Identification of proteins suppressing the functions of oncogenic phosphatase of regenerating liver 1 and 3

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Abstract. The phosphatase of regenerating liver (PRL) family, including PRL-1, PRL-2, and PRL-3, comprises protein tyrosine phosphatases whose deregulation is associated with the tumorigenesis and metastasis of many types of cancer. However, the underlying mechanism is poorly understood. In this study, aiming to increase understanding of the molecular mechanisms underlying the functions of PRL-1 and PRL-3, a yeast two-hybrid system was employed to screen for their interacting proteins. Alignment with the NCBI BLAST database revealed 12 interactive proteins: Synaptic nuclear envelope protein 2, emerin, mannose 6-phosphate receptor-binding protein 1, low-density lipoprotein receptor-related protein 10, Rab acceptor 1, tumor protein D52-like 2, selectin P ligand (SELPLG), guanylate binding protein 1, transmembrane and ubiquitin-like domain-containing 2, NADH:ubiquinone oxidoreductase subunit B8, syndecan 4 and FK506-binding protein 8 (FKBP8). These proteins are associated with cell proliferation, apoptosis, immune response, cell fate specification and metabolic process in biological process categories, and involved in various signaling pathways, including Alzheimer's disease, Parkinson's disease, Huntington's disease, hypertrophic cardiomyopathy and cell adhesion molecules. Interactions of PRL-1 with the prey proteins SELPLG and FKBP8 were confirmed by immunoprecipitation or immunostaining. Furthermore, SELPLG and FKBP8 suppressed PRL-1- or PRL-3-mediated p53 activity. Identification of the proteins interacting with PRL family proteins may provide valuable information to better understand the mechanism of PRL-mediated signal transduction in cancer and other diverse diseases.

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Introduction

Phosphatases of the regenerating liver [PRLs; also known as the protein tyrosine phosphatase type IVA (PTP4A) family] were originally identified as immediate-early genes in the regenerating liver (1). The PRL family is a group of protein tyrosine phosphatases (PTPs) and plays a role in the development and metastasis of various cancers, including colorectal, prostate, breast, gastric and liver cancers, and particularly in metastatic cancers (2,3). The PRL family comprises three genes: PRL-1, PRL-2 and PRL-3. The overexpression of the PRL family has been frequently reported in various cancers, especially in metastatic cancers (4-8). Overexpression of PRLs in normal cells has been found to promote proliferation, migration, and invasion (4,8,9) whereas the reduction of PRLs in cancer cells using small interfering RNA (siRNA) has been shown to inhibit cell motility and metastatic characteristics in a mouse model (10).

PRLs affect a number of signaling pathways associated with cell growth and cancer development. During tumorigenesis, PRLs have been found to modulate integrin β1-extracellular signal-regulated kinase 1/2, phosphoinositide 3-kinase/AKT, keratin 8, C-terminal Src kinase, Rho GTPase, cyclin-dependent kinase 2, p53 and FK506-binding protein 8 (FKBP8) signaling pathways (9,11-19).

Although it is important to elucidate the role of PRLs in cancer progression and the signaling pathways they affect, a major challenge to the analysis of the detailed signaling mechanism of PRLs is the lack of a physiologically relevant substrate and knowledge of its regulation by physical interaction. Several PRL-interacting proteins such as activating transcription factor-7, β-subunit of geranylgeranyl transferase-II, cadherin 22, ezrin, elongation factor 2, keratin 8, integrin-α1, PRL-1 (trimer), PRL-3 (oligomer) and FKBP8 have been reported (1,11,16,20-27).

PRL family members have been identified to be useful biomarkers and therapeutic targets in cancer as well as in metastatic cancer due to the aforementioned properties (1,3,27). However, little is known about the proteins that bind to PRL

and regulate PRL function or are regulated by PRL. Therefore, in the present study, to screen for novel PRL-interacting proteins, yeast two-hybrid methodology was applied using PRL-1 and PRL-3 as bait. The identification of PRL-binding proteins may be useful in providing a novel insight into the mechanisms of tumorigenesis and other diseases, and might eventually lead to the development of more effective therapies.

Materials and methods

Cell culture, plasmid and reagents. HEK293T, HeLa and U2OS cell lines (American Type Culture Collection, Manassas, VA, USA) were cultured under an atmosphere of 5% CO_2 at 37˚C in Dulbecco's modified Eagle's medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Gibco-BRL; Thermo Fisher Scientific, Inc., Waltham, MA, USA). cOmplete™ Mini Protease Inhibitor Cocktail tablets and Phosphatase Inhibitor Cocktail tablets were obtained from Roche Applied Science (Penzberg, Germany). Antibody against high availability (HA) probe was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA; cat. no. SC-805) and antibodies against Flag® M2 (cat. no. F3165) and β-actin (cat. no. A5441) were purchased from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany).

Flag-PRL-1 and Flag-PRL-3 (12,19,28) were digested with restriction enzymes (*Eco*RI/*Xho*I) and cloned into the yeast expression vector pLexA (Clontech Laboratories, Inc., Mountainview, CA, USA) to form pLexA-PRL-1 and pLexA-PRL-3, respectively. The authenticity and correct orientation of the cloned sequence were then confirmed by restriction digestion and polymerase chain reaction (PCR).

Two cDNA clones encoding FKBP8 and SELPLG from pJG4-5 (Clontech Laboratories, Inc.) were inserted into a pcHA vector (Addgene vector database) to express their proteins in mammalian cells. Prey genes were digested with restriction enzymes (EcoRI/XhoI) and cloned into the mammalian expression vector pcHA. Insertion of the prey genes were confirmed by restriction enzyme digestion and nucleotide sequencing.

PCR. The DNA used for the PCR was obtained from bacterial plasmid DNA (Bioneer Corporation, Daejeon, Korea). PCR was performed with the following primer pairs: PRL-1 forward, 5'-TACACACAATCCAACCAATG-3', and reverse, 5'-AATTAATGCTAGGGCAACAA-3', and PRL-3 forward, 5'-TCATTGAGGACCTGAAGAAG-3', and reverse, 5'-CTCAGCCAGTCTTCCACTAC-3'. PCR pre-mix was used for the reaction (Bioneer Corporation). In each reaction, 20 μl final reaction mixture contained 10 μl Premix *Taq*, 0.8 ml PCR forward primer (10 mm), 0.8 ml reverse primer (10 mm), 2 μl DNA (100 ng/μl) and dH_2O. Subsequently, the reaction mixture was incubated at 95˚C for 5 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 45 sec with 20 cycles. 1.5% agarose gel was used for electrophoresis of the PCR product.

Screening of a HeLa library and selection of proteins interacting with PRL-1 and PRL-3. The cDNA from a HeLa library (Clontech Laboratories, Inc.) was sub-cloned into pJG4-5 vectors (Clontech Laboratories, Inc.) for yeast two-hybrid screening. The EGY48 yeast strain (Clontech Laboratories, Inc.) was transformed with pLexA-PRL-1 or pLexA-PRL-3 by a small-scale yeast transformation protocol (28) and plated onto synthetic defined (SD)/-Trp1 (without yeast gene Trp1) medium (Sigma-Aldrich) and grown at 30˚C for 2-4 days. Selected clones were grown in 2 ml yeast extract peptone dextrose medium containing ampicillin at 30˚C overnight with shaking. The yeast strain expressing LexA-PRL-1 or PRL-3 bait protein was transformed with the HeLa cDNA library fused to the GAL-4 activation domain by the lithium acetate method (large-scale yeast transformation protocol) (28). The cDNA library was screened using a yeast two-hybrid system (Matchmaker LexA two-hybrid system; Clontech Laboratories, Inc.) to detect interacting proteins, according to the manufacturer's protocol. Positive clones were selected and assayed for lacZ reporter activity using a filter β-galactosidase assay with X-Gal. Plasmids from positive yeast clones were isolated and transformed into competent cells. Plasmids isolated from competent cells were transformed into XL1-blue competent cells (Agilent Technologies, Inc.- Santa Clara, CA, USA) for analysis of the insert size and for sequencing. The interaction between LexA-PRl-1 or PRL-3 and positive clones was confirmed by small-scale yeast transformation.

DNA sequences were determined (Bioneer Corporation) and nucleotide sequence databases were searched for homologous sequences by Basic Local Alignment Search Tool (BLAST) analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Transfection, immunoprecipitation and immunoblot analysis. PRL-1 or PRL-3 expression vectors were transfected into each cell line (HEK293T, HeLa and U2OS) using Lipofectamine Plus (Gibco-BRL; Thermo Fisher Scientific, Inc.), using the manufacturer's protocol. After 48 h, the cells were washed and lysed with lysis buffer containing 150 mM NaCl, 0.1% Nonidet P-40 and 50 mM Tris-Cl (pH 7.4). Detergent-insoluble materials were removed via centrifugation (1,000 x *g*), and the clear lysates were incubated with anti-Flag® M2 antibody (1:500) and Protein G Plus Agarose beads for 4 h (Santa Cruz Biotechnology, Inc.). The beads were washed three times with lysis buffer (29). For immunoblotting, coprecipitates or whole cell extracts were resolved via 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and subsequently transferred to nitrocellulose membranes. The membranes were immunoblotted with anti-HA (1:10,000) and anti-Flag® M2 (1:2,000) antibodies and then developed with an enhanced chemiluminescence detection system (Thermo Fisher Scientific, Inc.).

Immunofluorescence analysis. U2OS cells (50,000) were plated on coverslips pretreated with 0.1% gelatin in 12-well dishes, then transfected with indicated expression vectors (HA-SELPLG, HA-FKBP8 and/or Flag-PRL-1) and incubated for 2 days. The transfected cells were washed with phosphate-buffered saline (PBS), fixed for 20 min in 4% (w/v) paraformaldehyde, permeabilized for 10 min at room temperature with PBS containing 0.3% (v/v) Triton X-100, and further incubated for 10 min in 1% bovine serum albumin (Sigma-Aldrich). Samples were subsequently incubated for 1 h with primary antibodies anti-HA (1:10,000) and anti-Flag® M2

(1:2,000), washed three times with PBS, and then incubated with Alexa Fluor 488-conjugated goat antibody against mouse IgG and Alexa Fluor 594-conjugated goat antibody against rabbit IgG (Molecular Probes; Thermo Fisher Scientific, Inc.). The coverslips were mounted on glass slides in Vectashield medium (Vector Laboratories, Inc., Burlingame, CA, USA). Images were acquired using a Leica 6000 microscope (Leica Microsystems, Inc., Buffalo Grove, IL, USA). For DAPI staining, 1 ml DAPI (3 μM) in staining buffer (100 mM Tris, pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% Nonidet P-40) was added to each cell sample and incubated for 15 min at room temperature.

Dual-luciferase assay. HeLa cells were transfected with pRGC-luc (28), along with each expression vector (HA-SELPLG, HA-FKBP8, Flag-PRL-1 and/or Flag-PRL-3) as indicated using Lipofectamine Plus. The cells were lysed, and the luciferase activity was evaluated using a dual luciferase assay kit (Promega Corporation, Madison, WI, USA). The data were normalized to the expression levels of a cotransfected *Renilla* luciferase activity reporter control.

Functional classification, pathway analysis and protein interaction network. The 12 identified proteins were sorted by pathway and the Gene Ontology (GO) categories using the DAVID database. SELPLG was selected in the Biocarta pathway. For the network of the PRL-1, PRL-3 and prey proteins, the cellular protein interaction network was constructed based on the screened proteins in this study and in the STRING database.

Results

Screening of interacting proteins with PRL-1 or 3 using a yeast two-hybrid system. The PRL family plays a significant role in the development and cancer metastasis, and shares a high degree of sequence similarity. Notably, PRL-3 has >75% amino-acid sequence similarity to PRL-1, with a conserved function (1,27,30).

To screen novel PRL-interacting proteins, human PRL-1 and PRL-3 were used as bait in a yeast two-hybrid system. Flag-PRL-1 and Flag-PRL-3 were digested with restriction enzymes (*Eco*RI/*Xho*I) and the inserts were cloned into the yeast expression vector pLexA (Fig. 1A). To confirm the cloning, PCR products of full length PRL-1 and PRL-3 from pLexA-PRL-1 and pLexA-PRL-3 were identified by nucleotide electrophoresis (data not shown). In addition, the inserts of PRL-1 and PRL-3 from pLexA-PRL-1 and pLexA-PRL-3 were investigated by nucleotide electrophoresis following digestion with same restriction enzymes (Fig. 1B). Also, the sequence and the orientation of the inserts were confirmed by sequencing analysis. Finally, the expression of the PRL-1 bait in yeast EGY48 was confirmed by western blotting (Fig. 1C).

A HeLa cDNA library was transformed in yeast EGY48 strains transformed with pLexA-PRL-1 or pLexA-PRL-3 bait vector expressing PRL-1 or PRL-3 and cultured at 30˚C for 2-4 days until colonies appeared. Finally 38 blue colonies were observed on SD/-His/-Leu/-Trp/X-Gal plates, the colonies were inoculated in SD/-Leu/-Trp liquid medium and the plasmids were extracted. Purified plasmids were retransformed in yeast EGY48 strains containing pLexA-PRL-1 or PRL-3 bait vector and blue colonies were observed again on SD/-His/-Leu/-Trp/X-Gal plates (data not shown). Plasmids isolated from yeast were transformed into XL1-blue competent cells for further analysis of the insert size and for sequencing. Inserted fragments of library plasmids were mostly between 500 and 2,000 bp in size. Identity of the prey was determined by performing BLAST search analysis. The results of the BLAST search against the human gene database indicated that 12 genes interact with PRL-1 or PRL-3: Synaptic nuclear envelope protein 2 (SYNE2), emerin (EMD), mannose 6-phosphate receptor-binding protein 1 (perilipin 3; PLIN3), low-density lipoprotein receptor-related protein 10 (LRP10), Rab acceptor 1 (RABAC1), tumor protein D52-like 2 (TPD52L2), selectin P ligand (SELPLG), guanylate binding protein 1 (GBP1), transmembrane and ubiquitin-like domain-containing 2 (TMUB2), NADH:ubiquinone oxidoreductase subunit B8 (NDUFB8), syndecan 4 (SDC4) and FKBP8 (Table I) were identified. Among them, 9 prey proteins were isolated from screening using PRL-1 bait and 6 prey proteins were obtained from screening using PRL-3 bait. There were 3 prey proteins, namely NDUFB8, FKBP8 and SDC4, that were identified from both PRL-1 and PRL-3 baits (Fig. 1D).

In vivo binding and colocalization. From among the 12 candidate genes interacting with PRL-1 or PRL-3, two cDNA clones encoding for FKBP8 and SELPLG were inserted into pcHA vector to express their proteins in mammalian cells. Prey genes were digested with restriction enzymes (*Eco*RI/*Xho*I) and cloned into the mammalian expression vector pcHA. Insertion of the prey genes was confirmed by restriction enzyme digestion and nucleotide sequencing (Fig. 1E).

To confirm their binding in a yeast-independent interaction assay, coimmunoprecipitation experiments were performed. HEK293T cells were co-transfected with Flag-PRL-1 and HA-FKBP8 or HA-SELPLG constructs, and cell extracts were then subjected to immunoprecipitation with anti-Flag antibody, followed by immunoblotting analysis with an anti-HA antibody. HA-tagged FKBP8 and SELPLG were detected in anti-Flag-PRL-1 immunoprecipitates (Fig. 2A).

The localization of bait proteins and prey proteins was then examined. U2OS cells were transfected with Flag-PRL-1, and HA-FKBP8 or HA-SELPLG. Localization of FLAG tagged-PRL-1 was visualized with anti-FLAG primary antibody and Fluor 488-conjugated goat antibody against mouse IgG and localization of HA-tagged preys was visualized with anti-HA antibody and Alexa Fluor 594-conjugated goat antibody against rabbit IgG.

In cells, PRLs are typically associated with the plasma membrane and early endosome (1,27,30). An important mechanism responsible for this localization is prenylation, a post-translational lipid modification that commonly targets proteins to membranes (3,27,30). Fig. 2B and Table II show that PRL-1 localization is observed in the endosome, early endosome, endoplasmic reticulum, spindle, cytoskeleton, plasma membrane, microtubule cytoskeleton and intracellular non-membrane-bounded organelle. SELPLG is visible in the membrane fraction, insoluble fraction, plasma membrane, and is integral to the plasma membrane while FKBP38

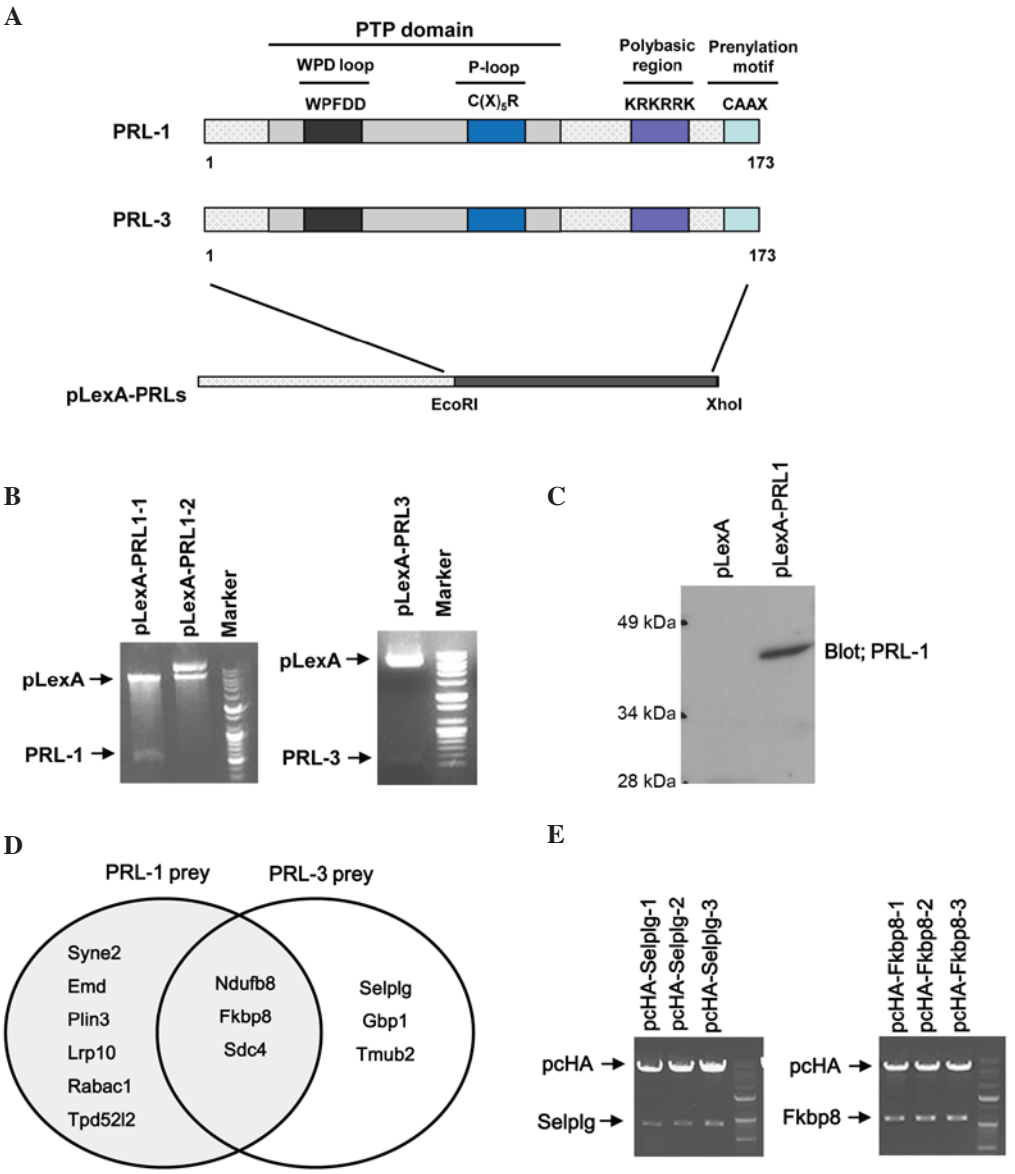

Figure 1. Screening of interacting proteins with PRL-1 or -3 using a yeast two-hybrid system. (A) Schematic representation of the PRL-1 and PRL-3 cloning strategy for the yeast two-hybrid assay. Full length PRL-1 and -3, containing a PTP domain, polybasic region and prenylation motif were inserted into pLexA bait vector. (B) Construction and identification of the bait vectors. Gel electrophoresis images of the vector (pLexA) and bait genes (PRL-1 and PRL-3). The plasmids were digested with *Eco*RI and *Xho*I, and samples were loaded onto agarose gel. (C) Expression of bait fusion protein in yeast. EGY48 yeast strain transformed with pLexA-PRL-1 or empty vector (pLexA) as a control were lysed and resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis for western blotting using anti-PRL-1 antibody. (D) Diagram of screening results and cloning of prey genes in mammalian expression vector. Twelve prey genes were identified from screening; 9 genes were isolated from screening using PRL-1 bait and 6 genes were isolated from PRL-3 bait screening. (E) Selplg and Fkbp8 prey cDNAs were inserted into pcHA vector for mammalian expression and digested with *Eco*RI and *Xho*I restriction enzymes. PRL, phosphatase of regenerative liver. PTP, protein tyrosine phosphatase.

is observed in the mitochondrial envelope, endoplasmic reticulum membrane, plasma membrane, endomembrane system and nuclear envelope-endoplasmic reticulum network (Fig. 2B and Table II). The expression of SELPLG and FKBP38 appears to be partially colocalized with PRL-1. In the presence of preys, changes in the localization of PRL-1 were not observed, suggesting that the expression of these preys does not affect the prenylation and localization of PRL-1.

SELPLG and FKBP8 inhibit the functions of PRL-1 and PRL-3. Having verified the binding of FKBP8 and SELPLG with PRL-3 protein, the next important question is whether FKBP8 and SELPLG affect the functions of PRL-1 and PRL-3 in cells. The roles of PRL-1 and PRL-3 are associated with the downregulation of p21 transcription as well as the activity of p53 (28). Therefore, the effects of two prey proteins on the downregulation of p53 reporter activities mediated by PRL-1 and PRL-3 were investigated. HeLa cells were transfected with each prey protein and/or Flag-PRL-1 (or Myc-PRL-3) and p53-luciferase reporter (pRGC-luc) (Fig. 3). When p53-luc was transfected with PRL-1 or PRL-3, inhibition of luciferase activity was observed (Fig. 3A), as shown previously (28). However, SELPLG and FKBP8 markedly attenuated the PLR-1-mediated p53-luc inhibition (Fig. 3A). Also, similar results were observed when SELPLG

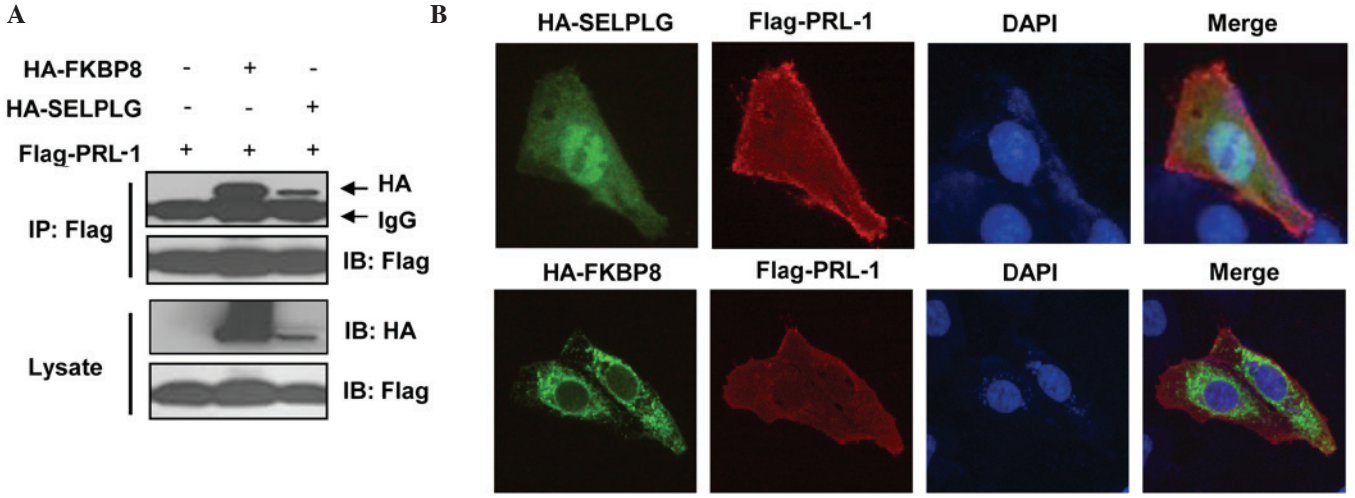

Figure 2. *In vivo* binding and colocalization. (A) FKBP8 and SELPLG interact with PRL-1. Flag-PRL-1 and/or HA-FKBP8 or HA-SELPLG were transfected into HEK293T cells. The cells were treated with MG132 for 4 h prior to harvesting, and 48 h later, the cells were prepared for co-IP and western blot analysis. (B) Colocalization of FKBP8 or SELPLG with PRL-1. Flag-PRL-1 and HA-FKBP8 or HA-SELPLG were transfected in U2OS cells. Then, 48 h later, the cells were prepared for immunofluorescence analysis. Images were acquired using a Leica 6000 microscope (magnification, x200). FKBP8, FK506-binding protein 8; SELPLG, selectin P ligand; PRL-1, phosphatase of regenerating liver 1; HA, high availability; DAPI, 4',6-diamidino-2-phenylindole; IP, immunoprecipitation; IB, immunoblotting.

and FKBP8 were introduced with PRL-3 (Fig. 3B). These findings reveal that SELPLG and FKBP8 inhibit the ability of PRL-1 and PRL-3 to reduce p53 reporter activity and imply that SELPLG and FKBP8 inhibit the cellular functions of PRL-1 and PRL-3.

Functional classification, pathway analysis and protein interaction network. The identified proteins were sorted according to pathways and GO categories using the DAVID bioinformatics resource. Pathways for SELPLG were identified using the BioCarta pathway database (data not shown). Pathways for NDUFB8, EMD, SELPLG and SDC4 were identified using KEGG pathway analysis and contained oxidative phosphorylation, Alzheimer's disease, Parkinson's disease, Huntington's disease, hypertrophic cardiomyopathy, arrhythmogenic right ventricular cardiomyopathy, dilated cardiomyopathy, cell adhesion molecules, adhesion and diapedesis of granulocytes, cells and molecules involved in local acute inflammatory response, and extracellular matrix (ECM)-receptor interaction (Table III). Among the 12 proteins, there were 9 proteins involved in diverse biological processes including vesicle transport, protein folding, cell proliferation, apoptosis, immune response, cell fate specification and metabolic process (Table IV). Cellular component data showed that the localizations of the 12 proteins mostly or partly matched with those of PRL-1 or PRL-3 (Table II).

A PRL-1 and PRL-3-prey proteins interaction network was constructed using the STRING database (Fig. 4). SDC4, PLIN3, SYNE2, TPD52L2, EMD and FKBP8 were indicated to by the most closely-related and specific node proteins associated with PRL-3, whereas SELPLG, GBP1, RABAC1 and NDUFB8 were the most remarkable node proteins associated with PRL-1. TMUB2 and LRP10 did not show any indirect interactions with PRL-1 or PRL-3 (Fig. 4). These notable node proteins appear to be particularly important in the regulation and organization of PRL-1 and PRL-3 in the prey proteins interaction network.

Discussion

The PRL family comprises a group of PTPs that play an important role in the development and metastasis of various types of cancer (12). The family members, which include PRL-1, PRL-2 and PRL-3, share a high degree of sequence similarity and show similar functional characteristics. It has been reported that several signaling pathways involved in cell growth and cancer development are affected (regulated by) PRLs (3,4). However, the mechanisms by which PRLs regulate signaling or interact with direct binding partners to mediate their effects remains to be clearly elucidated.

In the present study, 12 proteins interacting with PRL-1 or PRL-3 were identified using a yeast two-hybrid system. GO biological process data indicated that these proteins are mostly associated with nuclear envelope organization, endomembrane organization and nucleus organization (Table IV). Cellular components data suggest that they are located at membrane parts, integral to membrane, intrinsic to membrane, envelope, nuclear membrane, contractile fiber part, myofibril, organelle membrane and nuclear envelope (Table II). Molecular functions of 6 genes were classified as protein binding (data not shown). They were also found to be involved in various signaling pathways such as oxidative phosphorylation, Alzheimer's disease, hypertrophic cardiomyopathy, ECM-receptor interaction and cell adhesion molecules in KEGG pathways (Table III).

FKBP8 is a member of the FKBP family of proteins, and is widely expressed in cancer cell lines (31,32). In cancer, FKBP8 has potential antitumor effects via the regulation of anti-invasive syndecan 1, proinvasive matrix metalloproteinase 9 (33,34), mechanistic target of rapamycin, Rheb-GTP (35) and PRL-3 (28). Results of our previous study showed that FKBP8

Table I. List of the identified preys from screening.

Prey no.	Bait	Symbol	Full name	No. of clones
1	PRL-1	SYNE2	Synaptic nuclear envelope protein 2	4
2	PRL-1	EMD	Emerin	2
3	PRL-1	PLIN3	Mannose 6-phosphate receptor-binding protein 1	4
4	PRL-1	LRP10	Low-density lipoprotein receptor-related protein 10	2
5	PRL-1	RABAC1	Rab acceptor 1	2
6	PRL-1	TPD52L2	Tumor protein D52-like 2	3
7	PRL-3	SELPLG	Selectin P ligand	4
8	PRL-3	GBP1	Guanylate binding protein 1	2
9	PRL-3	TMUB2	Transmembrane and ubiquitin-like domain-containing 2	2
10	PRL-1, PRL-3	NDUFB8	NADH:ubiquinone oxidoreductase subunit B8	4
11	PRL-1, PRL-3	FKBP8	FK506-binding protein 8	6
12	PRL-1, PRL-3	SDC4	Syndecan 4	3

PRL, phosphatase of regenerating liver.

Table II. Analysis of the cellular components associated with the identified proteins, based on the cellular components gene ontology categories of DAVID.

Gene	Cellular components
FKBP8	Mitochondrial envelope, endoplasmic reticulum membrane, plasma membrane, nuclear envelope-endoplasmic reticulum network
NDUFB8	Mitochondrion, mitochondrial envelope, endoplasmic reticulum, integral to membrane, NADH dehydrogenase complex
RABAC1	Golgi apparatus, plasma membrane, synaptic vesicle, integral to membrane, cell junction, membrane-bounded vesicle, synapse
EMD	Nuclear envelope, endoplasmic reticulum, spindle, cytoskeleton, endomembrane system, microtubule cytoskeleton, nuclear membrane
GBP1	Plasma membrane, internal side of plasma membrane, plasma membrane part
LRP10	Coated pit, endomembrane system, integral to membrane, intrinsic to membrane
PLIN3	Endosome, Golgi apparatus, lipid particle, plasma membrane, internal side of plasma membrane, monolayer-surrounded lipid storage body
SELPLG	Cell fraction, membrane fraction, insoluble fraction, plasma membrane, intrinsic to plasma membrane
SYNE2	Nuclear envelope, cytoskeleton, plasma membrane, endomembrane system, integral to membrane, nuclear membrane
SDC4	Golgi apparatus, plasma membrane, adherens junction, focal adhesion, cell surface, cell-substrate junction, membrane raft, anchoring junction
TMUB2	Integral to membrane, intrinsic to membrane
TPD52L2	Perinuclear region of cytoplasm
PRL-1	Endosome, endoplasmic reticulum, spindle, cytoskeleton, plasma membrane, microtubule cytoskeleton
PRL-3	Endosome, early endosome, plasma membrane

DAVID, Database for. Annotation, Visualization, and Integrated Discovery.

binds to PRL-3, and suppresses PRL-3-mediated p53 activity and cell proliferation (28). The present study also provided evidence that FKBP8 binds to PRL-1, and suppresses the function of PRL-1, in addition to that of PRL-3.

SELPLG is a glycoprotein that acts as a counter-receptor for the cell adhesion molecules P-, E- and L-selectin expressed on myeloid cells and T lymphocytes (36). In leukocyte trafficking during inflammation, SELPLG tethers leukocytes

Table III. Signal pathway analysis of the identified proteins, based on the pathway categories of DAVID.

Gene	Signaling pathway
NDUFB8	Oxidative phosphorylation, Alzheimer's disease, Parkinson's disease, Huntington's disease
EMD	Hypertrophic cardiomyopathy, arrhythmogenic right ventricular cardiomyopathy, dilated cardiomyopathy
SELPLG	Cell adhesion molecules, adhesion and diapedesis of granulocytes, cells and molecules involved in local acute inflammatory response
SDC4	ECM-receptor interaction, cell adhesion molecules

DAVID, Database for. Annotation, Visualization, and Integrated Discovery; ECM, extracellular matrix.

Table IV. Biological process analysis of the identified proteins, based on the biological process gene ontology categories of DAVID.

Gene	Biological process
FKBP8	Cell fate specification, regionalization, protein folding, apoptosis, smoothened signaling pathway, pattern specification process, dorsal/ventral pattern formation, neural tube patterning and development, regulation of BMP signaling pathway, chordate embryonic development
NDUFB8	Oxidative phosphorylation, mitochondrial electron transport, NADH to ubiquinone, phosphorus metabolic process, energy derivation by oxidation of organic compounds, phosphorylation, cellular respiration, oxidation reduction
EMD	Muscle system process, muscle contraction, nucleus organization, nuclear envelope organization, muscle organ development, endomembrane organization, membrane organization, nuclear envelope reassembly
GBP1	Immune response
LRP10	Lipid transport, endocytosis, membrane invagination, lipid localization, membrane organization, vesicle-mediated transport
PLIN3	Vesicle-mediated transport
SELPLG	Cell motion, leukocyte adhesion, cell-cell adhesion, cell migration, biological adhesion, cellular extravasation, cell motility, leukocyte migration, leukocyte tethering or rolling, localization of cell
SDC4	Regulation of muscle contraction, regulation of phosphate metabolic process, regulation of phosphorylation, positive regulation of catalytic activity, regulation of kinase activity, regulation of system process, regulation of molecular function, regulation of transferase activity
TPD52L2	Regulation of cell proliferation
PRL-1	Protein amino acid dephosphorylation, phosphate metabolic process, cell cycle, regulation of cell migration, regulation of locomotion, regulation of cell motion
PRL-3	Protein amino acid dephosphorylation, phosphorus metabolic process, phosphate metabolic process

DAVID, Database for. Annotation, Visualization, and Integrated Discovery; BMP, bone morphogenetic protein.

to activating platelets or selectin-expressing endothelia. SELPLG requires post-translational modification by tyrosine sulfation and addition of the sialyl-Lewis-x tetrasaccharide for its high-affinity binding activity. Aberrant expression of and polymorphisms in the SELPLG gene are associated with defects in the innate and adaptive immune response.

In the present study, 12 potential PRL-1/3 binding proteins were identified, including 11 novel binding partners and a known binding partner, FKBP8. SELPLG and FKBP8 proteins were shown to directly bind to PRL-1 and inhibit the downregulation of p53 reporter activities mediated by PRL-3 and PRL-1. These results demonstrate that SELPLG and FKBP8 may be regulators of the oncogenic proteins PRL-1 and PRL-3 and can have a marked impact on cell proliferation.

It is possible that the 12 PRL-binding proteins positively or negatively regulate PRL function (FKBP8 and SELPLG) or may be regulated by PRLs. In regard to this hypothesis, further studies are underway to reveal those mechanisms.

In conclusion, multiple PRLs binding proteins were screened using a yeast two-hybrid system. The identified proteins are associated with diseases including Alzheimer's disease, Parkinson's disease, Huntington's disease, hypertrophic cardiomyopathy, arrhythmogenic right ventricular cardiomyopathy and dilated cardiomyopathy, suggesting that the PRL family may be involved in diverse diseases as well as cancer. Furthermore, these findings may provide valuable information for better understanding the interactions between the PRL family and target proteins, and revealing new biological functions of PRLs.

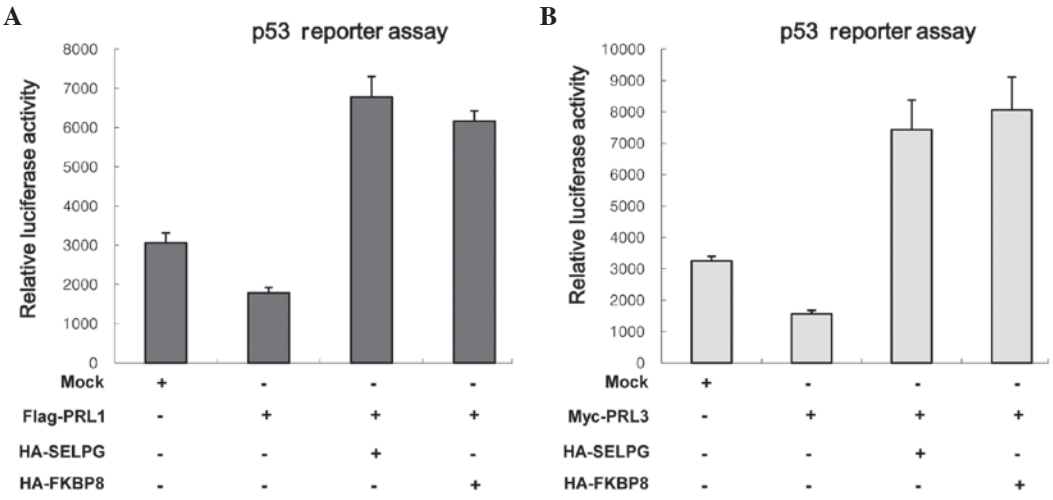

Figure 3. SELPLG and FKBP8 suppress the function of PRL-1 and -3. (A) The effect of PRL-1 on the p53 reporter is attenuated by SELPLG and FKBP8. The p53 reporter vector (100 ng) was transfected with PRL-1 (200 ng) plus HA-FKBP8 or HA-SELPLG (0 or 400 ng) as indicated. (B) The effect of PRL-3 on the p53 reporter is recovered by SELPLG and FKBP8. The p53 reporter vector (100 ng) was transfected with PRL-3 (200 ng) plus HA-FKBP8 or HA-SELPLG (0 or 400 ng) as indicated. A *Renilla* luciferase reporter was included in all transfection mixes and employed for normalization. The relative luciferase activity (fold by luciferase value) was calculated by dividing each normalized average luciferase value by the normalized average mock luciferase value. The data are expressed as the means ± standard deviation (n=4). FKBP8, FK506-binding protein 8; SELPLG, selectin P ligand; PRL, phosphatase of regenerating liver.

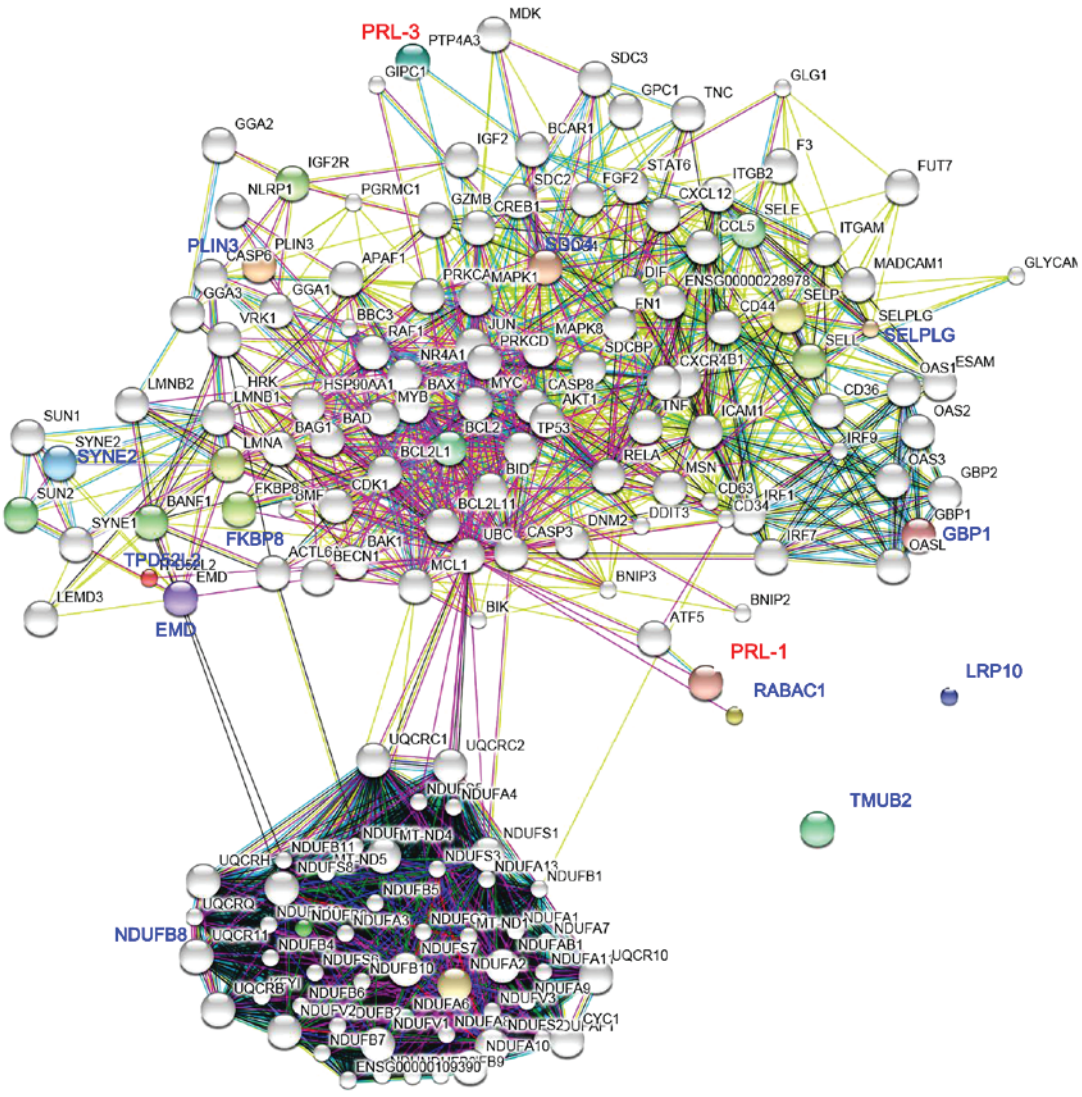

Figure 4. PRL-1, PRL-3 and prey proteins network. The PRL-1, PRL-3-to-cellular protein interaction network was constructed based on the screened proteins in this study using the Search Tool for the Retrieval of Interacting Genes/Proteins database. PRL, phosphatase of regenerating liver.

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