*, detection of the phosphoenzyme intermediate by SDS-PAGE with the Phostag reagent that slows the migration of phosphorylated proteins. PRLs become fully phosphorylated in the presence of the synthetic substrate OMFP. Phosphocysteine is hydrolyzed by boiling and dependent on the catalytic cysteine (44). *E*, phosphorus NMR detection of phosphocysteine in PRL-2 confirms the hours-long lifetime (44).

steady-state rate at different substrate concentrations. When k_3 is much larger than k_2, the kinetic terms simplify to the standard forms where K_M is $(k_2 + k_1)/k_1$ and k_{cat} is k_2. On the other hand, when k_2 is larger than k_3, the k_{cat} reduces to k_3 and the apparent K_M is decreased by dividing by the burst ratio (k_2/k_3). This has the curious effect of improving the apparent affinity of enzymes with burst kinetics for their substrates.

The remarkable stability of the PRL phosphocysteine intermediate leads to almost complete conversion to the phosphoenzyme form when substrate is present (Fig. 2*D*). At saturation, the ratio of the two forms is equal to the burst ratio. Detection of the phosphoenzyme intermediate is most easily accomplished by SDS-PAGE gel electrophoresis with the Phostag reagent that decreases migration of phosphorylated proteins (44). The instability of phosphocysteine allows the presence of phosphocysteine to be confirmed by heating prior to electrophoresis. Phosphocysteine can also be detected by its phosphorus NMR signal, which has been used to measure its half-life (Fig. 2*E*) (44).

A common misconception about PRLs is that the low steady-state activity is due the absence of a true, *bona fide* substrate. As the V_{max} depends only on the rate of hydrolysis (k_3), it is by nature independent of the substrate. At saturation, all substrates have the same turnover number. This may explain why attempts to identify PRL substrates have failed to reach a clear consensus. While these issues do not apply to the burst phase, it is difficult to observe and frequently ignored in assays of PRL activity. As a result, PRLs appear to have low activity and ill-defined substrate specificity.

PRL phosphatase activity is surprisingly easy to detect if the level of PRL cysteine phosphorylation is measured instead of substrate dephosphorylation. Phostag gel analysis of PRL-3 allowed detection of phosphatase activity even with nonphysiological substrates (47). Low levels of phosphoenzyme intermediate formed when PRL-3 was incubated with a variety of low-molecular-weight diphosphate and triphosphate-containing compounds. This likely explains the observation that the PRLs are partially phosphorylated when purified from *E. coli*, even though the bacteria are unlikely to possess PRL-specific substrates (44, 47).

Interactions with CNNM proteins

A major step forward in understanding the function of PRLs was the discovery that they tightly bind CNNM (CBS-pair domain divalent metal cation transport mediators) proteins (48, 49) (Fig. 3*A*). CNNM proteins are membrane proteins that mediate magnesium transport and are associated with the diseases of magnesium metabolism: familial dominant hypomagnesemia (50) and Jalili syndrome (51). CNNMs have also been implicated in growth control and energy metabolism through regulation of magnesium levels (48, 49, 52). Studies of CNNM magnesium transport showed that PRL binding inhibits transport (48, 53) suggesting that PRL oncogenicity is a consequence of the growth-promoting effects of elevated cytoplasmic magnesium levels (54, 55). A role for PRLs in magnesium homeostasis was confirmed by observations that PRL levels are regulated by magnesium availability, diet, and circadian rhythms (44, 49, 56–60).

There are four CNNM proteins in humans, CNNM1, CNNM2, CNNM3, and CNNM4, composed of an uncharacterized N-terminal extracellular domain, a transmembrane DUF21 domain, a CBS-pair (cystathionine-β-synthase) domain, and cyclic nucleotide-binding homology (CNBH) C-terminal domain (61, 62). The proteins are dimers and mediate magnesium transport through the conserved

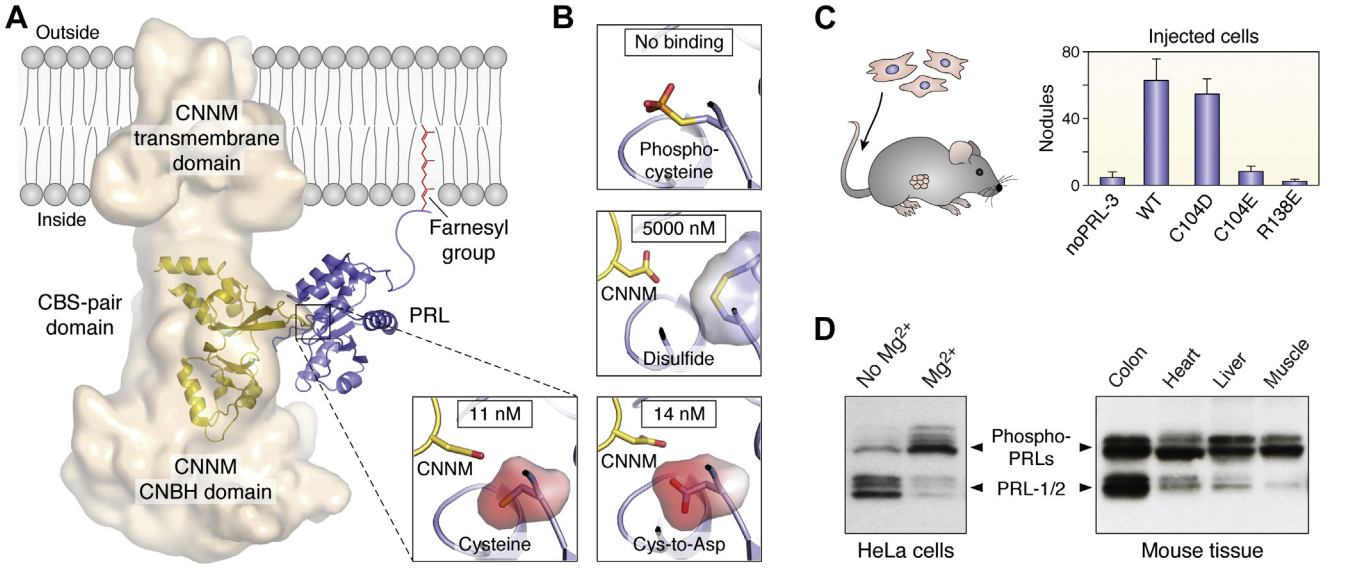

Figure 3. CNNM proteins bind PRLs *via* the catalytic site. *A*, model of PRL bound to a Mg^{2+} transporter CNNM protein. The model is based on structures of bacterial and human CNNM proteins and their complexes with PRLs (44, 47, 62, 63, 93–97). *B*, the PRL catalytic site controls the affinity of CNNM binding. A negative charge from either the catalytic cysteine or a substituted aspartic acid is required for high affinity (58). Oxidation or phosphorylation of the cysteine decrease or prevent binding (44). *C*, CNNM binding, but not phosphatase activity, is required for PRL-3 promotion of lung metastases by B16 melanoma cells (58). The PRL-3 C104D and R138E mutants are reciprocally deficient in either catalytic activity or CNNM binding while the C104E mutant is doubly deficient. *D*, phosphorylation of PRL-1 and -2 *in vivo*. The extent of phosphorylation changes in different culture media and tissues (58).

transmembrane domain. The CBS-pair domains regulate transport activity *via* binding of magnesium, nucleotides, and PRLs. Structurally, CBS-pair domains are composed of two CBS motifs with a mixed αβ-fold and an ATP-binding site at the dimerization interface.

PRL-CNNM complexes

Nine structures of complexes of PRL and CNNM CBS-pair domains have been determined (44, 47, 63) (Fig. 3*A*). The first structure was PRL-2 bound to the CBS-pair domain from CNNM3 and showed that binding is mediated by an extended CNNM loop that inserts into the PRL catalytic site (44). Subsequent structures with other PRLs and CBS-pair domains showed that the recognition mechanism is shared across all members of both families (Table 1). The most important binding interaction is between a CNNM aspartic acid and the PRL arginine in the P-loop. The negative charge of the aspartic acid carboxyl group mimics the phosphate group of a substrate and is positioned above the catalytic cysteine by hydrogen bonds with the positively charged arginine. Mutation of either the aspartic acid or arginine residue completely prevents binding.

Isothermal titration calorimetry measurements have shown that there is no specificity in the interactions between the different PRL and CNNM isoforms *in vitro*. All three PRLs bind CBS-pair domains, and the interaction loop is conserved across the four members of the CNNM family. The binding affinities are roughly 10 nM and very sensitive to modifications of the catalytic site (Fig. 3*B*) (47, 58). Substitution of the catalytic cysteine by either alanine or serine decreases the affinity as does disulfide bond formation (47, 63) (Fig. 3*B*). Despite this, all the crystal structures of complexes of PRL and CNNM CBS-pair domains except one contain either mutations of the catalytic cysteine or the cysteine disulfide. This occurs because, even with a 100-fold loss of affinity, the protein concentrations used to crystallize the complexes are higher than the K_d of binding.

Pseudophosphatase activity

Confirmation that CNNM binding rather than phosphatase activity is responsible for some of the biological functions of PRLs required the identification of mutations that selectively inactivate phosphatase or pseudophosphatase activity. The majority of mutations in the catalytic site disrupt both functions. The PRL-3 C104S mutation has been widely used as a negative control. Finding a mutant that blocks CNNM binding was straightforward as there are protein–protein contacts outside the catalytic site that are required for binding. Finding the reciprocal mutation was more difficult, but eventually it was observed that substitution of the catalytic cysteine by aspartic acid retained binding while inactivating catalytic activity (58). Not typically considered a conservative substitution, the aspartic acid preserves the negative charge of the thiolate in the active site (Fig. 3*B*). Remarkably, the cysteine-to-aspartic acid mutation increases the thermal stability of PRLs by 8 to 10 °C due to relaxation of the conformational strain required to shift the cysteine pK_a in the wild-type enzyme (58).

The PRL-3 C104D mutation provided the first unambiguous evidence that PRL pseudophosphatase activity is responsible for a biological function (58). The mutant inhibited CNNM4-associated magnesium transport in cells and promoted tumor formation in a murine model of cancer metastasis (Fig. 3*C*). In contrast, the R138E mutation, which disrupts binding but not catalysis (with small-molecule substrates), was inactive in both assays. While the R138E mutation could disrupt PRL-3 interactions with protein substrates, the C104D mutation unequivocally shows that at least two biological functions of PRLs do not require catalytic activity. The importance of phosphatase activity *versus* CNNM binding for other functions should become clearer as researchers test these mutants in different cellular and animal models.

Regulation of PRLs

PRLs are regulated at the gene, mRNA, and protein level. A variety of transcriptional factors bind and regulate the transcription of PRL genes in response to different stimuli and growth signals, see (10). PRL-1 was first identified due to the increase in mRNA and protein levels in the liver following partial hepatectomy (4). Similarly, PRL-3 levels are dramatically increased in metastatic colon cancer (11). Of particular relevance, considering that PRLs bind magnesium transporters, is the increase in PRL protein levels in response to magnesium depletion in cultured cells (44, 49, 57–60). The change in protein levels is also accompanied by changes in the levels of cysteine phosphorylation (44, 58) (Fig. 3*D*).

Reactive oxygen species (ROS) have been widely studied as a mechanism of regulation of CBPs, including PRLs (3, 64, 65). The catalytic cysteine is highly susceptible to oxidation, which can lead to temporary or permanent inactivation of catalytic activity. There are five possible oxidized forms of the sulfur ranging from addition of 1, 2, or 3 oxygen atoms to sulfenamide and disulfide bond formation (66, 67). In addition, cysteine modifications by nitric oxide (S-nitrosylation) and sulfhydryls (persulfidation) have been reported for some phosphatases (68, 69).

Relatively little is known about the oxidation of PRLs in cells. They are easily oxidized and form disulfides even under mildly reducing conditions *in vitro* (25, 27, 70). High concentrations of reducing agents are required to maintain the catalytic cysteine residue fully reduced. In cells, they are partially oxidized in addition to their extensive phosphorylation (70, 71). PRLs are particularly sensitive to inhibition by redox-active compounds that promote their oxidation or inhibit their reduction (71–73).

The biological consequences of PRL oxidation are not fully understood. Disulfide bond formation blocks both CNNM binding and catalysis, but it may also protect PRLs from overoxidation to the sulfinyl (SO_2H) or sulfonyl (SO_3H) forms (66, 67). Disulfide bond formation is reversible unlike the more oxidized states. Cysteine phosphorylation could be similarly

protective. Much more work is needed to characterize the modifications of PRLs and CBPs *in vivo*.

PRLs as molecular switches

The formation of phosphocysteine can be thought of as a mechanism for regulating PRL activity. It is a reversible modification, changes in response to stimuli (magnesium availability) and inhibits both catalytic activity and CNNM binding. Endogenous PRLs are stably phosphorylated in cultured cells and the levels change in response to magnesium in the culture medium (44) (Fig. 3*D*). Unphosphorylated PRLs slowly accumulate upon magnesium withdrawal and are rapidly phosphorylated upon readdition of magnesium (44, 58). PRLs are also highly phosphorylated in tissues (58). CNNMs were recently shown to bind ARL15, a member of the ARF-like family of G proteins (74).

Inhibitors of PRL phosphatase activity

Due to their oncogenicity, PRLs have long been the target of searches for inhibitors (75, 76). This has not been an easy task due to the shallow catalytic pocket, low activity, and ill-defined substrate specificity. Pentamidine, an antiparasitic drug, was the first proposed inhibitor, but it is not specific or potent (35, 77). Compounds based on rhodanine, a chemical group frequently involved in nonspecific interactions, have also been developed (78, 79). Redox-active compounds such as hydrogen peroxide are effective inhibitors both *in vitro* and *in vivo* (27, 71). Thienopyridine and a related compound, JMS-053, have been proposed to be PRL-specific inhibitors although their specificity is debated due to their ability to promote oxidation (72, 73).

Compounds that block protein–protein interactions rather than catalytic activity have also been proposed. Small molecules that inhibit PRL trimerization reportedly prevent cell proliferation and migration but not phosphatase activity (80). Cai *et al.* (81) developed a FRET-based screening method to detect potential inhibitors of the binding of CNNM3 to PRL-2.

Surprisingly, the most promising approach has been the design of antibodies against PRL-3 for use in immunotherapy to kill PRL-3 expressing tumors (82). This unconventional approach is based on the presence of PRL-3 antigens on the cell surface, which leads to antibody-dependent cytotoxicity or phagocytosis of the tumor cells. The humanized antibody, PRL3-zumab, reduced tumor relapse in an animal model and has been approved for Phase 2 clinical trials against solid tumors in Singapore, United States, and China (83).

Perspectives and conclusions

There are many unanswered questions about the biological function of PRLs but also signs that the gaps in our understanding are closing. One promising development is the realization that CNNM proteins are associated with another family of proteins, the melastatin-related transient receptor potential ion channels, TRPM6 and TRPM7 (61, 84–87). Like CNNMs, TRPM6/7 are important for magnesium homeostasis and have been implicated in cancer (55, 88–90). The convergence of these two well-established research fields promises new insights.

Finally, while many groups still consider PRLs to function primarily as phosphatases, there is increasing acceptance that PRLs also act as pseudophosphatases and that the two activities are inherently linked. CNNM binding blocks PRL catalytic activity and PRL catalytic activity generates the phosphoenzyme intermediate that is unable to bind CNNMs. Identifying the mechanisms that link these to magnesium levels and cell signaling pathways are key challenges for the future.

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Abbreviations—The abbreviations used are: CBP, cysteine-based phosphatase; CBS, cystathionine-β-synthase; CDC14, cell division cycle 14; DUSP, dual-specificity phosphatase; PRL, Phosphatase of regenerating liver; PTP, protein tyrosine phosphatase.

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