Somatic Lamins in Germinal Vesicles of Goldfish (*Carassius auratus*) Vitellogenic Oocytes

Akihiko Yamaguchi1 and Yoshitaka Nagahama*

Laboratory of Reproductive Biology, Department of Developmental Biology, National Institute for Basic Biology, Okazaki 444-8585, Japan

1Present address: Laboratory of Marine Biology, Department of Animal and Marine Bioresource Science, Faculty of Agriculture, Kyushu University, Fukuoka, Japan

**ABSTRACT.** In fish and amphibians, B-type lamins are divided into somatic (B1, B2) and oocyte-type (B3) lamins. In this study, we purified nuclear lamins from rainbow trout erythrocytes, raised an anti-lamin monoclonal antibody (L-200) that recognizes goldfish somatic-lamins, and isolated cDNAs encoding goldfish B-type lamins (B1 and B2) from a goldfish cell culture cDNA library. Goldfish B-type lamins are structurally similar to lamins found in other vertebrates with minor amino acid substitutions in the conserved region. Western blot analysis showed that goldfish oocytes contained mainly GV-lamin B3 as well as some somatic lamins. Laser-confocal microscope observations revealed that lamin B3 was present only in GV nuclear lamina, whereas somatic lamins were present in dense fibrillar structures throughout nuclear gels of isolated GVVs. Similar nuclear filamentous structures were also observed in GVVs of paraffin embedded oocytes. Epitope mapping indicated that L-200 recognized a conserved region containing a short stretch of the Y(E/Q)(Q/E)LL. A similar motif is also present in other cytoplasmic intermediate filaments (i.e., vimentin, desmin, peripherin and GFAP). Taken together, these findings suggest that lamins or lamin-related intermediate filaments are an important component of the interior architecture of goldfish vitellogenic oocyte nuclei (GVs).

**Key words:** actin/intermediate filament/meiosis/lamina/nuclear matrix

The inner nuclear membrane of the vertebrate nucleus is intimately associated with a fibrous mesh, or lamina, composed of lamins that are classified into type V intermediate filaments (IFs) (Aebi et al., 1986; McKeon et al., 1986; for review see Stuurman et al., 1998). IFs have a central α-helical longitudinal rod-domain consisting of repeated heptads which separate the non-helical head and tail domains thus forming a two stranded typical coiled-coil structure (for review see Parry and Steinert, 1995). In contrast, lamins contain 42 extra amino acids (aa) in their rod domain of six heptad repeats (Nigg, 1989; Weber et al.,1989; Mical and

Monteiro, 1998), a nuclear localization signal (NLS) (Loewinger and McKeon, 1988; Moir et al., 1995) and a CaaX box (C, cystein; a, aliphatic amino acid; X, variable) which plays a role in chemical modifications such as isoprenylation, carboxyl methylation and proteolytic trimming (Holtz et al., 1989; Moir et al., 1995). Lamins are classified as A- or B-type based on their primary sequence and/or solubility properties at mitosis. During interphase, lamins are present in an insoluble form. During metaphase, however, they depolymerize and become dispersed throughout the cytoplasm. A-type lamins depolymerize into oligomers and become completely soluble (Gerace and Blobel, 1980), whereas B-type lamins remain associated with membranous structures (Gerace and Blobel, 1980; Chaudhary and Courvalin, 1993). In frogs, four different lamins (LI to L III and LA) have been isolated and characterized throughout development. B-type lamins are subdivided into three groups, B1, B2 and B3. Lamins B1 and B2 have been identified in somatic cells of frogs (Krohne et al., 1987; Höger et al., 1990), chicks (Vorburger et al., 1989; Peter et al., 1989) and...
mammals (Höger *et al*., 1988, 1990; Pollard *et al*., 1990). Lamin B3 is the only lamin specifically expressed in animal oocytes. Based on its primary structure, lamin B3 belongs to the B-type lamins in that it becomes soluble during both meiotic and mitotic metaphases (Benavente *et al*., 1985; Newport *et al*., 1990), although small amounts (5–7%) of B3 and some minor B-type lamins have been reported to remain associated with membrane fragments (Lourim and Krohne, 1993; Lourim *et al*., 1996). Lamins likely play important roles in DNA binding (Ludérus *et al*., 1992; 1994), changes in chromatin organization (Höger *et al*., 1991; Taniura *et al*., 1995), nuclear structure (Furukawa and Hotta, 1993) and DNA replication (Newport *et al*., 1990; Meier *et al*., 1991; Jenkins *et al*., 1993; Moir *et al*., 1994; Ellis *et al*., 1997; Spann *et al*., 1997). Consequently, the differences between meiotic oocyte-lamin composition and mitotic somatic lamins may have important implications for meiotic nuclear structure and function.

In addition to the lamina, a second filamentous network, i.e. the nuclear matrix, has been identified in vertebrate nucleii (Berezny and Coffey, 1974; Capco *et al*., 1982; Fey *et al*., 1986). Lamins are a structural component of the nuclear matrix as well as the peripheral lamina (Hozak *et al*., 1995). Lamins appear not only with the inner nuclear membrane but also with internal nucleus foci coinciding with DNA replication in some S-phase nuclei (Moir *et al*., 1994). Lamin foci were also identified in G1 phase nuclei (Bridger *et al*., 1993). Intermediate filament-like structures and various other classes of filaments are present in the nuclear matrix (Berezney and Coffey, 1974; Capco *et al*., 1982; Jackson and Cook, 1988; He *et al*., 1991), suggesting that lamins are a likely candidate for internal nuclear matrix filaments. Internal matrix filaments were present in reconstituted sperm pronuclei in *Xenopus* cell free extracts after removal of lamin B3 (Jenkins *et al*., 1993; Zhang *et al*., 1996).

In this paper, we characterized an anti-somatic lamin monoclonal antibody generated against purified rainbow trout erythrocyte lamins. Laser-confocal microscope analysis revealed a densely packed interior filamentous nuclear structure in goldfish oocyte nuclei, whereas only the peripheral nuclear lamina was recognized by an anti-lamin B3 monoclonal antibody. These results indicate that lamin-like filaments play a role in supporting nuclear structures in the interior of GVs of goldfish oocytes.

**Materials and Methods**

**Fishes and cell strains**

Rainbow trout (*Oncorhynchus mykiss*) were obtained from the Gifu prefecture hatching center in Japan. Goldfish were bought from Yamato-Koriyama and kept at 15°C in laboratory aquaria. Epithelioma papillosum of goldfish cells (EPG) were cultured in L15 medium (Sigma) containing 10% fetal calf serum at 18°C and subcultured every 2–3 weeks.

**Isolation of nuclei and preparation of nuclear lamina**

Rainbow trout blood was collected by syringe with 1/10 volume of an anti-coagulant solution [0.2 M EDTA, 100 μM (p-amidino-phenyl) methanesulfonyl fluoride hydrochloride (p-APMSF)]. Erythrocytes were collected and washed in 75 mM NaCl, 25 mM EDTA (adjusted pH 7.4 by NaOH) by centrifugation (3,000 rpm for 10 min) three times. After the final spin, cells were lysed in nuclei isolation buffer [NIB: 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 5 mM EDTA, 1 mM spermine, 1 mM dithiothreitol (DTT), 100 μM p-APMSF, 0.25 M sucrose, 0.1% Triton X-100]. Nuclei were then collected by centrifugation at 3,000 rpm for 5 min and washed twice in NIB at 2,000 rpm for 5 min. 2×10⁹ nuclei were isolated from 10 ml of packed erythrocytes. Goldfish erythrocyte nuclei were isolated according to the methods above with the substitution of citric acid solution (pH 5.0) as the anti-coagulant. EPG cultured cells were collected by scraping and suspended in NIB. The suspension was passed through a 27 gauge needle to lyse the cells. Crude nuclei were collected by centrifugation at 10,000×g and washed with NIB several times. Isolated nuclei were then washed with NIB and finally resuspended in NIB containing 50% glycerol (v/v) for storage at −80°C.

Somatic nuclear lamins were prepared from isolated rainbow trout erythrocyte nuclei. Nuclei (2×10⁹) were resuspended in 20 ml nuclei swelling buffer (20 mM MES, 1 mM DTT, 100 μM p-APMSF, 0.25 M sucrose, pH 6.5) and aliquoted into eight polycarbonate tubes on ice for 15 min. 5,000 units of deoxyribonuclease II (DNase II) from beef spleen (Bioenzyme) were then added to each tube for digestion of DNA. After incubation for 1 hr at room temperature, an equal volume of 2×extraction buffer (20 mM Tris, 2 mM EDTA, pH 8) was added. The resulting nuclear ghosts were pelleted at 25,000×g for 30 min. Pellets were then suspended in 40 ml detergent buffer (20 mM MES, 0.3 M NaCl, 2 mM EDTA, pH 8) and stirred for 30 min at 4°C. The nuclear lamina fraction was collected by centrifugation (25,000×g). Finally, the lamina were rinsed in 10 mM Tris-HCl (pH7.4) and stored at −80°C. The insoluble lamina pellet was then used as an antigen to produce an anti-somatic lamin monoclonal antibody (L-200). Germinal vesicles (GVs) were isolated from goldfish oocytes according to the methods of Yamaguchi *et al*. (2001).

**Fluorescence microscope and laser scanning microscope observations**

Isolated goldfish GVs were transferred into siliconized microtubes, rinsed with 50 mM phosphate buffer (PB: 32.5 mM Na₂HPO₄, 17.5 mM NaH₂PO₄) and fixed in 3.7% formaldehyde/50 mM PB for 30 min at room temperature. After three PBS washes, GVs were permeabilized in PBS plus 1% NP40 for 30 min at 37°C. After three PBS washes, GVs were incubated with the primary antibodies (1:500) with gentle agitation at 4°C overnight. Af-
ter three PBS rinses, GVs were then incubated in PBS plus 1% BSA and 0.1% Tween-20 containing the secondary antibody (1:500, BioSource International, Camarillo, CA, USA, FITC conjugated mouse IgG) for 1 hr at room temperature. When double staining (lamin and actin) was carried out, rhodamine conjugated phalloidin (Molecular Probes, Eugene, OR, USA) was used to stain F-actin. After three PBS washes, GVs were placed in slide wells (0.5 mm depth) and mounted with an equal volume of PBS.

Goldfish ovaries containing vitellogenic follicles were dissected, cut into small fragments and fixed in MEMFN (100 mM MOPS, 2 mM EGTA, 1 mM MgSO₄, 4% formaldehyde prepared from paraformaldehyde, 0.2% NP-40, pH 7.4) for 3 hr at room temperature. Fixed ovarian fragments were stored in 70% ethanol at 4°C, serially dehydrated in ethanol (70, 80, 90, 95, 99.5, 100%) and lemosol (Wako Pure Chemicals, Osaka, Japan). Samples were embedded in paraffin (Sakura Finetec, Torrance, CA, USA). Six-μm sections were prepared and processed conventionally. After serial rehydration (xylene, 100, 95, 90, 80, 70, 50% ethanol, PBS), slides were immersed in blocking solution (10% heat denatured fetal calf serum in PBS) for 1 hr at 4°C. Anti-lamin primary antibody (L-200, 1:500) was then added and the sections were incubated overnight at 4°C. After washing with PBS, samples were incubated with rhodamine B conjugated anti-mouse IgG F(ab')2 (Immuno- tech, Marseille, France, 1:500) for 1 hr at room temperature. Slides were mounted in 50% (v/v) glycerol in PBS. Whole images were obtained with a laser confocal scanning microscope LSM510 Carl Zeiss, Germany) equipped with epifluorescence optics, appropriate filter sets and an Argon/Krypton laser.

**Extraction of proteins, electrophoresis and Western blotting**

Nuclear membrane fractions were mixed in urea (8 M), and then vigorously agitated for 15 min. After centrifugation at 10,000×g for 30 min, supernatants and pellets were analyzed by SDS-PAGE. Protein samples were prepared in sample buffer to the final concentration of 1000 nuclei/μl. Ten μl were loaded on the gels. Separated proteins were visualized by Coomassie brilliant blue staining.

Nuclei isolated from goldfish oocytes (GVs), erythrocytes and cultured cells were extracted with urea, mixed with an equal volume of 2×SDS sample buffer, and electrophoresed on SDS-PAGE (containing 8% polyacrylamide). Separated proteins were electrophoretically blotted onto the PVDF membranes (Millipore, Bedford, MA, USA). Western blot analysis was done by methods previously reported (Yamashita *et al*., 1991). Monoclonal antibodies (L200 and O3E6, see below) were used as primary antibodies. Alkaline-phosphatase-conjugated goat anti-mouse IgG (ZYMED, San Francisco, CA, USA) was used for visualization.

**Antibodies**

To produce an anti-fish lamin monoclonal antibody (L-200), nuclear lamin enriched residual nuclear membrane fractions from 5×10¹⁰ erythrocyte nuclei of rainbow trout (Fig. 1, lane 2) were injected into mice three times at two week intervals. Antigen was first injected in Freund’s complete adjuvant (Difco, Detroit, MI, USA). The second and third incomplete adjuvant injections were with or without adjuvant, respectively. Fusion and screening methods were according to Yamashita *et al.* (1991). Initial screenings were performed by ELISA plates, Xenobind (Denopore Corp., Hawthorne, NJ, USA) conjugated with electroeluted rainbow trout lamins (10 ng/ml) from SDS-PAGE gels. Finally, a fusion cell which produced L-200 was selected by Western blotting against crude extracts from isolated rainbow trout nuclei. Hybridoma supernatants were then used for immunoscreening or Western blot analysis.

An anti-goldfish lamin B3 monoclonal antibody (O3E6) was prepared according to the method above. One hundred μg *E. coli* produced the recombinant full-length lamin B3 that was used for the first injection. Subsequent injection contained 250 μg. The initial screening was carried out using ELISA plates coated with purified lamin B3 (10 ng/well). A hybridoma producing the anti-
lamin B3 monoclonal antibody was identified by Western blotting. Ascites fluid was then obtained by injecting mice with hybridoma cells producing either L-200 or O3E6. Mouse polyclonal anti-goldfish lamin B3 antibody (ascites) was raised against prepared GV-lamina.

**Screening of cDNA encoding goldfish lamins, DNA sequencing and expression in E. coli**

PolyA+ RNA from EPG cells was isolated with the FastTrack kit (Invitrogen, Carlsbad, CA, USA). Oligo-dT cDNA libraries were constructed with a λgt11 synthesis kit (Amersham-Pharmacia Biotech, Buckinghamshire, England) using EcoRI/Nol adapter primers (Promega, Madison, WI, USA) for the synthesis of first strand cDNA. A λZAP/EcoRI cloning kit (Stratagene, La Jolla, CA, USA) was also used to construct a cDNA library. L-200 was used to isolate goldfish somatic-lamin cDNA from the λgt11 cDNA expression library. Immunopositive partial cDNA clones were obtained, the largest of which was 2.9 kilobase (kb), sequenced (see below), and identified as B-type lamin. Probes were prepared with a random labeling kit (NEN Lifescience Products Inc., Boston, MA, USA) using the 2.9 kb insert to isolate a full-length cDNA clone from the λZAP cDNA library. A 3551-bp cDNA clone of lamin (C4-1) containing two pvu II sites inside the C4-1 coding region and one in the 3′ was obtained. After digestion by pvu II, DNA fragments were subcloned into pBluscript KS+ (Stratagene, La Jolla, CA, USA) and sequenced in both orientations with the Dye Terminator Cycle Sequencing Kit (Perkin Elmer ABI, Foster, CA, USA). EcoRI/mung bean deletion subcloning procedures were then used to sequence large fragments. Another immunopositive clone C7-1 (1.8 kb) was also sequenced after restriction enzyme (AccI, CluI, HindIII and SaeI) digestion, and subcloned into KS+. The results of sequencing revealed that it was a lamin B1 homologue.

To construct goldfish B-type lamin expression plasmids containing truncated coil 2 rod domains, PCR was carried out with the following combinations of primers: (1) N-terminal deletions–six forward primers with a NdeI restriction site 5′-GGGAATTCATATGGCGACCGCAACCCCGAG-3′ (N241); 5′-AATGATAAAGCGGTTCTCGCCG-3′ (N364); 5′-CTGTCATCGCTCAAGGGATG-3′ (N343); 5′-CTGCTGAGCTCAAAGCTG-3′ (N360); 5′-GAGATCAAAGCCTATCG-3′ (N369) and one reverse primer (5′-CCGGATCCATCACATGACCGCA-3′) with a BamHI site followed by a stop codon; (2) C-terminal deletions–five reverse primers introducing a BamHI site and stop codon (5′-CTCTAGAATCTCTCTCCGAC-3′ (CA378), 5′-GATCTCCATGCTGCGGCGAG-3′ (N364), 5′-GGCGGATCCCTGCTCTGCGG-3′ (CA534), 5′-CTGCTGCTGAGCTCAGCCG-3′ (CA345) and one forward primer containing a start codon and a NdeI restriction site (5′-GAGAATCTTGCGGGCGGCAACCCCGAG-3′) DNA encoding lamins with a truncated coil 2 were amplified by PCR using pBluscript SK- containing full length lamin (C4-1) as a template. The full coding region of lamin B3 was also amplified.

All PCR cycling conditions were as follows: 30 cycle–1 min at 95; 1 min at 55; 2 min at 72. Constructed PCR products were ligated to TA cloning vector, PCR2 (Stratagene, La Jolla, CA, USA) and verified by sequence analysis. Isolated plasmids were digested with NdeI and BamHI and inserts were subsequently ligated into isolated, pre-digested PET21a (Novagen, Madison, WI, USA). All expression plasmids were transformed into CaCl2 competent E. coli (strain BL21). Proteins with the expected SDS-PAGE mobility of truncated goldfish lamin B2 were detected in extracts from IPTG-induced E. coli cultures by Coomassie brilliant blue staining or Western blotting with L-200 as described above.

**Results**

**Characterization of fish somatic lamins**

Lamins are major salt- and detergent-resistant proteins with molecular weights between 60 to 80 kD. They can be easily extracted with 6–8 M urea or high pH solutions. We used rainbow trout erythrocytes to purify nuclear lamins because contamination by cytoplasmic proteins is easily minimized. Nuclear membrane fractions had four major proteins of 43 kD, 50 kD, and 67 kD and 70 kD (Fig. 1, lane 1). After non-ionic detergent treatment, residual nuclear membrane fractions contained only the 67 kD and 70 kD bands. The 50 kD protein was extracted by Triton X-100 and could not be detected by Coomassie staining (Fig. 1, lane 2). Similar to other vertebrate somatic lamins, the 67 and 70 kD peptides became soluble after treatment with 8 M urea and sodium carbonate (pH 11), but were resistant to non-ionic detergent (2% Triton X-100) treatment, thus identifying them as rainbow trout erythrocyte nuclear lamins.

The monoclonal antibody L-200 recognized two lamin proteins and some unknown peptides (43–47 kD in the bracket lane 2) which may be proteolytic peptides of the 70 kD lamin or intermediate filament proteins associated with nuclear lamina (Fig. 2A). L-200 also recognized two doublet bands in goldfish erythrocytes (Fig. 2B, lane 1), a single band in EPG culture cell nuclei (lane 2), and two other bands from isolated GVs (lane 3). L-200 did not recognize goldfish GV-lamin B3 (Mr 67 kD, lane 4), although it was recognized specifically by the polyclonal GV-lamin B3 antibody. Isolated GVs contained more oocyte-lamin B3 than somatic-lamins (lanes 3 and 4). These results show that molecular weights of goldfish somatic lamins differ according to cell type as well as from GV-lamin B3.

**Immunoscreening of goldfish lamins**

A lamin cDNA was isolated from goldfish EPG cells by immunoscreening with L-200. Sequence analysis of the 2.9 kb insert from λgt 11 plaque (C8-4) indicated sequence similarity with B-type nuclear lamins. A second full-length lamin cDNA isolated using random-labeled probes against C8-4 confirmed the sequence. The deduced amino-acid sequence
was highly homologous to B-type lamins, particularly lamin B2. Based on a partial sequence, immunopositive clone C7-1 had high similarity to lamin B1. All clones had an \(/g97\)-helix rod domain of heptad repeats, a NLS and a CaaX box. The presence of these characteristics of IFs (Fig. 3) suggests that the structure of lamins is very conserved among all vertebrates. Amino acid sequence alignment between goldfish B2 and B3 shows that both \(/g97\)-helical rod domain sequences are similar (50% homology), while C-terminal tail sequences have less homology (28% homology between B2 and B3, 30% between B1 and B3) indicating that, like \textit{Xenopus} B type lamins (B1, B2 and B3), goldfish B-type lamins can be classified into three subtypes.

The N-terminal sequence of C8-4 included the coil-2 domain (a.a. position 255 of lamin B2 on Fig. 3), suggesting that mab L-200 recognizes a downstream epitope. We then used PCR and bacterial expression techniques to identify the epitope that mab L-200 recognizes. Twelve lamin B2 constructs (one full length and eleven truncated coil-2) were expressed (Fig. 4A) and separated on SDS-PAGE to confirm deletions (Fig. 4B, Coomassie staining). L-200 recognized truncated lamin B2 peptides with deletions from the N-terminus to residue 344 inside coil 2, but not peptides with larger deletions (Fig. 4C, lanes 1–7). L-200 also recognized truncated lamin peptides deleted from the C-terminus to residue 363 inside coil 2, but not to residue 353 (Fig. 4C, lanes 8–12). These results indicate that L-200 recognizes a short region inside the coil 2 rod-domain (residues 353–363) consisting of eleven amino acids (QQLNEYQELLD), many of which are conserved Y(Q/E)(E/Q)LLD among lamins (Table I). Western blot analysis revealed that L-200 recognized mouse lamins A and B, \textit{Xenopus} lamin B3, the meiosis-specific nuclear structural protein MNS1, and goldfish lamins B1 and B2. L-200 did not, however, recognize goldfish lamin B3 (Table I). All proteins have the characteristic coiled-coil structure of IFs. L-200 also recognized \textit{Xenopus} lamin B3 from isolated \textit{Xenopus} GVs and its oligomeric form from egg extracts (data not shown). Therefore, we conclude that this epitope is conserved among most lamins and lamin-related proteins and is exposed in both the assembled and disassembled forms.

**Recognition of dense filamentous structures in oocyte nuclei by L-200**

As described above, goldfish GVs contained lamin B3 and minor somatic lamins. Two monoclonal antibodies (L-200 and O3E6) were used to examine their distribution in isolated GVs. Laser confocal microscope analysis using anti-B3 (O3E6) revealed that lamin B3 was distributed only in nuclear lamina (Fig. 5B). In contrast, L-200 stained the nuclear gel, except nucleoli, and nuclear lamina (Figs. 5C and 5D). F-actin is abundant in the nuclear gel under isolated conditions (Yamaguchi and Nagahama, unpublished). Double staining with rhodamine-phalloidin, a staining reagent for F-actin, and L-200, revealed different distributions for F-actin and lamin B1/B2 (Fig. 6). F-actin localized in the peripheral region of nucleoli, while filamentous structures were distributed throughout the nucleoplasm. These observations showed that the filamentous structure recognized by L-200 is not an F-actin bundle. Furthermore, staining of paraffin-embedded vitellogenic oocytes confirmed that the filamentous structure was not an artifact generated during the GV-isolation step. L-200 clearly recognized dense filamentous structures of GVs in oocytes in the early vitellogenic stage when peripheral nucleoli were observed (Fig. 7). This filamentous structure was most abundant in the nucleoplasm, with the exception of the nucleoli. O3E6 immunostained lamina proteins from isolated GVs (Fig. 5), but signals could not be detected after paraffin embedding.

**Discussion**

**Characteristics of fish lamins**

Vertebrate lamin isoforms have been classified based on biochemical and structural criteria. For example, A-type lamins have a neutral isoelectric point, while B-type lamins (B1, B2) are more acidic (Gerace and Burke 1988; Nigg,
Fig. 3. Amino acid sequence alignments of goldfish lamins. Amino-acid sequences from full length cDNAs of lamins B2 and B3 and partial lamin B1 sequence were aligned. Asterisks indicated conserved amino-acid residues between B2 and B3 or all three lamins. Characteristic lamin sequences are underlined. Serine residue of SPTRI/L is a putative goldfish lamin cdc2 targeting site. FNKT in coil 1b and DKA V in coil 2 correspond to the lamin B2 specific tetra peptide sequences (FHRS, DKAA) that have not been found in vertebrate lamin B1. QQLNEYQELLD (B2) is the epitope region recognized by the anti-lamin B1/B2 monoclonal antibody (L-200). The nuclear localization signal (NLS) and CaaX box sequences are also underlined.
Somatic Lamins in Germinal Vesicles

1989; Peter et al., 1989). Oocyte-type B3 lamin is less acidic than other B types, but is not neutral (Yamaguchi et al., 2001). We isolated three cDNAs encoding goldfish lamin B1, B2 and B3. The regions of highest similarity among the goldfish B-type lamins were at either end of the short rod segment (about 30 a.a.). These two rod ends are also highly conserved among other intermediate filaments (for review Parry and Steinert, 1995). Deletions and/or point mutation within these two regions showed that these rods play crucial roles in the assembly state of higher order structures (Coulombe et al., 1991; Heald and McKeon, 1990; Hatzfeld and Weber, 1991; Letal and Fuchs, 1995; McCormick et al., 1993; Stuurman et al., 1996). The presence of these conserved regions therefore indicates that they are also important for higher order structures in fish. All known vertebrate nuclear lamins, including fish lamins, have a p34<sup>cd2</sup> targeting motif (SPTRI/L) within the N-terminal head domain that induces lamina disassembly by mitotic/meiotic phosphorylation.

Sequence analysis of other vertebrate lamins indicates that there are no conspicuous differences between lamin B1 and B2 except for the conservation of two tetra peptides (FHRS in coil 1b; DKAA in coil 2) in B2 lamins, but not B1 lamins (Höger et al., 1990). Our sequencing results of goldfish B2 revealed that FNKT and DKAV replaced FHRS and DKAA, respectively. This minor amino acid substitution probably does not affect the overall domain structure. A similar change was also found in goldfish B3. For example, the nuclear localization signal (NLS) residing within the C-terminal domain consists of an invariant lysine (K) followed by three other basic residues (K or R), a hydrophobic residue, and an acidic residue (E or D). The goldfish lamin B3 NLS (RKRKHE) substitutes R for the first K and replaces a hydrophobic residue with an H. Nevertheless, NLS functions properly in *Xenopus* oocytes and also goldfish EPG cells (data not shown). The conserved L-200 epitope was also modified in goldfish lamin B3 (Table I and see next section). With the exception of these minor changes we did not find any unique sequences in goldfish B-type lamins.

**Fig. 4.** Epitope mapping for monoclonal antibody L-200. (A) Schematic diagram of goldfish lamin B2 deletion mutants, showing the NLS site, CaaX motif and reactivity with L-200. (B) *E. coli* lysates (5 μg) stained with Coomassie-brilliant blue (upper panel) or immunoblotted with L-200 (lower panel). L-200 reacts with full length lamin B2, four N-terminal deletion mutants (lanes 1-5) and two C-terminal deletion mutants (lanes 8 and 9). Lane numbers correspond to the schematic diagram in (A).
A. Yamaguchi and Y. Nagahama

Characterization of anti-lamin monoclonal antibody L-200

We raised an anti-lamin monoclonal antibody using purified nuclear lamina as the antigen. Deletion analysis revealed that the anti-lamin antibody L-200 recognized an epitope inside the coil 2 region in both the disassembled and assembled forms. L-200 immunoprecipitated the disassembled form from *Xenopus* egg extracts (unpublished data) and also detected the assembled form by confocal microscopy. Goldfish lamin B3 was the only vertebrate lamin that L-200 could not recognize. A His (histidine) substitution in goldfish lamin B3 (EQLEHQNILLD) for the well conserved Y (QLNEYQELLLD) in other vertebrate lamins and cytoplasmic intermediate filaments (Table I) may have prevented recognition by L-200. This antibody also recognized meiosis-specific nuclear structural protein MNS1 in mouse spermatocytes (Furukawa et al., 1994). In coil 2, MNS1 contains the sequence YEQLL (206–210). Other cytoplasmic intermediate filaments (i.e., vimentin, desmin, peripherin and GFAP) also have similar motifs (YQ(D/E)LL; Y(E/Q)(Q/E)LL) suggesting that this sequence represents the L-200 epitope (Table I). L-200 detected several peptides (mw 42–47 kD) and lamins in Western blots of isolated nuclei or nuclear lamina (Fig. 2A, lane 2). These peptides may be intermediate filament proteins precipitated with nuclear lamins during preparation of nuclear lamina. Taken together, these results suggest that L-200 recognizes the conserved sequence Y(E/Q)(Q/E)LL in the α-helical coiled-coil domain of several kinds of nuclear or cytoplasmic skeletal intermediate-type filaments. Further Western blot analyses of various lamin-related intermediate filaments using L-200 are required to confirm this suggestion.

Filamentous structures interior to germinal vesicles

DNA-free nuclear matrices, visualized under nearly physiological ionic conditions after nuclease digestion (Jackson and Cook, 1988), were shown to contain individual nuclear filaments about 10 nm wide with 23 nm axial repeats. An intermediate-type smooth 9–13 nm filament network, exposed after extraction with ammonium sulfate (0.25 M) and high salt (2 M NaCl), serves as the core structure around which other proteins associate to form the complete matrix (He et al., 1991).

Lamins are present in the interior of the nuclei as well as in the lamina. Nevertheless, electron microscope studies revealed that lamins are not the core filament, but are present in the nodes or knobs on the diffuse nucleoskeleton (Hozak et al., 1995). Early observations of reconstituted sperm pronuclei in *Xenopus* cell free extracts revealed that the internal matrix filaments are not comprised of lamin B3, but that the

---

Table I. ALIGNMENT OF EPITOPE SEQUENCES RECOGNIZED BY L-200.

<table>
<thead>
<tr>
<th>IF</th>
<th>Epitope</th>
<th>Blot</th>
<th>Accession</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFlamB1</td>
<td>QQLDDYEYQELLD</td>
<td>Y</td>
<td>AB034199</td>
</tr>
<tr>
<td>GFlamB2</td>
<td>QQLNEMYQELLD</td>
<td>Y</td>
<td>AB034198</td>
</tr>
<tr>
<td>GFlamB3</td>
<td>EQLEHQNILLD</td>
<td>N</td>
<td>AB034197</td>
</tr>
<tr>
<td>MlamB1</td>
<td>QQLSDEYQELLD</td>
<td>Y</td>
<td>P14733</td>
</tr>
<tr>
<td>MlamB2</td>
<td>QQLAEYQELLD</td>
<td>Y</td>
<td>P21619</td>
</tr>
<tr>
<td>MlamA</td>
<td>QQLDEYQELLD</td>
<td>Y</td>
<td>P48678</td>
</tr>
<tr>
<td>XelamB3</td>
<td>QQLETYQELLD</td>
<td>Y</td>
<td>P10999</td>
</tr>
<tr>
<td>MNS1</td>
<td>RQGEAYQELLD</td>
<td>Y</td>
<td>D14849</td>
</tr>
<tr>
<td>GFvim</td>
<td>RHLREYQDLLN</td>
<td>I</td>
<td>150484</td>
</tr>
<tr>
<td>MMvim</td>
<td>RHLREYQDLLN</td>
<td>P</td>
<td>20152</td>
</tr>
<tr>
<td>Xxvim</td>
<td>RHLREYQDLLN</td>
<td>NP</td>
<td>003371</td>
</tr>
<tr>
<td>ZFvim</td>
<td>RHLREYQDLLN</td>
<td>A</td>
<td>AAC98491</td>
</tr>
<tr>
<td>MMdesmin</td>
<td>RHLREYQDLLN</td>
<td>P</td>
<td>31001</td>
</tr>
<tr>
<td>Xedesmin</td>
<td>RHLREYQDLLN</td>
<td>P</td>
<td>23239</td>
</tr>
<tr>
<td>ZFdesmin</td>
<td>RHLREYQDLLN</td>
<td>A</td>
<td>AAB03217</td>
</tr>
<tr>
<td>Mperifer</td>
<td>RHLREYQDLLN</td>
<td>P</td>
<td>15331</td>
</tr>
<tr>
<td>Xeperi</td>
<td>RHLREYQDLLN</td>
<td>P</td>
<td>0A34591</td>
</tr>
<tr>
<td>MMGFAP</td>
<td>RHLQERYQDLLN</td>
<td>P</td>
<td>P03995</td>
</tr>
</tbody>
</table>

*L-200 recognized intermediate-type filaments (IF), goldfish (GF) B1 and B2, mouse (MM) lamin A and B (Furukawa and Hotta 1993), Xenopus (Xe) lamin B3, mouse meiosis specific nuclear structural protein (MNS1) (Furukawa et al., 1994), but not GF3 (See Fig. 2B in this article) by western blot analysis. Underlining indicates conserved sequences. This conserved epitope motif is also conserved in other IFs such as vimentin (vim), desmin, peripherin (peri) and glial fibrillary acidic protein (GFAP). Note: GF3 contains unique amino-acid residues (filled triangles) within the conserved region. Accession numbers, positive reactions (Y) or negative reactions (N) are also shown. No blot letter indicates filaments that were not tested.

**Fig. 5.** Confocal microscope analysis showing the distribution of lamins in isolated GV. An isolated goldfish GV was stained with (A) control mouse serum, (B) anti-B3 specific mab O3E6, or (C) anti-somatic lamin mab L-200 followed by a FITC-conjugated anti-mouse IgG. Panel D is a DIC image of panel C. O3E6 recognized only nuclear lamina, while L-200 recognized structures inside the nuclear gel as well as the lamina. Bar = 100 μm.
matrix surface is composed of B3 (Zhang et al., 1996). These results are similar to our confocal microscope observations on isolated goldfish GVs. Although previous reports suggested involvement of other intermediate-type filaments in the interior GV structure, we could not determine whether these internal filaments are composed of lamins or novel filamentous proteins containing the conserved epitope recognized by L-200. Other lamin-specific antibodies with less conserved antigens are needed to resolve this issue.

Nuclear actin, another candidate for nuclear filament structures, has been found in various cell types (Amankwah and Deboni, 1994; Pafenov et al., 1995; Clubb and Locke 1998; Wasser and Chia, 2000). Recently, filamentous structures containing nuclear actins, not cytoplasmic F-actins, were immunologically identified in the interior of Xenopus oocytes (Gonsior et al., 1999). The distinct distribution of F-actin and lamin-related filaments in isolated goldfish GVs also indicates that the dense internal filaments are not F-actin (Fig. 5). Collectively, these observations suggest that a novel intermediate filament may be present in the goldfish GV nuclear matrix.

Acknowledgments. We thank Dr. M. Yoshimizu of Hokkaido University for kindly providing the EPG cells. Laser confocal microscopy (Zeiss LSM510) was performed at the Laboratory of Electron Microscopy at Okazaki National Research Center. This research was aided in part by Grants-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Culture, and Sports, Japan (07283104).

References


Somatic Lamins in Germinal Vesicles


(Received for publication, August 29, 2001 and in revised form, October 11, 2001)