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In vitro characterization of the RS motif in N-terminal head domain of goldfish germinal vesicle lamin B3 necessary for phosphorylation of the p34cdc2 target serine by SRPK1☆

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ABSTRACT

The nuclear envelopes surrounding the oocyte germinal vesicles of lower vertebrates (fish and frog) are supported by the lamina, which consists of the protein lamin B3 encoded by a gene found also in birds but lost in the lineage leading to mammals. Like other members of the lamin family, goldfish lamin B3 (gfLB3) contains two putative consensus phosphoacceptor p34cdc2 sites (Ser-28 and Ser-398) for the M-phase kinase to regulate lamin polymerization on the N- and C-terminal regions flanking a central rod domain. Partial phosphorylation of gfLB3 occurs on Ser-28 in the N-terminal head domain in immature oocytes prior to germinal vesicle breakdown, which suggests continual rearrangement of lamins by a novel lamin kinase in fish oocytes. We applied the expression-screening method to isolate lamin kinases by using phosphorylation site Ser-28-specific monoclonal antibody and a vector encoding substrate peptides from a goldfish ovarian cDNA library. As a result, SRPK1 was screened as a prominent lamin kinase candidate. The gfLB3 has a short stretch of the RS repeats (9-SRASTVRSSRRS-20) upstream of the Ser-28, within the N-terminal head. This stretch of repeats is conserved among fish lamin B3 but is not found in other lamins. *In vitro* phosphorylation studies and GST-pull down assay revealed that SRPK1 bound to the region of sequential RS repeats (9–20) with affinity and recruited serine into the active site by a grab-and-pull manner. These results indicate SRPK1 may phosphorylate the p34cdc2 site in the N-terminal head of GV-lamin B3 at the RS motifs, which have the general property of aggregation.

1. Introduction

The nuclear lamina supports the inner nuclear membrane and contributes to size, mechanical stability, and nuclear shapes. The major structural proteins are nuclear lamins that belong to the intermediate filament protein family and classified into A- (lamin A and lamin C) or B-type (lamins B1 and B2) based on their cDNA sequence homologies, biochemical properties, and behavior during mitosis in vertebrates. B-type lamins are expressed ubiquitously during development; in contrast, lamin A expression is developmentally regulated. In lower vertebrates (fishes, amphibians, and birds), three genes encoding B-type lamins (B1, B2, and B3) are known. Little information is available on the structure and/or assembly state of the lamins within nuclei, although they form filamentous structures in some types of cells. Fish and amphibian oocytes are giant cells with huge nuclei, called germinal vesicles (GV), that are approximately 100,000-fold larger in volume than typical somatic nuclei, with condensed chromosomes in late meiotic prophase (diplotene), but having similar DNA content. This arrangement enables easy manipulation of intact lamina structures without chromatin and other proteins. The composition of lamins in oocytes differs from that of those in somatic cells. The protein lamin B3 is the major component of the GV-lamina [1–3], in which 10-nm IF-like filaments form a lattice of remarkable organization [4,5] and form compressed superstructures in native GV-lamina [6].

A major function of lamin is to help maintain the structure of the nuclear envelope. Nuclear lamina must be disassembled during mitosis and reassembled after mitosis. Lamins are intermediate filaments (IFs), and like other IFs (e.g., [7]), they consist of longitudinal alpha-helical rod domain flanked by a non-alpha-helical amino head, which is not predicted to fold into a distinct conformation, as well as a globular tail domain. Lamins have highly conserved Ser-containing

Abbreviations: ASF/SF2, alternative splicing factor/splicing factor 2; CLK, cdc2-like kinase; GV, germinal vesicle; LB3, lamin B3; RS repeat, arginine/serine repeat; SRPK1, serine arginine-rich protein kinase 1.

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phosphoacceptor sites (P-sites) within two regions that flank either end of the rod domain (i.e., Ser 22 and Ser 392 on human lamin A/C), although invertebrates' lamins have only P-site on N-terminal head in *Drosophila* and absence in *C. elegans*. These sites are phosphorylated during mitosis, thereby causing morphological changes involving lamina disassembly concomitant with nuclear envelope breakdown [8–11]. The mitotic cdc2 kinase has been identified as being responsible for this phosphorylation, controlling higher-order assemblies, such as longitudinal association with polar head-to-tail polymers. It is thought that phosphorylation at this site interferes with head-to-tail interaction between lamins [9,12,13]. Other kinases are also involved in lamin disassembly or disruption at an equivalent position. Human cytomegalovirus (HCMV)-encoded protein kinase UL97, a calcium-dependent protein kinase C (PKC), phosphorylates lamin A/C on sites targeted by cdc2 kinase during virus nuclear egress to mediate dissolution of nuclear lamina [14,15]. These findings suggest that lamin disassembly (or the inhibitory effect for lamin polymerization) is mainly regulated by phosphorylation at specific sites by various kinases in certain cell types and/or a cell-cycle-dependent manner [16,17], although phosphorylation of sites other than cdc2 sites can also cause lamin depolymerization [18–21].

GV-lamin B3 (LB3), as well as other A- and B-type lamins, contains two conserved cdc2-targeting serine residues on the N- and C-terminal regions flanking the rod domain. Germinal vesicle breakdown (GVBD) is a prominent event during oocyte maturation, when lamina disassembly, spindle formation, and chromosome condensation occurs concomitantly with nuclear envelope breakdown. This lamina disassembly is believed to be regulated by phosphorylation of a specific site on lamin B3 by cdc2 kinase, in much the same manner as the somatic lamins [2,22]. However, the phosphorylation status of LB3 before and during oocyte maturation remains unclear. Recently, we raised an anti-phospho-site-specific monoclonal antibody to investigate the phosphorylation state on the conserved cdc2 site (Ser-28) on goldfish LB3 (gfLB3). We found that before oocyte maturation, a part of gfLB3 was phosphorylated at the conserved cdc2 target site (Ser-28), which corresponds to Ser-22 in human lamin A, in the absence of the cdc2 kinase/cyclin B complex [23]. Furthermore, upon heterogeneous microinjection into *Xenopus* oocytes, gfLB3 with a substitution in Ser-28 for alanine (S28A) forms aggregates in the nuclear (GV) periphery. These results suggest that a novel lamin kinase phosphorylates the conserved cdc2 site (Ser-28) and regulates the localization of lamin in immature oocyte cytoplasm.

In this study, using an anti-phosphorylation Ser-28-specific monoclonal antibody (C7B8D), we applied the expression-screening method to isolate a novel lamin kinase from the goldfish ovarian cDNA library. As a result, SRPK1 was identified as a prominent candidate of Ser-28 lamin kinase.

2. Materials and methods

2.1. Fishes

Goldfish (*Carassius auratus*) were bought from dealers (Yamato-Koriyama, Nara Prefecture, Japan) and raised in the laboratory (Biotron Application Center, Kyushu University) at 17 °C. Wrasse (*Pseudolabrus sieboldi*) was caught near the Fishery Research Laboratory of Kyushu University, Fukuoka, Japan. Ovaries were immediately removed from both fishes, frozen in liquid nitrogen, and stored at −80 °C until RNA extraction.

2.2. Antibody

Anti-gfLB3 (pSer-28) specific monoclonal antibodies (C7B8D) were raised against BSA-coupled phospho-peptides (GASPSGVpSPTRLTRLQEK-C) and has been characterized previously [23]. The ascites (1:2000) or hybridoma supernatants were used for subsequent expression cloning and for detection of the phosphorylation of Ser-28 of gfLB3.

2.3. Constructs of PCR-directed mutagenic mutants of gfLB3

A cDNA coding a N-terminus head domain (corresponding to the region, 1–54 aa) of gfLB3 (AB034197) ligated with pGEM-T easy vector (Promega) was amplified by polymerase chain reaction (PCR) using a combination primer set of N-terminus primer introducing BamHl site (5′-GGATCCATGATCACCTCCACCCCGATG-3′) and C-terminus primer introducing XhoI site (5′-CTCGAGGCGCTCGATGTAGTTGGCCAG-3′). Cycling conditions were follows: 30 cycles, 1 min at 94 °C; 1 min at 55 °C; 1 min at 72 °C. This construct was used for an original template for PCR-directed mutagenesis with mutagenic primers (Table 1). Briefly, a template (100 pg) was amplified by a set of primer (10 pmol each) with DNA polymerase (PrimeStar, Takara). Cycling conditions were follows: 35 cycles 10 s at 98 °C; 15 s at 55 °C; 2 min at 72 °C (B to E, G, H to N), 10 s at 98 °C, 2 min at 68 °C 35 cycles (F and O). Mutagenic plasmids were confirmed by DNA sequencing with CEQ8800 (Beckman Coulter). The PCR fragments were double digested by BamHl and Xhol, then inserted into the corresponding sites of GST gene fusion vector pGEX-6P (GE HealthCare) in frame.

2.4. Expression of gfLB3 recombinant proteins and purification

Various N-terminal mutants derived from pGEX-gfLB3N-WT were expressed in *Escherichia coli* strain BL21 (GE HealthCare) after growing to an OD600 0.5 and inducing with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h at 25 °C. *E. coli* pellets were sonicated in PBS containing EDTA-free protease inhibitor cocktails (Roche) on ice. All GST fused gfLB3N proteins were purified with elution buffer (10 mM glutathione in 50 mM Tris–HCl pH 8.0) from the supernatants using glutathione sepharose 4B column chromatography (GE HealthCare). After dialysis against PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4), proteins were concentrated to 2 mg/ml with concentrator (AmiconUltra, Millipore), then stored at −80 °C. All GST-fused gfLB3 proteins were soluble in PBS. Protein concentration was determined by the absorbance at 595 nm using Bradford protein assay kit (Biorad) with BSA as standards.

E. coli produced full length (wild-type), and truncated gfLB3 (NΔ6, NΔ21) inserted into pET21 vector in frame were constructed, expressed and purified in the urea (6 M) according to Yamaguchi et al. [23]. Protein concentration was determined by the absorbance at 480 nm using 2-D Quant kit (GE Healthcare) with BSA as standards. After dialysis with sodium carbonate buffer (pH 11), soluble proteins (final conc. 0.2 mg/ml) were used as substrates for lamin kinase assay.

2.5. cDNA library and expression cloning

The strategies of expression cloning are according to Matsuo et al. [24] with some modification. Total RNA was prepared from goldfish ovaries with Isogen (Nippon gene, Tokyo, Japan) and polyA+ RNA was isolated with Oligotex dT30 (Takara). cDNA was synthesized with oligo-dT primer and inserted into EcoRl, Xhol sites of Uni-ZAPXR vector (Stratagene) and packaged with Gigapack III Gold packaging extracts (Stratagene). The XLI-Blue MRF' strain of *E. coli* which had been transformed with pGEX-gfLB3N-WT was cultured overnight in LB culture medium with 10 mM $MgSO_4$ for infection with expression library (1×10^5 pfu). After 3 h incubation at 42 °C, GST-gfLB3N-WT and library-originated proteins are co-expressed in the XLI-Blue by overlaying nitrocellulose membranes (Millipore) which had immersed in 10 mM IPTG on the plates. After the incubation overnight, the membranes were lifted and transferred in the 5% skim milk blocking solution in TTBS [20 mM Tris (pH 7.4), 150 mM NaCl, 0.1% Tween-20] and incubated for 1 h. After being washed the membranes twice with TTBS, once with TBS, for 15 min, membranes were incubated

Table 1
(Upper) Expression vector, mutagenic primers, and PCR templates for making gfLB3 RS motif mutant constructs. pGEX-gfLB3N-WT (1–54), named as construct A, was the initial template for mutagenesis (see also Fig. 1). Illustrations of RS motif after mutagenesis are shown in Figs. 7 and 8. Constructs, except L (*), were made as planned. L was made during PCR amplification with a set of primers, shown in brackets. After sequencing, it was used for experiments. (Lower) Expression vectors and primers used for making lamin kinase constructs. Vertical bars indicate the position at which deletion was induced. Underlined italic letters indicate that bases have been changed. Sites for restriction enzymes are underlined.

Mutant name of construct	Vector	Mutagenic primer name	Upstream primer sequence (5′–3′) downstream primer sequence (5′–3′)	Template plasmid	Amino acid sequence of RS motif (9–20) after mutagenesis
A			pGEX-gfLB3N-WT(1–54)		9-SRASTVRSSRRS-20
B		E7088A09	CGCCTGGA|CACGGTGGAGGCGCGGCT	A	SRASTV--SRRS
		E7088A08	CACCGTG|TCCAGGCGGAGCGGCGCG		
C		66934–002	GCGC*CGCTCC*GCACGGTGGAGGCGCGGCT	D	SRASTV----RS
		66394–001	CGTG*CGGAGC*GGCGCGTCTCCGTCCGGC		
D	pGEX-6P	E7088B03	ACGCGCC|CACGGTGGAGGCGCGGCT	A	SRASTV------
		E7088B02	CCACCGTG|GGCGCGTCTCCGTCCGGC		
E		E7088A10	GTGGAGGC|CTCCATCGGGGTGGAGGT	A	--ASTVRSSRRS
		E7088A11	GATGGAG|GCCTCCACCGTGCGCTCC		
F		E7088A10	GTGGAGGC|CTCCATCGGGGTGGAGGT	D	--ASTV------
		61211–003	GATGGAG|GCCTCCACCGTGGGCGCG		
G		E7088A10	GTGGAGGC|CTCCATCGGGGTGGAGGT	I	--ASTVRSSRSRRS
		E7088A11	GATGGAG|GCCTCCACCGTGCGCTCC		
H		E7088A10	GTGGAGGC|CTCCATCGGGGTGGAGGT	J	--ASTVRSSRSSSRRS
		E7088A11	GATGGAG|GCCTCCACCGTGCGCTCC		
I		E7088B05	CTCCG*GCGGGA*CCTGGAGGAGCGCACGGT	A	SRASTVRSSRSRRS
		E7088B04	CAGG*TCCCGC*CGGAGCGGCGCGTCTCCG		
J		E7088B07	CCG*GCGGGAGGAGGA*CCTGGAGGAGCGC	A	SRASTVRSSRSSSRRS
		E7088B06	CAGG*TCCTCCTCCCGC*CGGAGCGGCGCG		
K		E7088B09	CGGGAG*GCGGACCTGGA*GGAGCGCACGGT	A	SRASTVRSSRSASRRS
		E7088B08	CAGG*TCCGCCTCCCGC*CGGAGCGGCGCG		
L*		(E7088A10)	(GTGGAGGC|CTCCATCGGGGTGGAGGT)	J	SRASTV--SRRSASTVSRRS
		(E7088A11)	(GATGGAG|GCCTCCACCGTGCGCTCC)		
M		67521–002	GAGGAGCG|GCGGCTCTCCATCGGGGT	J	SR----RSSRSSSRRS
		67521–001	GAGCCGC|CGCTCCTCCAGGTCCTCC		
N		78985–002	GCC*AGCCGCTGCGGCAGC*GCTCCGCCTGGAGGAGCG	A	SRASTVRSSRRSAAAAA
		78985–001	AGC*GCTGCCGCAGCGGCT*GGCGCGTCTCCGTCCGGC		
O		78985–003	GCC*AGCCGCTGCGGCAGC*GCTCCGGCGGGAGGAGGA	J	SRASTVRSSRSSSRRSAAAAA
		78985–001	AGC*GCTGCCGCAGCGGCT*GGCGCGTCTCCGTCCGGC		

Name	Vector	Primer name	Sequence (5′–3′)
gfCLK4a	pGEX-6P	D6851C04	CGCGGATCCGCGACACGAATGCGTTCTCCTG
		D6851C05	CCGGAATTCCGGTGTTTTATGGCTTCATCC
gfSRPK1a	pCold I	D8651H04	GGGGTACCATGGAGAGAAAAGTTCTGGC
		D8651H05	CCGGAATTCCGGTGTTTTATGGCTTCATCC
gfSRPK1b		D6851C10	CCGGAATTCCGGAAAGTACTTGCACTGCAG
		D8719B07	GCTCTAGAGCCACTAGGTGGCGATCCAGG
gfDYRK2		D6851C12	CGCGGATCCGCGTCTTCCCCAGGACTGGGC
		D6851D01	CCGGAATTCCGGGCATCAGTCATTTGTGCC

with mAb C7B8D overnight at 4 °C. Following TTBS and TBS washes, membranes were incubated with HRP-coupled secondary antibody [1:20,000; goat F(ab')^2 fragment mouse IgG (H+L), Beckman Coulter] for 2 h at room temperature. Peroxidase conjugated products were visualized on X-ray film using chemiluminescence reagent (SuperSignal WestDura, Pierce). Positive plaques were isolated and plasmids (pBluscript SK-) were recovered by in vivo excision according to the user manuals. DNA sequencing was carried out after subcloning of the inserts into pBluscript SK- digested with appropriate enzymes using CEQ8800 (Beckman Coulter).

2.6. Expression of goldfish lamin kinases

Isolated cDNAs coding goldfish protein kinases (gfSRPK1-a,-b, gfDYRK2) with full length were ligated into pCold I expression vector (Takara) at the appropriate sites in frame using sets of primers for PCR (Table 1). Each constructed vectors were transformed into *E. coli* strains (Origami 2, Novagen; BL21, GE HealthCare) competent cells, then cultured in LB medium with IPTG (0.4 mM) for 24 h at 15 °C according to the manufacture's protocol. Bacterial cell pellets were sonicated in PBS with EDTA-free protease inhibitors (Roche) on ice and supernatants were collected. All kinases, expressed in soluble fractions confirmed by HRP-conjugated anti-6x His antibody (Nacalai), and stored in crude extracts or alternatively purified with elution buffer (150 mM imidazole, 50 mM sodium phosphate, 300 mM NaCl) using nickel based metal affinity TALON resin (Clonetech). The buffer exchange against PBS was conducted with concentrator (AmiconUltra, Millipore). Similarly, GST-fused gfCLK4a (pGEX-gfCLK4a) protein was expressed in *E. coli* strain BL21 by IPTG (0.4 mM) induction. All recombinant protein kinases were mixed with glycerol (30%, final concentration), divided in cocktail and rapidly frozen in liquid nitrogen, then stored at −80 °C until use.

2.7. In vitro lamin kinase assay

The frozen kinase cocktails or extracts, recombinant human SRPK1(Invitrogen) were diluted appropriate folds (see legends on figures) with dilution buffer (DB: 100 mM β-glycerophosphates, 15 mM $MgCl_2$, 5 mM EGTA, 20 mM HEPES pH 7.5) before measurements of kinase activity. The lamin kinase assay was started by mixing of 5 μl of kinase sample with the same volume of lamin kinase buffer [(final concentration, 50 mM Tris–HCl, 10 mM $MgCl_2$, 5 mM EGTA, 500 μM ATP, phosphatase inhibitors (phosSTOP, Clontech)] in the presence of various recombinant lamin substrates (0.2 mg/ml, final concentration). After appropriate incubation at 25 °C, the reaction was terminated by adding 2.5 μl of 5 × SDS sample buffer. 2.5 μl was subjected

to 7.5% (Wt, NΔ6, NΔ21) or 12.5% (GST-LB3N-Wt and its variants) SDS–PAGE, then Western blotting was carried out using anti-pSer mAb (C7B8D, hybridoma supernatants) followed by HRP-conjugated secondary IgG (Beckman Coulter), and then detected by chemiluminescence reagent (Nacalai) according to Yamaguchi et al. [23]. Image of chemiluminecence signals on X-ray film was obtained by scanning and the intensity was measured by NIH image.

2.8. 5′-RACE cDNA cloning of lamin B3 from wrasse (Pseudolabrus sieboldi) ovary

A cDNA library was constructed from wrasse ovarian polyA+RNA using Marathon cDNA construction kit according to manufacturer's protocol (Clonetech). 5′RACE was carried out using the following primer sets, 1st PCR, gene specific primer, GSP1 5′-AACAGCTCCGTGCTCTGGTGAAC-3′, adaptor primer, AP1 5′-CCATCCTAATACGACTCACTATAGGGC-3′, nested PCR, GSP2 5′-CGACTGGACATTTTATAGGCTGGTGAGC-3′, AP2 5′-ACTCACTATAGGGCTCGAGCGGC-3′. Cycling conditions were follows: 30 cycles, 30 s at 94 °C; 4 min at 68 °C. PCR products were ligated into pGEM-T-easy vector (Promega) and sequenced using CEQ8800 (Beckman Coulter).

2.9. GST-pull down

GST pull-down assays were done by incubating equal amounts (10 μg) of GST-gfLB3-N (wild-type and mutants) with partial purified His-gfSRPK1 in a buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM DTT, and 1% NP-40 for 30 min at 22 °C. Glutathione-sepharose 4B (10 μl, GE Healthcare) was added, and then incubated for 30 min at the same temperature. Samples were washed extensively after pull-down and separated by SDS–PAGE and visualized by Coomassie brilliant blue (C.B.B.) staining. Alternatively, Western blotting was carried out using anti-His-tag HRP-conjugated IgG (MBL), and then signal was detected by chemiluminescence reagent (Nacalai).

2.10. Phylogenic tree analysis

All of the protein sequences were obtained from the current release of GenBank and ENSEMBL database. Protein sequences were aligned and a phylogenic tree was constructed using ClustalW program (1.83) (http://www.ddbj.nig.ac.jp/) and visualized by tree view program (1.6.6) (http://taxonomy.zoology.gla.ac.uk/rod.html).

3. Results

3.1. Expression cloning of lamin kinases from the goldfish ovarian cDNA library

Previous experiments using Western blotting analysis with anti-gfLB3 (pSer-28)-specific monoclonal antibody, C7B8D, have shown that gfLB3 is phosphorylated at Ser-28 in immature oocytes [23]. Therefore, we used an expression screening method to obtain gfLB3 Ser-28 kinase from the ovarian cDNA library using the same mAb (Fig. 1). We used soluble GST-fused gfLB3-N-terminal head (1–54 aa) as a substrate for immunoscreening because full-length lamin proteins were recovered in insoluble inclusion bodies of *E. coli*. After partial sequencing analysis of 42 positive clones from the goldfish ovary (1×10^5 pfu), 26 clones encoding protein kinase domain were selected. They were classified as cdc2-like kinase (CLK4), serine arginine-rich protein kinase (SRPK1), dual specificity of tyrosine related kinase (DYRK2, 3), or non-specific serine/threonine protein kinase-coding proto-oncogene, PIM-3, by searching the homologs in the GenBank databases (Table 2). All isolated kinases belonged to

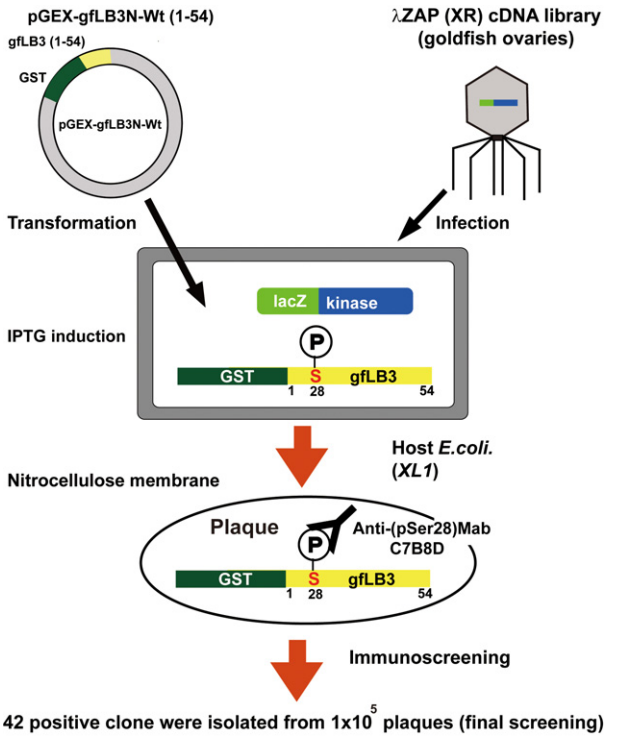

Fig. 1. Outline of expression cloning of gfLB3 Ser-28 kinases. *E. coli*, which expressed fusion protein (GST-gfLB3N-Wt), were infected with the lambda ZAP(XR) cDNA library derived from goldfish ovarian mRNA. GST-gfLB3N-Wt (1–54) and proteins from the library were inducted by IPTG. After transfer of the proteins on the nitrocellulose membrane, phosphorylated Ser-28 of GST-gfLB3N-Wt was detected on X-ray film by mAb C7B8D.

the CMGC kinase family (cyclin-dependent kinases [CDKs], mitogen-activated protein kinase [MAPKs], glycogen synthase kinases [GSKs], and CDK-like kinase [CLKs]), except for the PIM-3 kinase, which belongs to the CAMK (calmodulin-dependent protein kinase-related) group. Their overall protein structures, deduced from cDNA sequences, are shown in Fig. 2. Two clones were full length of cDNA (~3 kbp)-encoded kinases, with open reading frames of 634 and 646 aa (Fig. 3A). Because the presumed proteins were 64% and 63% identical to human SRPK1 (hsSRPK1), 91% and 66% identical to zebrafish SRPKla (zfSRPK1a), and 67% and 85% identical to zfSRPKlb by the database search, they were designated gfSRPKla and gfSRPK1b, respectively. Phylogenic tree analysis showed that SRPK1 genes are duplicated in the fish lineage, in contrast to the single copy in tetrapods (http://www.treefam.org/; TFI05334). Fish SRPK1a and tetrapod SRPK1 were grouped into the same cluster as gfSRPK1 (a, b) (Fig. 4). This suggests that fish SRPK1a is an ortholog for human SRPK1; SRPK1b remains a specific gene duplicated in the fish lineage. The deduced sequences of both clones contained conserved residues of the serine/threonine kinase (I–XI) kinase catalytic domain, divided by spacer regions characteristic of SRPKs. In contrast, little homology between the spacer regions of gfSRPK1a and gfSRPK1b was found. Both clones have two stretches (11–23 and 264–274) rich in basic amino acids, which may function as nuclear localization signals (NLS).

Similarly to SRPK1, 2 full-length clones encoding cdc2-like kinases (CLK) were isolated. A BLAST search against the zebrafish information database (http://zfin.org/; clk4a, ZDB-GENE-030131–576; clk4b, ZDB-GENE-050227–19) led us to designate the two isolated cDNAs as gfCLK4a and gfCLK4b, respectively. One cDNA clone (~2 kbp) contained an open reading frame of 486 aa, and the other clone (~1.7 kbp) contained an open reading frame of 472 aa (Fig. 3B). The human CLK family contains four genes (CLK1–4) [25]. No human CLK1 orthologs have been found in fish (TF101041). A phylogenic analysis

Table 2
The results of immunoscreening of gfLB3 (Ser-28) kinsase. DDBJ accession numbers (ID) are included.

The total number of phages						1×10^5 pfu
Positive plaques after final screening						42
Positive plaques encoding kinase domain						26
	Name of kinase	ID	group	subgroup	function	
CLK4a	cdc2-like kinase	AB765406	CMGC	SR kinase	splicing	12
CLK4b		AB765407				2
SRPK1a	serine-arginine rich protein kinase	AB765408	CMGC	SR kinase	splicing	1
SRPK1b		AB765409				3
DYRK2	dual specificity of tyrosine related kinase	AB765410	CMGC		cell cycle regulation	1
DYRK3		AB765411				2
PIM-3 kinase	serine/threonine-protein kinase pim-3	AB765412	CaMK		cell cycle regulation	5

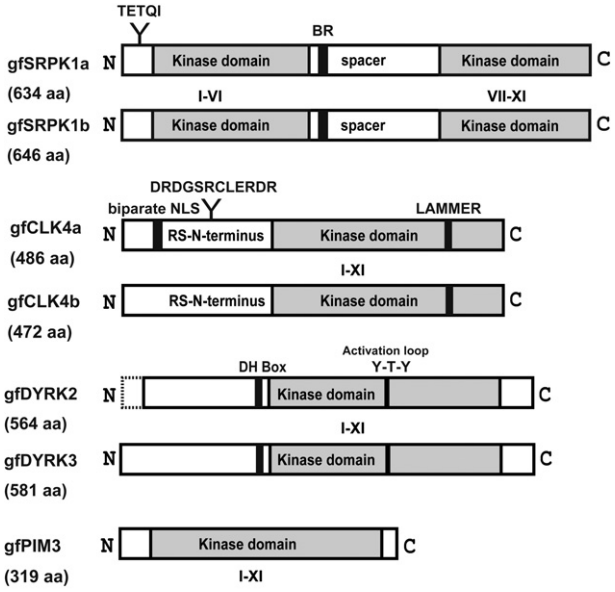

Fig. 2. Structures and deduced amino acid sequences of gfLB3 kinases, with respect to Ser-28, isolated by expression screening. Protein structures deduced from cDNA inserts of the seven positive phage clones. gfSRPK1a and gfCLK4a have unique sequences (TETQI and DRDGSRCLERDR) that are not found at the N-termini of SRPK1b and CLK4b, respectively (see Fig. 3). BR indicates a region rich in basic amino acids (K or R). gfCLK4a also has a bipartite NLS not found in CLK4b. gfDYRK2, 3 have a DH (DYRK homology)-box and the same activation loop (Y-T-Y) sequence. gfDYRK2 cDNA has a truncated N-terminus, lacking 24 aa in this clone, in comparison to that of zebrafish (95% aa identity). Black boxes indicate specific domains.

also demonstrated that fish CLK4a and 4b, as well as SRPK1, are duplicated genes that arose in the fish lineage (Fig. 4). The fish CLK4 group is distantly related to other tetrapod CLKs; therefore, we propose that it is renamed a fish-specific CLK that is of a primitive class in vertebrates. However, here, we use "CLK4" in respect to zebrafish database. Fourteen clones encoding gfCLK4a were isolated from the goldfish ovary (1×10^5 pfu), which shows that the CLK4a transcripts are more abundant than other kinases in fish ovaries. Similar to SRPK1, the deduced sequences of both clones contain a conserved kinase catalytic domain (I-XI) with the conserved signature motif LAMMER (4a, 396–401; 4b, 375–380), a DYRK-homology box (DH-Box: 4a, 150–165; 4b, 129–134), and an EF-hand calcium-binding domain (4a, 449–461; 4b, 428–440). In contrast, little homology between the N-terminal sequences is found. A bipartite type NLS (27–42) was found in gfCLK4a but not in CLK4b.

Dual specificity-related tyrosine kinases comprise four major groups (DYRK1–4 [26]) on the TreeFam database in vertebrates (TF314624). One N-terminal truncated clone (-4 kbp) and two full-length clones (-5 kbp) were isolated and identified as DYRK2 and DYRK3, respectively. The gfDYRK2 encoded a partial open reading frame of 564 aa; gfDYRK3 encoded a full open reading frame of 581 aa.

The pim family of proto-oncogenic Ser/Thr kinases consists of three members: Pim1, Pim2, Pim3 [27] (Accession ID, TF320810). Five clones (-2 kbp) encoding an open reading frame of 319 aa were identified as the PIM3 kinase. A SIK3 (salt inducible kinase 3, DDBJ ID AB765930), belonging to the AMPK (AMP-dependent protein kinase) family, was isolated from the same library by expression screening but was excluded from further analysis because the isolated clone was missing the kinase catalytic domain (data not shown).

Based on this expression screening, SRPK1, CLK4, DYRK2, and PIM3 kinases were all gfLB3 Ser-28 kinase candidates. Among these kinases, SRPK and CLK are classified as SR kinases that can modify arginine/serine (RS) dipeptide motifs. Both kinases have been reported to specifically phosphorylate splicing factors [28], a prototypical SR protein, and ASF/SF2 (also called human alternative splicing factor), and can also disassemble nuclear speckles [29,30]. However, no study has elucidated the phosphorylation of nuclear lamins by SR kinases, and therefore, we focused on the N-terminal head sequence of lamins to analyze why SR-kinases can phosphorylate Ser-28, a potential p34cdc2 target site of gfLB3.

3.2. *Discovery of RS repeats upstream of the p34cdc2 target serine of fish lamin B3*

Using alignments of N-terminal aa sequences of vertebrate LB3 available on ENSEMBL and NCBI databases, we found consecutive RS repeats only in fish LB3 (Fig. 5). The sequence and number or length of RS repeats in LB3 was different among species of teleosts. Wrasse and torafugu LB3s showed more RS repeats, flanked by single and plural Ala, respectively. Tetraodon (generally called green spotted puffer) had the longest consecutive RS repeat of LB3, in contrast to zebrafish LB3, which contained the least number of repeats. Two consecutive dipeptides were found in chicken LB3 but not in *Xenopus*. Comparison of the N-terminal sequence with that of other lamins (A, B1, and B2), including invertebrate lamins, showed that all lamins have high Ser and Arg contents at the N-terminus, which is not predicted to fold into a distinct conformation. Fish LB3 was characteristic for prominent RS repeat numbers with higher contents of Ser and Arg than somatic lamins (A, B1, and B2) on the N-terminal head, upstream of the conserved p34cdc2 target serine (Table 3). The gfLB3 had 4 SR repeats with high contents of Ser and Arg (44%). The fish LB3 group had a RS repeat-rich region but the length was less than that of ASF/SF2, LBRs [31,32], and other RS-containing proteins [31,33] that are expected to be the favored substrates of SR kinases.

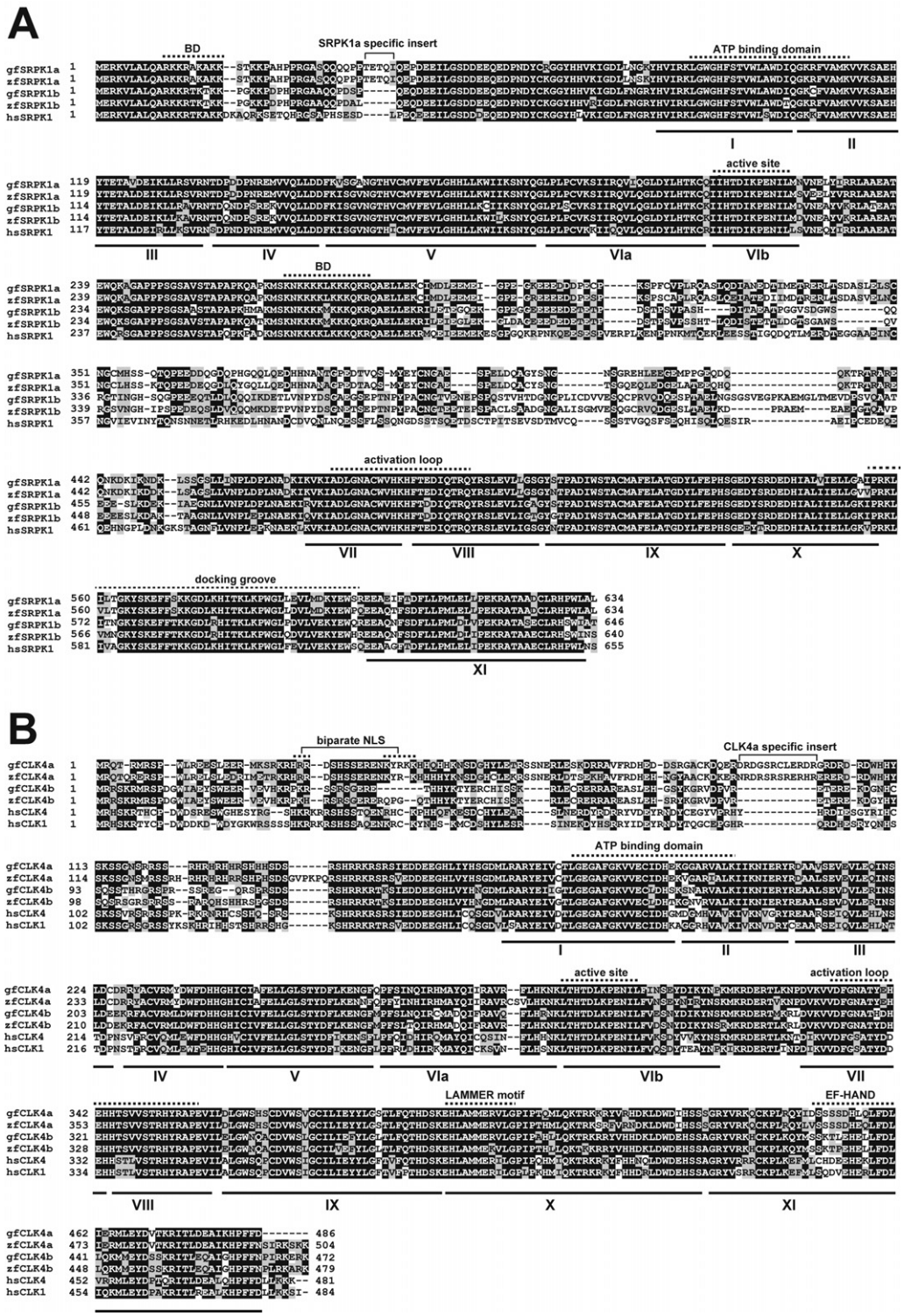

Fig. 3. Schematic representation of deduced amino acid sequences of (A) goldfish SRPK1 and (B) goldfish CLK4, in comparison with zebrafish and human. The deduced amino acid sequences of gfSRPK1 (a,b) and gfCLK4 (a,b) were aligned and compared with those of zebrafish and humans (hsSRPK1, CLK1, and CLK4). The same protein databases as Fig. 4 were used. Residues that are identical or similar are filled in black or in grey, respectively. The location of the ATP-binding domain, active site, activation loop (both kinases), docking acidic groove (SRPK1 specific; broken line), LAMMER, EF-Hand motif (CLK specific; broken line), and conserved kinase domains (I–XI; solid line) are shown.

Fig. 4. Phylogenic trees of SR kinases. (A) SRPK1 tree. (B) CLK tree. The fish CLK4 group is rooted from other CLKs at the base, indicating a fish-specific subgroup. Protein sequences are from the current release of ENSEMBL and GenBank databases. The protein IDs used for alignments of the sequence are beside the names. Extension trees were constructed from the zebrafish MAP kinase p38a (GenBank ID 30316123). Lines indicate genetic distance. Hsap, *Homo sapiens* (human); Mmus, *Mus musculus* (mouse); Ggal, *Gallus gallus* (chicken); Xtro, *Xenopus tropicalis* (frog); Olat, *Oryzias latipes* (medaka); Drel, *Danio rerio* (zebrafish); Frub, *Takifugu rubripes* (torafugu); Caur, *Carassius auratus* (goldfish). All boot strap analysis of amino acids involves 1000 replications of the data.

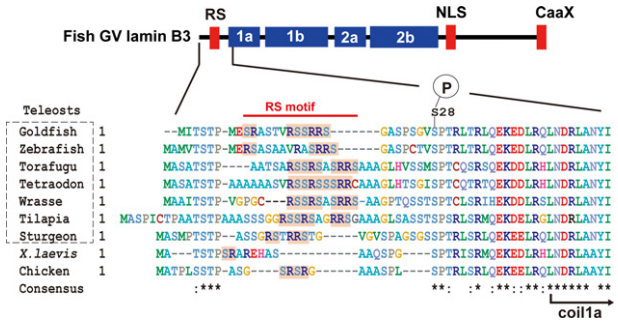

Fig. 5. Schematic representation of N-terminal amino acid sequences conserved in teleost LB3. (Upper) LB3 is divided into head and tail structures, flanking a central rod domain (1a, 1b, 2a, 2b). A unique RS/SR dipeptide-rich region (RS motif) was found on the N-terminal head of teleost LB3s but not in *Xenopus* LB3. Chicken LB3 has 2 repeats of SR. Nuclear localization signal (NLS) and CaaX motif on the tail are conserved among all LB3s. Ser-28 is a consensus phosphorylation site for M-phase p34cdc2 target Ser. LB3 sequences were obtained from the DDBJ, Genbank and ENSEMBL database, goldfish (*Carassius auratus*, AB034197); zebrafish (*Danio rerio*, AAL27546); Nile tilapia (*Oreochromis niloticus*, XP_003437552); torafugu (*Takifugu rubripes*, XP_003976315); tetraodon (*Tetraodon nigroviridis*, ENSTNIP00000005987 and translated sequence from the scaffold, SCAF7762 [region 30483078 to 30483128]); wrasse (*Pseudolabrus sieboldi*, AB765414); sturgeon (*Acipenser baerii*, CAD38130); *Xenopus* (*Xenopus laevis*, NP_001081545); chicken (*Gallus gallus*, XP_413842). A p34cdc2 target sequence (SP motif) is conserved among lamins of all vertebrates.

Table 3
Prominent RS repeat numbers with high contents of Ser and Arg on the N-terminal head domain of fish GV-lamin B3.

	Vertebrate lamins			
	B3	A	B1	B2
Goldfish	*4 (44%)	ND	1 (27%)	1 (30%)
Zebrafish	*3 (33%)	1 (18%)	1 (22%)	1 (19%)
Pufferfish (torafugu)	*4 (42%)[a]	0 (20%)	1 (14%)	0 (11%)
(*Tetraodon*)	*3 (36%)[a]	0 (13%)	1 (29%)	1 (26%)
Xenopus	1 (30%)	1 (18%)	2 (41%)[b]	2 (36%)
Chicken	*2 (35%)	1 (30%)	0 (15%)	0 (13%)
Mouse	-	1 (33%)	1 (17%)	0 (7%)

Invertebrate lamins	
Drosophila C	2 (36%)
Drosophila Dmo	1 (27%)
Sea urchin	1 (30%)
C. elegans	4 (40%)[c]

The number of RS/SR repeats and percentage of Arg and Ser upstream of the sequence of the conserved p34cdc2 target Ser, derived from vertebrate (upper) and invertebrate (lower) lamin databases. Lamin protein sequences were from the current version of Genbank or ENSEMBL database. The *Drosophila melanogaster* lamin C, (Q03427); the *Drosophila* lamin Dmo, (P08928); the sea urchin (*Strongylocentrotus purpuratus*) B-type lamin, (AAB34118); the *Caenorhabditis elegans* lamin, (CAA52188); the goldfish (*Carassius auratus*) LB1, (AB765413); the goldfish LB2, (AB034198); the zebrafish (*Danio rerio*) LB1, (AJ25201); the zebrafish LB2 (AJ005936); the torafugu (*Takifugu rubripes*) LA1, (ENSTRUP00000033112); the torafugu LB1 (ENSTRUP00000011709); the torafugu LB2, (ENSTRUP00000010260); the *Tetraodon nigroviridis* LA1, (ENSTRUP00000010260); the tetraodon LB1, (ENSTNIP00000005695); the tetraodon LB2, (ENSTNIP00000009942); the *Xenopuslaevis* LA, (P11048); the *Xenopus* LB1, (P09010); the *Xenopus* LB2, (P2191); the chicken (*Gallus gallus*) LA, (P13648); the chicken LB1, (P14731); the chicken LB2, (P14732); the mouse (*Mus musculus*) LA, (P48678); the mouse LB1, (P14733); the mouse LB2, (P21619). As for LB3, the sequences of Fig. 4 were used for analysis. The LB3 gene was lost in the mammalian lineage.

[a] Pufferfish (*Takifugu rubripes*, called torafugu, and *Tetraodon nigroviridis*) LB3s have RS repeats bracketed by single Ala, Arg, or Ser (see Fig. 2).

[b] *Xenopus* LB1 has an RS-rich region (10-RSSGRRSS-17) that possibly binds to SRPK1.

[c] *C. elegans* lamin does not contain the conserved p34cdc2 target Ser; therefore, the number of RS and the percentage of Arg and Ser were counted and calculated in the sequence region (1–35), respectively. Asterisks indicate lamins that contain more than two consecutive RS/SR repeats. ND indicates not done because of the absence of cDNA information.

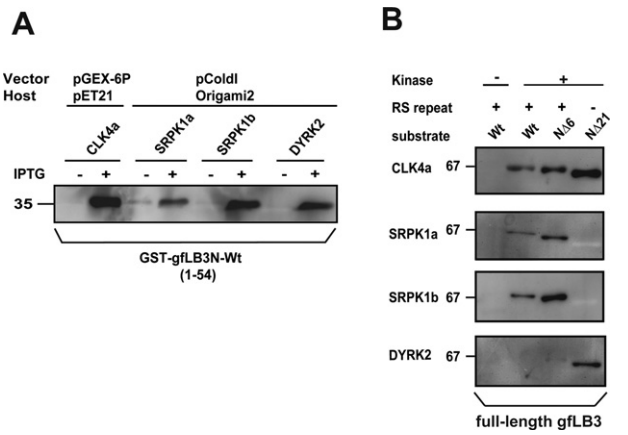

Fig. 6. Expression and preliminary characterization of recombinant protein kinases, gfCLK4a, gfSRPK1 (a,b), and gfDYRK2. (A) Ser-28 kinase activity of GST-gfLB3N-Wt. CLK4a was expressed as a GST-fused protein in *E. coli*. SRPK1 (a, b) and DYRK2 were expressed as His(6)-tag fused proteins. Active recombinant kinases were prepared in *E. coli* (IPTG+). Supernatants without IPTG induction have no kinase activity (IPTG-). (B) Phosphorylation of N-terminal truncated recombinant gfLB3 protein by four individual kinases. Purified recombinant full-length goldfish LB3 (Wt), NΔ6 (retains RS repeats), or NΔ21 (deleted RS repeats) was added to the reaction to characterize individual kinases. In both experiments, the supernatants from *E. coli* lysates expressing active recombinant kinases were diluted 50-fold in DB. Kinase reaction condition was 1 h at 25 °C. After the termination of the reaction with SDS-sample buffer, proteins (2.5 μl) were separated on SDS–PAGE (12% for GST-gfLB3N-WT, 7.5% for full length of gfLB3), and then transferred on the PVDF membrane. Lamin-antibody (C7B8D) complexes were visualized with chemiluminescence reagents on X-ray film for 30 s. Bars on the left indicate molecular masses (in kDa).

3.3. *RS repeat-dependent phosphorylation by SRPKl but not by CLK4*

Both SRPK and CLKs are known as SR kinases that interact with and phosphorylate consecutive RS repeats. Four goldfish Ser-28 kinase (SRPK1a and 1b, CLK4a, and DYRK2) extracts were prepared with activity from *E. coli* with GST-gfLB3N-Wt protein as a substrate (Fig. 6A), but other kinases (CLK4b, DYRK3, and Pim3) could not be recovered with activity from *E. coli.* Further screening was performed using purified full-length (Wt) and truncated gfLB3 proteins (NΔ6 and NΔ21) as substrates (Fig. 6B). In an *in vitro* kinase assay, gfCLK4a could phosphorylate Wt, as well as NΔ6 and NΔ21, in which the SR repeats were missing. In contrast, both SRPK 1a and 1b could phosphorylate Wt and NΔ6 but not NΔ21. DYRK2 could phosphorylate NΔ21 but not Wt or NΔ6, although it could phosphorylate Ser-28 of the GST-gfLB3N-Wt (Fig. 6B). In summary, SRPK1 was the only kinase in which RS repeats were required for phosphorylation of Ser-28, in contrast to CLK4 directly phosphorylating Ser-28. As a result, gfLB3 Ser-28 kinases isolated by expression screening were subdivided into RS repeat-dependent (SRPK1) or RS repeat-independent lamin kinases (CLK4 and DYRK2) groups.

3.4. *Site-directed mutagenic analysis of RS repeats and GST-pull down assay to identify binding motif of gfLB3*

One of the well-known SR proteins is ASF/SF2, which has 20 serines within the RS domain; of these serines, SRPK1 phosphorylates nearly a dozen serines adjacent to arginine [34]. We next addressed whether the short RS stretch of gfLB3 is sufficient for high-affinity binding of SRPK1 for phosphorylation of Ser-28. We constructed GST-gfLB3N with various RS repeats by using site-directed mutagenesis (B–M, Fig. 7A). Purified gfSRPK1a was used for an *in vitro* kinase reaction. Phosphorylation activity decreased in a stepwise manner, corresponding to the repeat number of RS (A–F) (Fig. 7B and C). Low activity was detected in the sequential RS null mutant (D, F). These results show that SRPK1 binds to the sequential RS repeats (15–20) to

phosphorylate Ser-28. In contrast, intense phosphorylation was detected on Ser-28 with extended RS repeats (G–K and M). The highest activity was detected in mutant J, which has the longest consecutive RS, aside from mutant M. One amino acid substitution of Ser to Ala (J to K marked by asterisk) slightly decreased the intensity. The intensity of mutant L, which had shorter RS repeats divided by additional tetrapeptides (ASTV), decreased abruptly. This finding indicates that the length of consecutive RS repeats is important for SRPK1 binding, whereas insertion of a neutral amino acid (Ala) has a lesser effect. The first RS (9–10) is also involved in gfSRPK1 binding because mutant E had a significantly low intensity, in spite of the presence of three repeats of RS (15–20); an additional repeat (G) compensated for the missing RS (9–10) of mutant E. These results indicate that gfLB3 has a functional stretch of RS repeats (RS motif), where SRPK1 binds to the region with RS repeats (9–20) to catalyze the phosphorylation of Ser-28 eight amino acids downstream of the RS repeat at the active site. Similar substrate specificity was detected by recombinant human SRPK1 (Fig. 7B, C). The mutant E, which deleted the first RS repeat seems to have different substrate specificity between goldfish and human SRPK1 on the histogram. A significant difference ($P < 0.05$) is that the first RS (9–10) was not necessary for hsSRPK1 but was necessary for gfSRPK1 (Fig. 7C; mutant E). This indicates that different substrate specificity at the binding motif, with a kinase domain sequence that is almost always the same (Fig. 3A).

When compared to the N-terminal sequence of fish LB3, the pufferfish (tetraodon) contains longer RS repeats, with a farther distance to the p34cdc2 site (13 aa away from the terminus of RS repeats), and the goldfish has 1.5 fewer RS repeats that are 8 aa away from the p34cdc2 site (Fig. 8A). We investigated the relationship between the length of RS repeats (binding site) and the distance to the target site of fish LB3. Two mutant substrates, N and O, were constructed by the addition of 5 Ala downstream of the wild-type RS motif of goldfish (A) and tetraodon (J), respectively (Fig. 8A); the phosphorylation rate was then compared. Time-course experiments showed that the incorporation of phosphate on Ser-28 of mutant N (Wt + 5 Ala) was less than that of Wt (A). In contrast, by using the puffer-type substrates with longer RS repeats, phosphorylation rates of Ser-28 of both substrates (J, O) were rapidly increased and reached the maximal level faster than the phosphorylation rates of Ser-28 of goldfish-type substrates (A, N) (Fig. 8B).

GST-pull down assay was carried out to confirm the SRPK1 binds to RS repeats. SRPK1 was not detected with mutant (F) in which RS repeats are missing completely. In contrast, the binding was detected between gfSRPK1 and GST-gfLB3 mutant protein (J) which has the longest RS repeats with the highest activity, but precipitated amounts of gfSRPK1 is much less than that of input (Fig. 9, see also Fig. 7B and C). A weak binding between SRPK1 and Wt (A) was also detected by Western blotting. Taken together, we conclude that SRPK1 bind to the RS repeats of gfLB3 with affinity, not stable docking to the particular sequence, and recruited serine into the active site. These results indicate that the longer RS repeats enhance the interaction of SRPK1 with substrates and that the complex pulls the far target Ser to the active site for phosphorylation (Fig. 10).

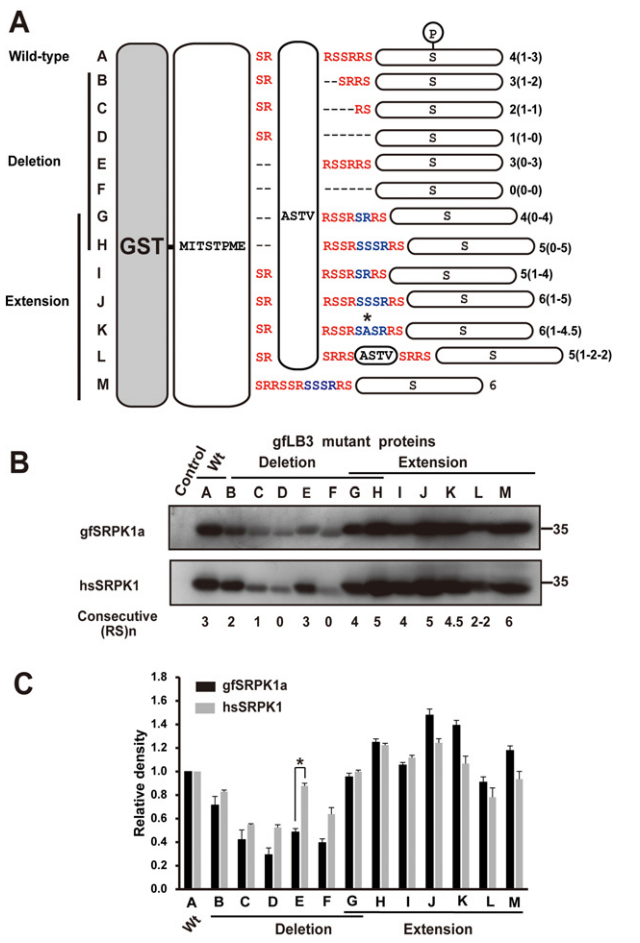

Fig. 7. Identification of RS motif on gfLB3. (A) Schematic illustration of domain organization of N-terminus of gfLB3 and different RS-mutant constructs generated to delineate the region responsible for SRPK1 activity. All recombinant GST-fused RS mutants were constructed according to primer sets in Table 1. The total number of RS repeats within the motif and the number of RS divided by tetrapeptides (ASTV) in the bracket are shown on the right. Asterisk indicates Ala, not Ser or Arg, the only difference in amino acid sequence between J and K. Blue characters indicate amino acids appended to the RS repeats of wild-type gfLB3 (Red characters). (B and C). All *E. coli* produced recombinant proteins (2 mg/ml), which were purified by affinity absorption on glutathione-sepharose. Goldfish SRPK1a protein was produced in *E. coli* strain BL21 and purified through metal affinity resin. The kinase cocktail was diluted 100-fold in DB. Alternatively, human recombinant SRPK1 (100 pg/μl, Invitrogen) was used for the comparison of the substrate specificity. Reactions were started by mixing the reaction buffer, incubated for 30 min at 25°C in a water bath, and terminated by mixing with SDS sample buffer. A control lane indicates no substrates. The efficiency of phosphorylation of each mutant substrate was visualized by Western blot analysis using C7B8D hybridoma supernatants. A set of samples was separated on single polyacrylamide gel (12%). Lamin-antibody complexes were visualized with chemiluminescence reagents on X-ray film for 30 s. The numbers below the pictures indicate the number of repeats of RS, corresponding to Fig. 7A. The efficiency of the phosphorylation of mutant substrates is comparable on a histogram (goldfish SRPK1a, black; human SRPK1, gray). Relative intensity was estimated as a ratio divided by the value of wild-type. Error bars represent the standard deviation from three individual experiments. Bars on the right indicate molecular masses (in kDa). Statistical analysis was performed using student *t*-test, regarding $p < 0.05$ as statistically significant. A significant difference was indicated by asterisk in the figure. No significant difference was detected except mutant E. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

4.1. Identification of RS/SR dipeptide repeats in fish lamin B3

SR proteins can be broadly classified into SR family proteins, such as splicing factors containing an RNA-recognition motif (RRM) and C-terminal RS domain, and SR-related proteins that are distinct from SR without RRM. Genome-wide computational sequence analysis showed that a number of SR-related proteins contain a minimum of two consecutive SR/RS repeats, functionally associated with processes other than splicing, including chromatin structure/remodeling, transcription, cell cycle, cell structure, and ion homeostasis [33,35]. Fish LB3 contained a minimum number of RS repeats, compared to other RS-rich proteins (Fig. 5). This study showed that 2 or 3 RS repeats are sufficient for binding of SRPK1 to activate phosphorylation at a neighboring target site. This finding suggests that SRPK1 interacts not only with known SR proteins but also with minor proteins with shorter RS repeats. Prominent RS repeats were not found in

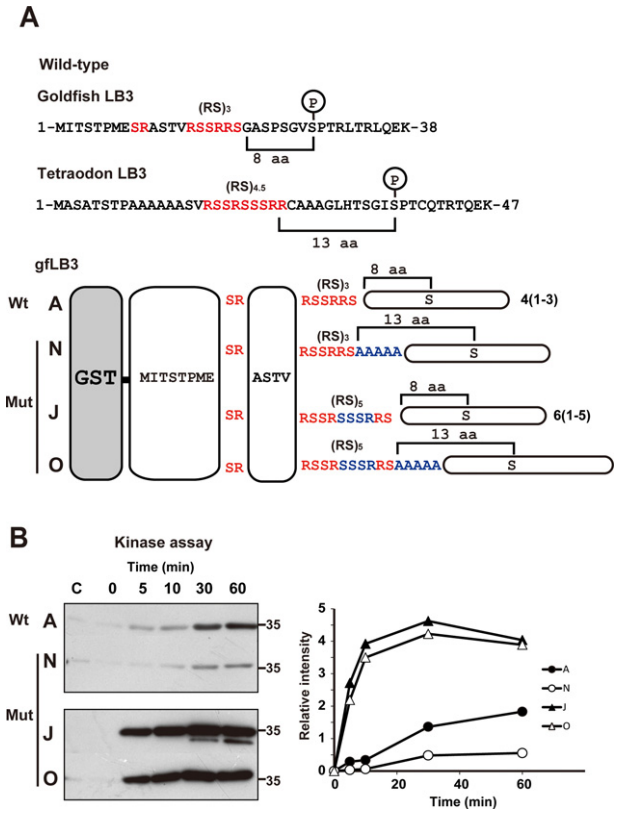

Fig. 8. The correlation of the length of RS repeats, with the distance to the target phosphorylation site. (A) The gfLB3 has a SRPK1 (or p34cdc2) phosphorylation site three repeats away from the RS motif, whereas tetraodon has a target Ser 13 aa away from the RS motif. Constructs of two mutant SR proteins (N, O) that have 5 additional consecutive Ala downstream of the RS repeats in wild-type A and mutant J, respectively. Red characters indicate RS repeats. Blue characters indicate amino acids appended to wild-type gfLB3. (B and C) Purified gfSRPK1a was diluted 50-fold with DB, and then used in a kinase assay. Reactions were started by mixing the reaction buffer, incubated at 25 °C in a water bath, and terminated by mixing with SDS sample buffer at each time. Samples were separated on polyacrylamide gels (12%). Lamin-antibody (C7B8D) complexes were visualized with chemiluminescence reagents on an X-ray film for 30 s. Relative intensity was plotted on the graph. Subscripts of the bracket (RS) indicate the number of the repetition of RS. Bars on the right indicate molecular masses (in kDa). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

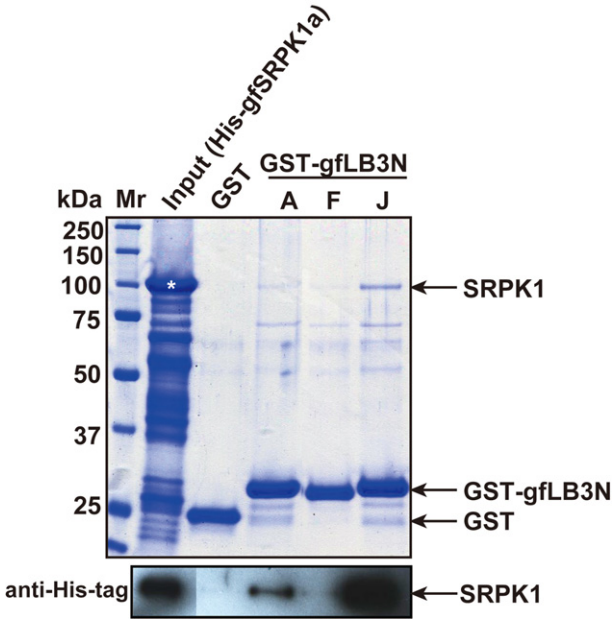

Fig. 9. SRPK1 binds to RS repeats of gfLB3. GST pull-down assay shows no binding with mutant (F) but significant binding with wild type (A) and mutant (J) which has longest RS repeats. Asterisk indicates the position of gfSRPK1 (input). SRPK1 precipitated with GST-gfLB3 were confirmed by Western blotting using anti-His-tag antibody. The equivalent amounts of proteins were separated on polyacrylamide gels (10% for C.B.B. staining, 7.5% for Western blotting), except one-50th quantity of His-gfSRPK1 protein was loaded for Western blotting.

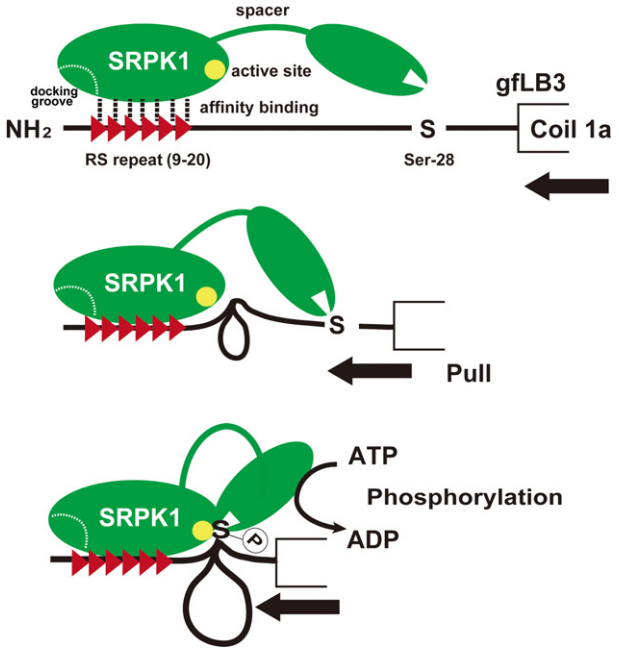

Fig. 10. Grab-and-pull model of substrate binding and phosphorylation of the p34cdc2 target site of gfLB3 by SRPK1. SRPK1 (green) binds with affinity towards the N-terminal RS motif (red triangles) of gfLB3 to pull the target serine (Ser28) into the active site for phosphorylation. Pulling activity is dependent on the binding affinity, which correlates with the number of consecutive RS repeats of gfLB3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

other lamins, except fish LB3, which indicates that the N-terminus of GV-lamin B3 differs from that of other lamins that have the same conserved cdc2 target site in front of the rod domain. The gfLB3 supports the nuclear membrane, but B1 and B2 are constituents of internal filamentous structure [36]. Different nuclear distribution of lamins might be dependent on having the RS motif or not. In contrast, *Xenopus* LB3 has a well-characterized GV-lamin in the absence of RS repeats, but XLB1 has RS-like motif that possibly interacts with SRPK1 (Table 3). This suggests that *Xenopus* lamins are controlled differently from those of fishes.

SR proteins tend to form aggregates because they have properties of intrinsically disordered proteins that likely lack a folded structure under physiological conditions [37]. Phosphorylation on the RS domain by multiple kinases has shown to be critical for RS domain-mediated protein interactions [31]. Thus, reversible molecular aggregation and dissociation seem to be regulated by phosphorylation/dephosphorylation in the RS domain [38,39]. Unphosphorylated RS repeats of LBR adopt short, transient helical conformation, whereas phosphorylation of Ser induces Arg claw-like structures [40]. The lamin B receptor (LBR) is the most thoroughly studied protein of the RS domain-containing proteins. The zebrafish LBR is the only characterized LBR in the fish group that contains three RS repeats neighboring an SP motif in exon 2, but the total number of RS/SR motifs is less than that in human LBRs [41]. LBR self-associates through its N-terminal domain and forms oligomers that are suggested to bind heterochromatin [42]. The RS domain of LBR is phosphorylated

by multiple kinases at the beginning of mitosis by nuclear translocated SRPK1 and potentially by Akt and Clk kinases [43]. Furthermore, Ser-71, which is located just upstream of the RS repeats, is phosphorylated by cdc2 kinase during the M phase [44]. This multiphosphorylation may result in dissociation of LBR to chromatin [45]. The gfLB3 Ser-28 is phosphorylated by M-phase cdc2 kinases *in vitro* [23]. Therefore, SRPK1 and p34cdc2 kinases may phosphorylate the same Ser-28 of the gfLB3 during the meiotic prophase and metaphase of fish oocytes. Interphase phosphorylation by SRPK1 prevents translated lamins from aggregation in the oocyte cytoplasm, and then the increase of phosphorylated form of lamins by cdc2 may contribute to lamina disassembly at GVBD. GV has a highly compressed nuclear structure in aggregation that is dependent on the RS motif, which is based on lamin, LBR, and other SR proteins. RS domain-mediated protein interaction supports the inner nuclear membrane to arrange the higher-ordered nuclear structure and to maintain nuclear activities, such as chromatin structure maintenance, transcription, and splicing, adjusting association/dissociation of molecules that are regulated by multiple kinases in fish oocytes.

4.2. Substrate specificity and the roles of fish SR kinases

We isolated and characterized two fish SR kinases, SRPK1 and CLK4, for the first time. Fish CLK4 has different substrate properties from SRPK1 because CLK4 phosphorylates the Ser-28 of a truncated gfLB3 with a deleted RS motif (NΔ21). Cdc2-like kinases (CLK) are conserved throughout eukaryotic evolution, from yeast to human CLK [46]. In a genome-wide search of splicing regulatory proteins, we found that the fish does not contain the orthologs of CLK1 but contain duplicated fish-specific CLK4a and 4b ([47] and Fig. 4). ASF/SF2 is a preferred substrate of mammalian CLKs. CLK phosphorylates RS domains but has a much broader specificity than SRPK1 [25,28]. CLK was able to phosphorylate histone H1 and MBP, although not to the same extent as ASF/SF2 [48]. Therefore, it is not surprising that there is different constrained substrate specificity, depending on the RS motif of gfLB3 between SRPK1 and CLK4.

Recent studies show that phosphorylation of numerous Ser residues by SRPK1 regulates their cellular distribution and activities, in cooperation with CLK [29,43,49–53]. SRPK1-mediated phosphorylation plays an important role in SR protein nuclear import and sequential phosphorylation by CLK, which cooperatively regulates subcellular localization [51,54]. Similarly, SRPK1 and CLK4 may cooperatively regulate phosphorylation of the cdc2 site in transport and subcellular localization of fish LB3 in immature oocytes. Further analysis of *in vivo* interaction and localization of SR-kinases with gfLB3 in oocytes is necessary to develop this study.

Inhibitor studies are effective in analyzing enzymatic kinetics. Inhibition of phosphorylation of SRp75 with SRPIN340, a specific inhibitor for SRPK1 and 2, prevent virus proliferation [55,56]. However, our *in vitro* kinase assay showed that significant inhibitory activity at the concentration of 1–100 μM was not detected in combination of purified kinases (gfSRPK1, hsSRPK1) with GST-gfLB3N-Wt or full-length gfLB3 (data not shown). This finding indicates that this inhibitor recognized the particular enzyme–substrate complex. Further analysis using the specific inhibitor is necessary for detailed study of the SRPK1–LB3 complex.

4.3. Phosphorylation mechanism of SRPK1

The gfSRPK1 had similar substrate specificity as that of the human, in respect to Ser-28 of gfLB3 (Fig. 7B and C), indicating that SRPK1 phosphorylation is evolutionarily conserved between yeast and humans, including fish [57]. Recent studies have revealed that SRPK1 possesses a docking groove distal from the active site that recognizes the RS domain [58]. The stable docking interaction is critical for regulation of phosphorylation of ASF/SF2. The docking motif is generally rich in basic residues, conforming to a consensus sequence (R-x-R/K-x-x-x-R), and binds the acidic groove of SRPK1 [51,59,60], located far from the active site [61]. However, the identical sequences were not found in any RS repeat sequences of fish LB3 (Fig. 5). GST-pull down assay showed affinity binding of SRPK1with RS repeats (Fig. 9). The binding strength is dependent on the length of RS repeats. Electrostatics where negatively charged surface interacts with positively charged RS motif seems to be enough to phosphorylate Ser-28. This finding is contrast to the results of previous studies that docking motif of ASF/SF2 plays an important role; the RS domain itself does not affect the mechanism of phosphorylation [51]. The sequence of the acidic docking groove of SRPK1 is highly conserved among species (Fig. 3A). It suggests that SRPK1 distinguish substrates with docking motif (i.e. ASF/SF2) from various substrates with short RS repeats only (i.e. LB3) properly.

SRPK1 docks near the C-terminus of the RS1 segment (docking motif) and phosphorylates the RS domain at multiple Ser residues, using directional (C-terminal to N-terminal) and possessive mechanisms [34,51,62,63], described by the proposed grab-and-pull model [34]. Our experimental results are consistent with this model (Fig. 10). The SRPK1 strongly binds to the short stretch of the RS motif (15–20). Also consistent with our results is the recent finding that LBR has a short conserved peptide sequence, RSRSPGR, that overlaps the RS repeats and may serve as a docking motif for SRPK1 [60]. This indicates that long and short RS repeats may constitute the docking and/or affinity binding motif. Similarly, an RS motif (9–20) was inserted to the best position for SRPK1 bound to initialize phosphorylation of Ser-28 of gfLB3. The length of sequential RS repeats determined the strength of interaction between SRPK1 and its substrates and pulled far Ser residues into the active site, in a similar manner to ASF/SF2 (Fig. 10). The positioning and numbers of the inserted RS repeats upstream of the p34cdc2 target Ser of the individual fish LB3 appeared to be determined by evolution of the fish lineage (Fig. 5).

In general, investigation into the phosphorylation specificity of SR proteins has been very difficult owing to the repetitiveness of sequence. However, our study suggests that analysis of the phosphorylation of a single amino acid by using a pSer-specific antibody may help in the analysis of the unique phosphorylation mechanism of minor proteins with short RS/SR dipeptides repeats, as well as typical SR proteins with long RS repeats.

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