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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