The lamina-associated polypeptide 2 (LAP2) isoforms β, γ and ω of zebrafish: developmental expression and behavior during the cell cycle

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Summary

Zebrafish lamina-associated polypeptides 2 (ZLAP2) β, γ and ω have in common an N-terminal region with a LEM domain, and in the C-terminal half of the molecule a lamina binding domain and a membrane spanning sequence. The maternally synthesized ω is the largest isoform and the only LAP2 present in the rapidly dividing embryonic cells up to the gastrula stage. ZLAP2ω levels decrease during development, concomitant with the increase of the somatic isoforms ZLAP2β and γ. In somatic zebrafish cells ZLAP2γ is the predominant isoform, whereas only small amounts of ZLAP2β are present.

During early embryonic development, ZLAP2ω becomes associated with mitotic chromosomes before anaphase. The surface of these chromosomes is decorated with vesicles, and each chromosome assembles its own nuclear envelope at the end of mitosis (karyomere formation). Ectopically expressed ZLAP2ω-green fluorescent protein (GFP) fusion protein targets vesicles to mitotic chromosomes in *Xenopus* A6 cells, suggesting that ZLAP2ω is involved in karyomere formation during early zebrafish development.

When ZLAP2β and γ were expressed as GFP fusion proteins in *Xenopus* A6 cells, the β- but not the γ-isoform was found in association with mitotic chromosomes, and ZLAP2β-containing chromosomes were decorated with vesicles. Further analysis of ZLAP2-GFP fusion proteins containing only distinct domains of the ZLAP2 isoforms revealed that the common N-terminal region in conjunction with β- or ω-specific sequences mediate binding to mitotic chromosomes in vivo.

Key words: Zebrafish LAP2, Karyomere, Chromatin binding, Lamina, Mitosis

Introduction

The nuclear envelope is composed of three distinct membrane domains: the outer nuclear membrane, which is in continuity with the endoplasmic reticulum; the membranous wall of the nuclear pore complex, which is located where the outer and inner nuclear membranes fuse to form the pore channel; and the inner nuclear membrane, which is associated with a meshwork of proteins, the nuclear lamina.

Nuclear intermediate filament proteins, the lamins, are the major structural elements of the lamina (for reviews, see Gant and Wilson, 1997; Krohne, 1998; Gruenbaum et al., 2000; Wilson et al., 2001). They are essential architectural proteins implicated in the postmitotic reorganization of the nucleus, including chromatin decondensation and replication (Hutchison et al., 1994; Moir et al., 2000; Lopez-Soler et al., 2001; Holaska et al., 2002; for a review, see Benavente, 1991).

The inner nuclear membrane is structurally and functionally distinct from the other two membrane domains. It contains specific integral membrane proteins (IMPs) that interact with the underlying lamina and the peripheral chromatin during interphase (for reviews, see Collas and Courvalin, 2000; Gruenbaum et al., 2000; Dechat et al., 2000a; Cohen et al., 2001; Goldman et al., 2002).

So far, the best investigated IMPs of the inner nuclear membrane are members of the lamina-associated polypeptide 2 (LAP2) family. In mammals, they have been shown to be generated by alternative splicing from a single gene (Harris et al., 1994; Berger et al., 1996). Six mammalian isoforms (LAP2α, β, γ, δ, ε and ζ) have been identified, and *Xenopus* homologues to the rat LAP2β have been cloned (Gant et al., 1999; Lang et al., 1999). All isoforms except the mammalian LAP2α and ζ are type II IMPs with a short carboxyterminus projecting into the perinuclear cistern and an N-terminal domain localized on the nucleoplasmic side of the inner nuclear membrane (for a review, see Dechat et al., 2000a). All mammalian isoforms possess the same N-terminal segment of 187 amino acids (LAP2 constant region) that is highly conserved in *Xenopus*. Two structural homologous globular domains of approximately 40-50 amino acids in length (LAP2-N and LEM-motif) (Cai et al., 2001) have been located within this sequence. The LEM-motif is also found in otherwise unrelated IMPs of the inner nuclear membrane, like emerin and

MAN1 (Lin et al., 2000). It interacts with the DNA binding protein BAF (barrier to autointegration factor) (Furukawa, 1999; Cai et al., 2001; Lee et al., 2001; Shumaker et al., 2001), suggesting that BAF is mediating the binding of LAP2 and emerin to chromatin. Nuclear reconstitution experiments in *Xenopus* egg extracts indicated that in vitro the LAP2 constant region and BAF are directly or indirectly required for membrane-chromatin attachment and lamina assembly (Gant et al., 1999; Shumaker et al., 2001; Segura-Totten et al., 2002). LAP2-N but not the LEM motif binds to DNA in vitro (Cai et al., 2001).

Other nuclear proteins binding to the mammalian LAP2β are germ-cell-less (Nili et al., 2001), HA95 (Martins et al., 2000) and B-type lamins (Furukawa et al., 1995; Furukawa et al., 1998). The lamina/B-type lamin binding domain has been localized in rat LAP2β between amino acids 298 and 373 (Furukawa et al., 1995; Furukawa et al., 1998). Part of this sequence is highly conserved in *Xenopus* LAP2 isoforms (Gant et al., 1999; Lang et al., 1999; Lang and Krohne, 2003).

LAP2α was found to be distributed throughout the nucleus and is not enriched in the lamina (Dechat et al., 1998). It possesses a nuclear targeting/chromatin binding domain (Vlcek et al., 1999), interacts with lamins A/C (Dechat et al., 2000b) and binds to mitotic chromosomes in in vitro nuclear reconstitution experiments (Vleck et al., 2002).

LAP2α, β and γ are expressed in the majority of mammalian cells, whereas in somatic cells of *Xenopus* only LAP2β isoforms have been detected (Lang et al., 1999). The absence of LAP2β from *Xenopus* eggs and embryos before gastrulation and the expression of a LAP2-related membrane protein of higher molecular weight in oocytes and eggs indicate that this class of IMPs is developmentally regulated in amphibia (Lang et al., 1999).

Here, we report on the molecular characterization of zebrafish LAP2 isoforms, on their expression pattern during embryonic development, and on their behavior during the cell cycle in the embryo and somatic cells.

Materials and Methods

Cells, transfection, animals and tissues

A6 cells (kidney epithelial cells of *Xenopus laevis*) were cultured according to standard procedures (27°C/5% CO_2). Cells were grown on coverslips and transfected with GFP expression vectors (see below) using Rotifect (Roth, Karlsruhe, Germany) according to the manufacturer's instructions. Cells were analyzed 20 to 48 hours after transfection.

Zebrafish ZF4 cells (Driever and Rangini, 1993) were cultured in Dulbecco's modified Eagle's medium/F12 (D-MEM/F12; Gibco BRL, Burlington, ON, Canada) supplemented with 15% fetal calf serum (FCS; Gibco BRL) and 1% antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin; Sigma, St Louis, MO) at 27 ºC/5% CO_2. AB9 is a cell line isolated from the caudal fin of zebrafish. AB9 cells were maintained in D-MEM supplemented with 15% FCS, 2 mM L-glutamine and 1% antibiotics under identical conditions.

Zebrafish embryos of defined stages were obtained as described by Kimmel et al. (Kimmel et al., 1995). Zebrafish embryos and small tissue pieces of zebrafish liver, testicle and ovary were directly homogenized and boiled in lysis buffer for analysis by SDS-PAGE.

cDNA isolation and sequence analysis

A zebrafish kidney cDNA library spotted on filters (library no. 575 of the Resource Center of the German Human Genome Project; Berlin) was screened by standard methods. A double-stranded cDNA fragment comprising the complete nucleotide sequence of the *Xenopus* LAP2 (Lang et al., 1999) (Accession No. Y17861) was [^{32}P]-labeled using the random priming DNA labeling kit ver. 1.1 (MBI Fermentas, St Leon-Roth, Germany) according to the manufacturer's instructions. Hybridization was performed as recommended by the supplier of the DNA filters. Both strands of the selected cDNA (zebrafish LAP2-B4, Accession No. AJ320189) were sequenced as described (Lang et al., 1999).

For northern blot analysis, total RNA of zebrafish embryos at the eight-cell stage, and at 3, 4.5, 7.5, 10, 24, and 48 hours postfertilization (hpf) was isolated using the Trizol reagent (Gibco BRL) as described (Gajewski and Krohne, 1999). Northern blot analysis was performed according to standard procedures as described (Sambrook and Russell, 2000), but using a modified Church buffer [1 mM EDTA, 0.5 M phosphate buffer (pH 7.0), 7% (w/v) SDS].

Expression of LAP2 in bacteria and antibody production

A PCR product of the zebrafish LAP2-B4 cDNA coding for amino acids 1-165 was cloned into the pET-21a expression vector (Novagen, Bad Soden, Germany). The targeted sequence was obtained using the following primers:

5′ AGCTTCATATGTTGGAATTTCTGGAAGAC 3′ (5′ end), and
5′ TCTTCCTCGAGGTCGCTGTACTGGTCTGAA 3′ (3′ end).

The sequence was expressed in *Escherichia coli* strain Bl 21 Codon plus (Stratagene, Heidelberg, Germany) and proteins were affinity purified by nickel-chelate affinity chromatography as recommended by the supplier (Qiagen, Hilden, Germany). Two guinea pig antisera against zebrafish LAP2 (ZLAP2) were generated as described previously (Cordes et al., 1991). ZLAP2-serum1 and ZLAP2-serum2 specifically react with all zebrafish LAP2 isoforms and, in addition, ZLAP2-serum1 binds weakly to LAP2 polypeptides from other vertebrates. Human autoimmune antibodies against LAP2 (MAN serum) (Paulin-Levasseur et al., 1996; Lang et al., 1999) and mouse monoclonal lamin antibodies X155 and X223 (Lourim and Krohne, 1993) have been previously described. Mouse monoclonal antibodies against GFP were obtained from Roche (Mannheim, Germany). To control for the specificity of guinea pig antisera, polyclonal ZLAP2 antibodies were affinity purified using bacterially expressed ZLAP2 (amino acids 1-165) coupled to CNBr-activated Sepharose^{TM}4B (Amersham Pharmacia, Freiburg/Germany).

RT-PCR, construction of expression vectors and immunoblotting

cDNA from zebrafish ovary and prim-5-stage embryos were prepared as follows. Three ovaries and 50-100 embryos were homogenized in 5 ml of solution D [4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7, 0.5% sarcosyl (*N*-laurosarkosimer), 7.2 μl of β-mercaptoethanol per ml solution D], and then mixed with 50 μl of 2 M sodium acetate (pH 4.0). Five ml of water-saturated phenol and 2.5 ml chloroform/isoamylalcohol (24:1) were added, mixed and incubated on ice for 20 minutes. After centrifugation at room temperature for 10 minutes at 13,000 ***g***, the upper phase was re-extracted with 5 ml chloroform/isoamylalcohol and centrifuged. The RNA was precipitated overnight with two volumes of ethanol, pelleted by centrifugation (30 minutes, 4°C) and washed once with 80% ethanol.

The pellet was redissolved in 300 μl of 'DNAse-Mix' (15 μl of New England Biolabs restriction buffer #2 or #3, 3 μl of RQ DNAse I, 1.5 μl of RNAsin, 1.5 μl of 100 mM DTT, 279 μl of water) and incubated at 37°C for 30 minutes. The solution was then mixed with 30 μl of 2 M sodium acetate (pH 4.0), extracted with phenol/chloroform (see above) and then precipitated overnight with two volumes of ethanol. The embryonic RNA was pelleted, and washed (see above). The

Table 1. Primers used for the construction of ZLAP-GFP expression vectors

GFP construct*	Vector	Primer
1-214GFP	pEGFP-N1	5¢ TTCTCGAGTGACATG TTGGAATTTCTGGAAGA 3¢
(2)		5¢ AAGGTACCCTAGTGTGTTGCCCACTGCGT 3¢
1-360GFP	pEGFP-N1	5¢ TTCTCGAGTGACATG TTGGAATTTCTGGAAGA 3¢
(2)		5¢ AAGGTACCTTTTGTCGATCACTGGAAGGC 3¢
1-502GFP	pEGFP-N1	5¢ TTCTCGAGCTTGACATGTTGGAATTCCTGGAA 3¢
(3)		5¢ CATTGGTACCATCATTGTCTCCTTCATATCCAT 3¢
214-314GFP	pEGFP-N1	5¢ GATCCTCGAGATGGAGGATGTGGAGGAGGAGGA 3¢
(3)		5¢ CTCTGGTACCTTGTCAGAGGATGAGGCCTC 3¢
315-460GFP	pEGFP-N1	5¢ TCTCCTCGAGATGGATTTCTCTGAGCCCTCAATAGTG 3¢
(3)		5¢ ACTGGGTACCTCTTTTTGTCGATCACTGGAAG 3¢
214-502GFP	pEGFP-N1	5¢ GATCCTCGAGATGGAGGATGTGGAGGAGGAGGA 3¢
(3)		5¢ CATTGGTACCATCATTGTCTCCTTCATATCCAT 3¢
GFP503-657	pEGFP-C2	5¢ AGACAGAATTCGTTGAAAAGGTGTCAGCCAT 3¢
(3)		5¢ TATTAGGATCCTTATTTGCTGGTACTGTCATC 3¢
Full-length ZLAP2g, b, w	pEGFP-N1	5¢ TTCTCGAGCTTGACATGTTGGAATTCCTGGAA 3¢
(1, 2, 3)		5¢ AATGGTACCTTGCTGGTACTGTCATCTGTGCCG 3¢

*The amino acids contained in the GFP fusion protein are indicated and the position of the GFP in the fusion protein. Numbers in brackets denote the ZLAP2 cDNA that has been used for amplification: (1) ZLAP2g; (2) ZLAP2b; (3) ZLAP2w.

pelleted oocyte RNA was dissolved in 500 µl of water, mixed with 500 µl of 8 M lithium chloride and precipitated for 6 hours at –20°C. After centrifugation and washing, the pellet was redissolved in 50 µl of water and the RNA was tested on an agarose gel.

The cDNA was prepared from 5 µg total RNA using the Supercript II reverse transcriptase from Gibco according to the manufacturer's instructions. It was then used as a template for PCR amplification. The following primer sequences containing a consensus Kozak site and an in-frame stop codon were selected from the LAP2-B4-cDNA and used for the amplification:

5′ CTTGACATGTTGGAATTTCTGGAAGAC 3′ (5′ end), and

5′ TTATTTGCTGGTACTGTCATCTGTGCC 3′ (3′ end).

The PCR products were inserted into the pCR 2.1 TOPO vector (Invitrogen, Karlsruhe, Germany). Inserts that had been controlled by sequencing were then excised with *Kpn*I and *Xho*I and cloned into the pBluescript KS vector. Distinct sequences of the three zebrafish LAP2 cDNAs present in the pBluescript KS vector were amplified by PCR and cloned into the eucaryotic expression vectors pEGFP-C2 and pEGFP-N1 (Clontech, Heidelberg, Germany). The PCR primers used are listed in Table 1. Coupled in vitro transcription/translation of cDNAs, SDS-PAGE and immunoblotting were performed as described (Lang et al., 1999).

Microscopic procedures

Transfected *Xenopus* A6 cells grown on coverslips were fixed for 30 minutes with phosphate buffered saline (PBS: 137 mM NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, 7 mM Na_2HPO_4, pH 7.4) containing 1.25% glutaraldehyde or 3% formaldehyde. For visualization of chromatin, fixed cells were directly stained for 20 minutes with HOECHST 33258 (2.5 µg/ml PBS). Cells were not dried before mounting to preserve the three-dimensional structure. Some coverslips were processed for immunofluorescence after methanol/acetone fixation as described previously (Lang et al., 1999).

Zebrafish embryos at the 32-128 cell stage, at the late blastula stage (4 hpf) and the 13-somite stage (22 hpf) were manually dechorionated, fixed for 40-60 minutes in 3% formaldehyde/PBS, followed by an extraction with 1% Triton-X 100/PBS for 1 hour. The following steps were performed at 4°C. Tissue pieces and embryos were incubated with PBS containing 0.5-1% bovine serum albumin (PBS/BSA), followed by an overnight incubation with the primary antibody (ZLAP2-serum1, ZLAP2-serum2 diluted 1:300 in PBS/BSA or affinity purified antibodies from ZLAP2-serum1). Specimens were then washed three times each for 1 hour and incubated with secondary antibodies [anti-guinea pig immunoglobulin (Ig) G coupled to Texas Red diluted 1:200 in PBS/BSA] for 5 hours. For visualization of chromatin, preparations were stained with HOECHST 33258 (2.5 µg/ml) during the last 2 hours of the incubation with the secondary antibodies. Washed embryos (see above) were mounted with PBS containing 50% glycerol. The secondary antibodies used (anti-guinea pig IgG coupled to Texas Red; Dianova/Germany) did not stain any structure in zebrafish embryos when the primary antibody was omitted (data not shown). Digital images of transfected cells, living and fixed embryos were taken with a Confocal Laser Scanning Microscope (CLSM; TCS SP, Leica, Heidelberg, Germany) and with a Zeiss Axiophot (Zeiss, Jena, Germany) equipped with a CCD camera (software: CamWare V1.00).

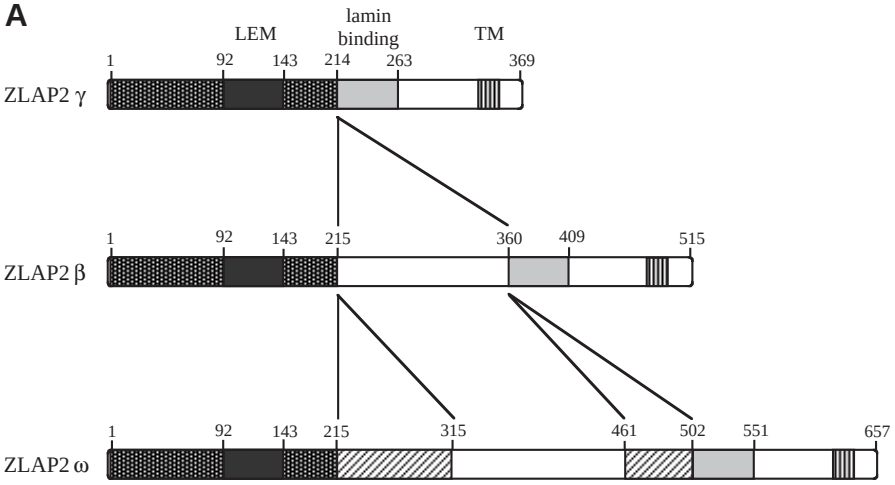
For electron microscopic inspection, embryos were dechorionated, fixed at 4°C for 18 hours with 6.25% glutaraldeyde buffered with 0.1 M phosphate buffer (pH 7.4), washed for 30 minutes with 0.1 M phosphate buffer (pH 7.4), and then fixed for 2 hours with 2% OsO_4 in 50 mM cacodylate (pH 7.2). Embryos were incubated overnight with 0.5% uranyl acetate (in H_2O), dehydrated, embedded in Epon812 and ultrathin sectioned. To allow the electron microscopical analysis of *Xenopus* A6 cells expressing ZLAP2-GFP fusion proteins, cells were grown on CELLocate coverslips (Eppendorf, Hamburg, Germany), fixed 1-2 days after transfection with 1.25% glutaraldehyde in PBS and screened under the fluorescence microscope. Cells were subsequently fixed for 45 minutes with 2.5% glutaraldehyde in PBS and then processed as described for the embryos. Sections were analyzed with a Zeiss EM10 (Zeiss/LEO Oberkochen, Germany). Adobe Photoshop and PowerPoint were used for the preparation of figures.

Preparation of membranes from ovary, extractions of membranes and transfected cells

All buffers were used at 4°C and the experiments were carried out at 4°C unless indicated otherwise. All buffers contained 0.1 µg/ml of trypsin inhibitor and 0.2 mM phenylmethylsulfonyl fluoride. Sucrose-purified total membranes from zebrafish ovaries were prepared as described (Gajewski et al., 1996). Membrane aliquots were mixed with 1 ml of 4 M or 6 M buffered urea (1.5 mM KH_2PO_4, 7 mM Na_2HPO_4), and incubated for 10 minutes at 20°C. Samples were then fractionated by centrifugation (120,000 ***g***, 60 minutes) into pellet and supernatant. The pellet was washed once with PBS and the proteins of the supernatant were precipitated with chloroform/methanol (Schmidt et al., 1994).

Transfected cells grown in Petri dishes (35 mm diameters) were washed three times with PBS and harvested. Cells were then

A



B

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gamma   MLEFLEDPSVLTKDKLKSALLANNVALPNGDQRKDVYVQLYLKNLTVQNKKSSGSPDVFS 60
beta    MLEFLEDPSVLTKDKLKSALLANNVALPNGDQRKDVYVQLYLKNLTVQNKKSSGSPDVFS 60
omega   MLEFLEDPSVLTKDKLKSALLANNVALPNGDQRKDVYVQLYLKNLTVQNKKSSGSPDVFS 60

gamma   SDEELPPAPVVSNRSRSGRKATRKTDKVRPDDVDVTELSNEGLKDLLLKYGLNAGPIVAS 120
beta    SDEELPPAPVVSNRSRSGRKATRKTDKVRPDDVDVTELSNEGLKDLLLKYGLNAGPIVAS 120
omega   SDEELPPAPVVSNRSRSGRKATRKTDKVRPDDVDVTELSNEGLKDLLLKYGLNAGPIVAS 120

gamma   TRKVYEKRLQKLLDQGPPVAVALPSETSQTDGNQNGNNDSDQYSDREEEPVAPAPVTVPE 180
beta    TRKVYEKRLQKLLDQGPPVAVALPSETSQTDGNQNGNNDSDQYSDREEEPVAPAPVTVPE 180
omega   TRKVYEKRLQKLLDQGPPVAVALPSETSQTDGNQNGNNDSDQYSDREEEPVAPAPVTVPE 180

gamma   PEVEAELIPVVERPVRSRGKTPVTSRTRSGQHTR---------------------------- 214
beta    PEVEAELIPVVERPVRSRGKTPVTSRTRSGQHTRD--------------------------- 215
omega   PEVEAELIPVVERPVRSRGKTPVTSRTRSGQHTREDVEEEDWPVLNVKRKLKRSSHRPDQ 240

gamma   ------------------------------------------------------------
beta    ------------------------------------------------------------
omega   MVPASDDTENSELSSAECFAVSEDRRRTGPGAGRRETRPLLSDRTSKLSSKSESLRRRSA 300

gamma   ------------------------------------------------------------
beta    ---------------FSEPSIVKEVSVSLMRMKVQPLTVPKDPKPSKRYSMSATESTKRP 260
omega   PVRSVLNEASSPDKDFSEPSIVKEVSVSLMRMKVQPLTVPKDPKPSKRYSMSATESTKRP 360

gamma   ------------------------------------------------------------
beta    VSSLNNKHDENTADIPSPPHRQSSREPLVSLINTACVEVDDQGMQDVLSCRSANGGLLAQ 320
omega   VSSLNNKHDENTADIPSPPHRQSSREPLVSLINTACVEVDDQGMQDVLSCRSANGGLLAQ 420

gamma   ------------------------------------------------------------
beta    GVRSAAVSKLSKPVLSQSKPSKPLVDMMCCLSPSSDRQKE---------------------- 360
omega   GVRSAAVSKLSKPVLSQSKPSKPLVDMMCCLSPSSDRQKEESCGSPQTHPKSRHSKITPF 480

gamma   ----------------------VEKVSAIDQTPRAVERDVLKEIFPTENLNTPTGISATC 252
beta    ----------------------VEKVSAIDQTPRAVERDVLKEIFPTENLNTPTGISATC 398
omega   LSQITPVRGLDNKLMDMKETMMVEKVSAIDQTPRAVERDVLKEIFPTENLNTPTGISATC 540

gamma   RRPIRGAAGRPLIDTWLNESRQRLSDLKQTTSSSSFSSTSSYTESRSVPRVSSIPLSASK 312
beta    RRPIRGAAGRPLIDTWLNESRQRLSDLKQTTSSSSFSSTSSYTESRSVPRVSSIPLSASK 458
omega   RRPIRGAAGRPLIDTWLNESRQRLSDLKQTTSSSSFSSTSSYTESRSVPRVSSIPLSASK 600

gamma   STAPPTVKSRSRRSLPVWVQLVLLSAVAGFLFFIYQAMETNDVGLFKQSGTDDSTSK 369
beta    STAPPTVKSRSRRSLPVWVQLVLLSAVAGFLFFIYQAMETNDVGLFKQSGTDDSTSK 515
omega   STAPPTVKSRSRRSLPVWVQLVLLSAVAGFLFFIYQAMETNDAGLFKQSGTDDSTSK 657
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Fig. 1. Isoforms of lamina-associated polypeptide 2 in the zebrafish (ZLAP2). (A) ZLAP2γ, ZLAP2β and ZLAP2ω. The position of the LEM motif (LEM), lamin binding domain (lamin binding), transmembrane domain (TM) and the position of individual amino acids is marked for each isoform. The insertion sites of the β- and ω-specific sequences are marked by lines. (B) Amino acid sequence comparison (single letter code) of ZLAP2γ, β and ω. Bold printed letters represent amino acids identical in the three proteins. Proteins contain 369 (γ), 515 (β) and 657 (ω) amino acids. Gaps have been introduced to allow an optimal alignment of the three sequences. The sequences have been published under Accession Nos AJ320192 (γ), AJ320191 (β) and AJ320190 (ω) in the EMBL Nucleotide Sequence Database.

resuspended in 1 ml of 6 M or 8 M buffered urea (1.5 mM KH_2PO_4, 7 mM Na_2HPO_4) and incubated for 10 minutes. All further steps were carried out as described above.

Results

Characterization of zebrafish LAP2 isoforms

We screened a zebrafish kidney cDNA library with a fragment of a previously characterized *Xenopus* LAP2β cDNA (Lang et al., 1999) and isolated one positive clone with a length of 2477 nucleotides (clone B4; GenBank Accession No. AJ320189). DNA sequencing and amino acid prediction revealed that the 5′-end was highly homologous to the *Xenopus* LAP2β. The 3′-end was coding for a putative transmembrane domain and exhibited similarities with the C-terminal end of *Xenopus* LAP2β. The middle part of the cDNA showed little homologies to other LAP2 isoforms and contained a stop codon. To verify whether the 5′ and 3′ ends of the coding region of clone B4 belong to the same gene, we carried out polymerase chain reactions with two different cDNA preparations from *Danio rerio*. Using the same set of primers, we could amplify DNA fragments of 1114, 1554 and 1980 nucleotides, which all contained an open reading frame with a start and stop codon. Comparison of the predicted amino acid sequences revealed that we had amplified three zebrafish LAP2 isoforms (ZLAP2) with lengths of 369 (ZLAP2γ), 515 (ZLAP2β) and 657 (ZLAP2ω) amino acids (Fig. 1A,B). All three proteins possessed the same N-terminal domain (amino acids 1-214) with a LEM module, and the same C-terminal sequence (155 amino acids) with a putative transmembrane domain (TM) and a segment (lamin binding) exhibiting high homologies to the lamina binding domain of *Xenopus laevis* and rat LAP2β (Lang and Krohne, 2003; Yang et al., 1997). Segments of the common N-terminal (amino acids 1-165 of ZLAP2; 63% identity with mouse LAP2γ) and C-terminal [amino acids 229-264 in ZLAP2γ; 72% identity with mouse LAP2γ (see Lang and Krohne, 2003)] ZLAP2 domains exhibited high sequence homologies with all other known LAP2 except for the mammalian LAP2α. Comparison of ZLAP2β and ZLAP2γ revealed that the β isoform contained a characteristic insertion of 146 amino acids (β domain) with little homology to the comparable regions of *Xenopus* and mammalian LAP2β. The third protein, ZLAP2ω, was identical to ZLAP2β except for two additional insertions of 101 and 42 amino acids (ω-

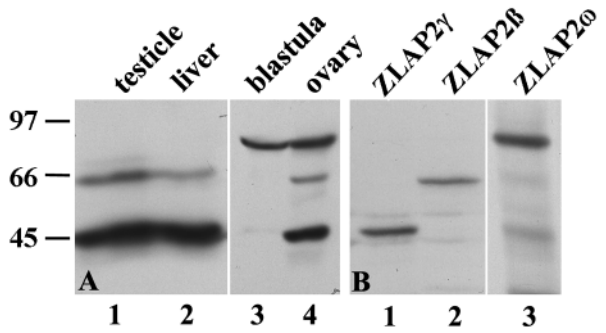


Fig. 2. Characterization of ZLAP2-specific antibodies and proteins encoded by ZLAP2 cDNAs. (A) Total proteins from zebrafish testicle (lane 1), liver (lane 2), blastula (lane 3) and ovary (lane 4) were separated by SDS-PAGE (11% acrylamide) and immunoblotted with the ZLAP2-serum1. A typical radiogram is shown. (B) Coupled in vitro transcription and translation of the cDNAs coding for ZLAP2γ (lane 1), ZLAP2β (lane 2) and ZLAP2ω (lane 3). [^{35}S]-Methionine-labeled polypeptides were separated by SDS-PAGE (11% acrylamide) and visualized by fluorography. The additional smaller polypeptides (B, lane 3) with mobilities of ZLAP2γ and ZLAP2β probably result from the internal start of translation at triplets coding for methionine at positions 241 and 447/448 of ZLAP2ω. Molecular masses of reference proteins (in kDa) are marked in (A).

domains) flanking the β domain. The ω-domains exhibited no homology to all known LAP2 isoforms including the mammalian LAP2α. The exchange of one amino acid in the conserved C-terminal domain of ZLAP2ω (V643A) (Fig. 1B) is a fault of the polymerase during the PCR.

We generated ZLAP2 antibodies (ZLAP2-serum1 and ZLAP2-serum2) specific for epitopes in the common N-terminal domain to verify whether proteins with predicted molecular weights of 40,447 Da (ZLAP2γ), 56,278 Da (ZLAP2β) and 72,225 Da (ZLAP2ω) are expressed in zebrafish. When total proteins of testes, liver, ovary (Fig. 2A) and two zebrafish cell lines (Fig. 7C) were analyzed by SDS-PAGE and immunoblotted using the ZLAP2-specific antibodies, two polypetides with relative mobilities of 45,000 and 63,000 were detected. A third immunoreactive polypeptide of 84,000 was present in embryos of the blastula stage and ovary but was absent from somatic cells (Fig. 2A) and from male germ cells (data not shown). Polypeptides synthesized from the three cloned cDNAs by coupled in vitro transcription/translation showed identical mobilities (Fig. 2B), demonstrating that the proteins detected by immunoblotting represent ZLAP2γ (M_r 45,000), β (M_r 63,000) and ω (M_r 84,000). Antibodies from ZLAP2-serum1 and ZLAP2-serum2 both reacted specifically with the three ZLAP2 isoforms, whereas only antibodies from serum1 weakly recognized, in addition, the *Xenopus* and mammalian LAP2 isoforms (data not shown).

To verify that the three identified zebrafish LAP2 isoforms possess properties characteristic for integral membrane proteins, we extracted ovary membrane fractions and cultured cells with urea. When cultured zebrafish cells (AB9 cells and ZF4 cells) were extracted with 6 M and 8 M urea, ZLAP2β and γ were largely recovered in the membrane pellet (approximately 80-90%, data not shown). Identical results were obtained when *Xenopus* A6 cells expressing the C-terminal domain common to all three ZLAP2 isoforms as a

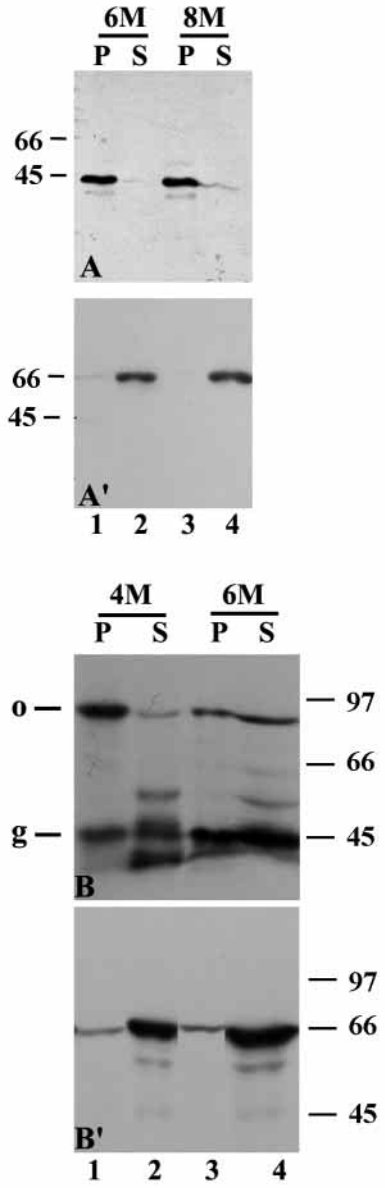


Fig. 3. Biochemical properties of a GFP-ZLAP2ω fusion protein, and of full-length ZLAP2ω. A GFP-ZLAP2ω fusion protein (amino acids 503-657 of ZLAP2ω; GFP503-657) expressed in *Xenopus* A6 cells (A), and ZLAP2ω from zebrafish ovaries (B) was analyzed. The GFP was fused to the aminoterminus of this LAP2 deletion mutant. Proportional amounts of proteins of pellet fractions (P) and supernatants (S) were separated by SDS-PAGE and immunoblotted with GFP antibodies (A) or ZLAP2-specific antibodies (ZLAP2-serum1). The quality of the fractionation was controlled by immunoblotting with antibodies against lamin B2 (A', B'). Lamins should be recovered in the supernatant after urea extraction. (A, A') GFP-ZLAP2ω fusion protein; *Xenopus* A6 cells extracted with 6 M and 8 M urea (A, GFP antibody; B', lamin B2 antibody). (B,B') Membranes of zebrafish ovaries purified by sucrose step gradient centrifugation were extracted with 4 M and 6 M urea. (B, ZLAP2-serum1; B', lamin B2 antibody). The positions of ZLAP2ω (o) and ZLAP2γ (g) are marked in (B) as well as molecular masses of reference proteins (in kDa).

GFP fusion protein (e.g. amino acids 503-657 of ZLAP2ω; GFP503-657) have been extracted with urea (Fig. 3A). GFP503-657 was retained in the membrane pellet, whereas

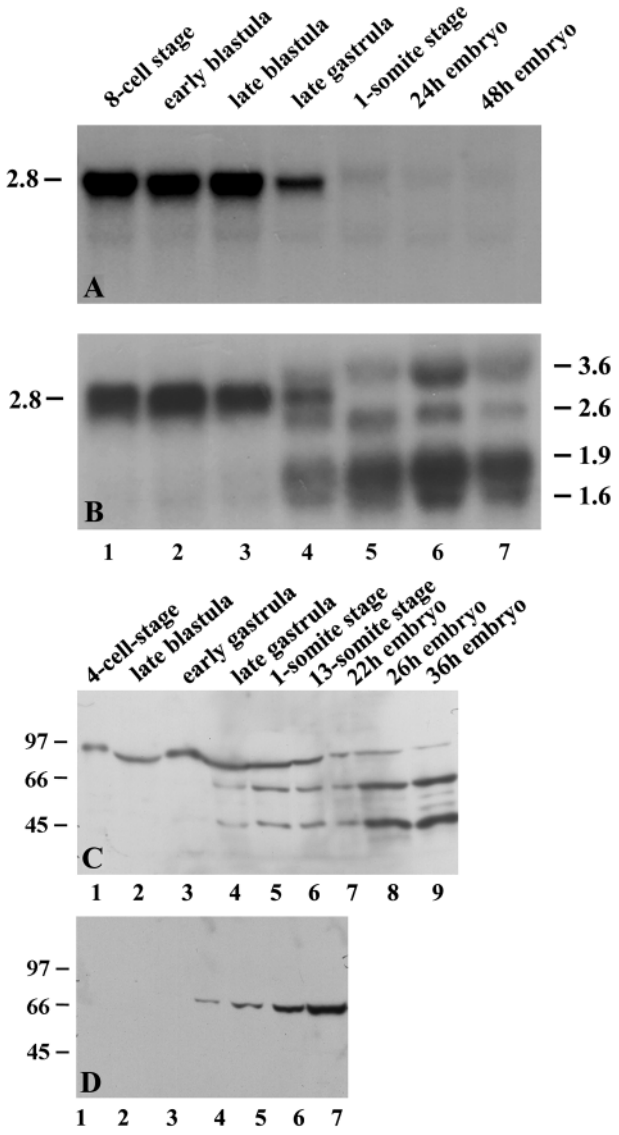


Fig. 4. Expression of ZLAP2 isoforms during zebrafish development. (A,B) Northern blot analysis of the ZLAP2 expression. Total RNA of *D. rerio* embryos at the eight-cell stage (lane 1), early blastula (3 hpf, lane 2), late blastula (4.5 hpf, lane 3), late gastrula (7.5 hpf, lane 4), one-somite stage (10 hpf, lane 5), at the age of 24 hours (24 h embryo, lane 6) and 48 hours (48 h embryo, lane 7) were hybridized with in vitro synthesized [^{32}P]-labeled RNA complementary to ZLAP2 mRNAs. (A) A sequence exclusively present in the ZLAP2ω mRNA (nucleotides 642-942 of ZLAP2ω) or (B) a probe complementary to the complete ZLAP2γ mRNA were used for hybridization. The hybridized mRNAs have estimated sizes of 3600 (3.6), 2800 (2.8), 2600 (2.6), 1900 (1.9) and 1600 (1.6) nucleotides. (C) Total proteins of nine developmental stages were separated by SDS-PAGE (11% acrylamide) and immunoblotted with ZLAP2-serum1. In each lane, total proteins of 2.5 embryos were loaded. The developmental stage is specified on top of each lane. Expression of ZLAP2β and γ was first seen in the late gastrula (lane 4), and ZLAP2ω was only detectable in minor amounts in embryos aged 36 hours (36 h embryo; lane 9). The apparent different mobilities of ZLAP2ω in some lanes is caused by abundant yolk proteins that have slightly lower mobilities than ZLAP2ω. (D) Total proteins of seven developmental stages identical to those in lanes 1-7 of (A) were separated by SDS-PAGE (11% acrylamide) and immunoblotted with lamin antibody X155 reacting in zebrafish with lamin B2. In each lane, total proteins of 2.5 embryos were loaded. Lamin B2 is first detectable in the late gastrula (lane 4). Molecular masses of reference proteins (in kDa) are marked (C,D).

peripheral membrane proteins like lamins were recovered in the supernatant (Fig. 3A'). In transfected *Xenopus* A6 cells, GFP503-657 was localized at the nuclear envelope, and variable amounts were detectable in the endoplasmic reticulum (Lang and Krohne, 2003). Extractions of whole cellular membranes of zebrafish ovaries with 4 M urea confirmed that ZLAP2ω and γ possesses properties characteristic for integral membrane proteins (Fig. 3B,B'). During the extraction with higher urea concentrations (6 M, Fig. 3B, lanes 3 and 4), apparently a significant amount of very small membrane fragments had been formed that could not be pelleted. A similar behavior of integral membrane proteins of *Xenopus* ovary extracts in the presence of urea has been observed previously (Gajewski and Krohne, 1999; Lang et al., 1999). Also, we noted that the nucleoplasmic domain of ZLAPω is more susceptible to degradation than that of the β and γ isoforms. Therefore, we always detected immunoreactive bands with lower molecular weights on immunblots of the supernatants. Our urea extractions show that the three ZLAP2 isoforms are integral membrane proteins.

The analysis of ZLAP2 expression during development revealed that the isoforms are developmentally regulated both at the mRNA (Fig. 4A,B) and at the protein levels (Fig. 4C), comparable to the situation in amphibia (Lang et al., 1999). In embryos at the eight-cell stage and in embryos during the blastula stage (Fig. 4A, lanes 1-3), we detected only an mRNA band of 2.8 kb that reacted with an ω-specific probe. Lower amounts of the ω-mRNA were also detectable in embryos at the late gastrula stage (7.5 hpf) but not in the older developmental stages tested (Fig. 4A, lanes 5-7). When the complete ZLAP2γ was used for hybridization (Fig. 4B), the ω-mRNA was detected as well as additional mRNAs in embryos at the late gastrula (7.5 hpf) and in older developmental stages. These mRNA bands had sizes of 3.6, 2.6, 1.9 and 1.6 kb. We suggest that these mRNAs in older embryos (Fig. 4B, lane 4) are coding for ZLAP2β and γ. It is not unusual that LAP2 mRNAs possess long non-translated 3′ ends. For the mammalian LAP2β mRNAs, sizes of 3.5 and 2 kb have been reported (Furukawa et al., 1995). We cannot rule out that some of the mRNAs detected (Fig. 4B, lanes 4-7) are coding for additional LAP2 isoforms with mobilities on SDS-PAGE close to that of ZLAP2β, γ or ω.

Immunoblots supported the interpretation of our northern blot data. At the protein level, LAP2ω was the only isoform present in embryos up to the early gastrula stage (Fig. 4C). By contrast, LAP2β and γ were absent from early developmental stages and first detectable at the late gastrula stage (Fig. 4C, lane 4). Interestingly, the expression of LAP2β/γ and somatic B-type lamins started at the same time point during development (Fig. 4D, lane 4) [for the characterization of the B-type lamin specific for the female germ line of the zebrafish see Yamaguchi et al.; see also Hofemeister et al. (Yamaguchi et al., 2001; Hofemeister et al., 2002)]. The amount of LAP2ω per embryo decreased with the progression of development concomitant with the increase of somatic LAP2 isoforms. In embryos aged 36 hours, LAP2ω was barely detectable. In all

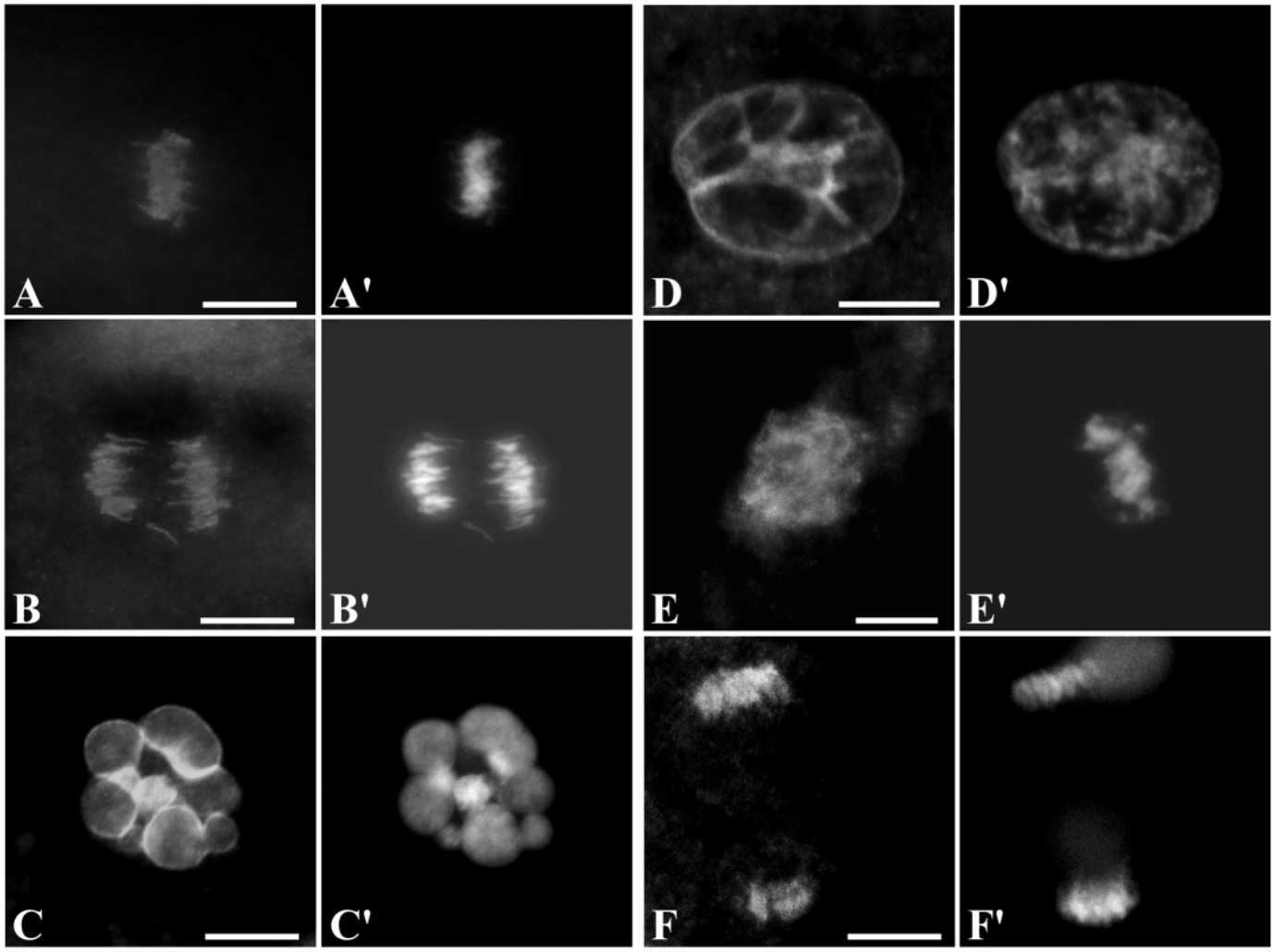


Fig. 5. Distribution of LAP2 during the cell cycle in zebrafish blastomeres. Localization of ZLAP2 in whole-mount preparations of embryos by indirect immunofluorescence microscopy with polyclonal antibodies specific for ZLAP2 (A-F; A'-F', DNA staining by Hoechst). Embryos at the cleavage period (A,B; 1.5 hpf), early blastula stage (C,D; 2.5 hpf) and late blastula stage (E, F; 4 hpf) have been analyzed. Cells at metaphase (A-A',E-E'), anaphase (B-B',F-F'), the karyomere stage (C-C') and interphase (D-D') are shown. (D) The nuclear envelope of the interphase nucleus (D) is highly invaginated, resulting in an apparent intranuclear staining with LAP2 antibodies. This morphology is characteristic for nuclei that have been formed by the fusion of karyomeres. Digital images were taken with a CLSM (C-C',D-D',E-E',F) and a Zeiss Axiophot (A-A',B-B',F'). Bars, 10 μm.

embryonic stages tested, the β and γ isoforms were expressed at the same level, whereas LAP2γ was the predominant polypeptide in somatic tissues of adult organism (see Fig. 2A), as well as in two zebrafish cell lines tested (Fig. 7C). Owing to the lack of ZLAP2ω-specific antibodies, we cannot exclude that this isoform is expressed in the adult organism in specialized cells of some organs as it has been shown for a *Xenopus* B type lamin (lamin L_{III}/B3) (Benavente et al., 1985).

The behavior of ZLAP2 isoforms during the cell cycle in the embryo and somatic cells

To learn more about the properties of the ω-isoform in comparison with LAP2β and γ, we analyzed cleavage stages of embryos and cultured cells. The immunolabeling of embryos at developmental stages expressing exclusively LAP2ω with our LAP2-specific antibodies indicated that the ω isoform is localized at the nuclear envelope during interphase, as depicted in Fig. 5D. The inspection of mitotic cells from embryos aged 1-2 hours and 4 hours revealed the staining of anaphase chromosomes (Fig. 5B,F). The staining of metaphase chromosomes was clearly visible in the early cleavage stages (Fig. 5A; 1-2 hpf) but not in older embryos (Fig. 5E; 4 hpf). The electron microscopic inspection of blastomeres from the same developmental stages showed that variable numbers of vesicles, which often appeared flattened, were associated with mitotic chromosomes. On metaphase chromosomes, fewer vesicles were seen (data not shown) than on chromosomes at anaphase (Fig. 6A). In addition, numerous vesicles were regularly seen in the cytoplasm bordering the chromosomes (Fig. 6A). The morphology of reforming nuclei at telophase during the blastula stage (Fig. 5C) indicated that a nuclear envelope had been assembled around single or few chromosomes (Fig. 5C; karyomere formation) before the

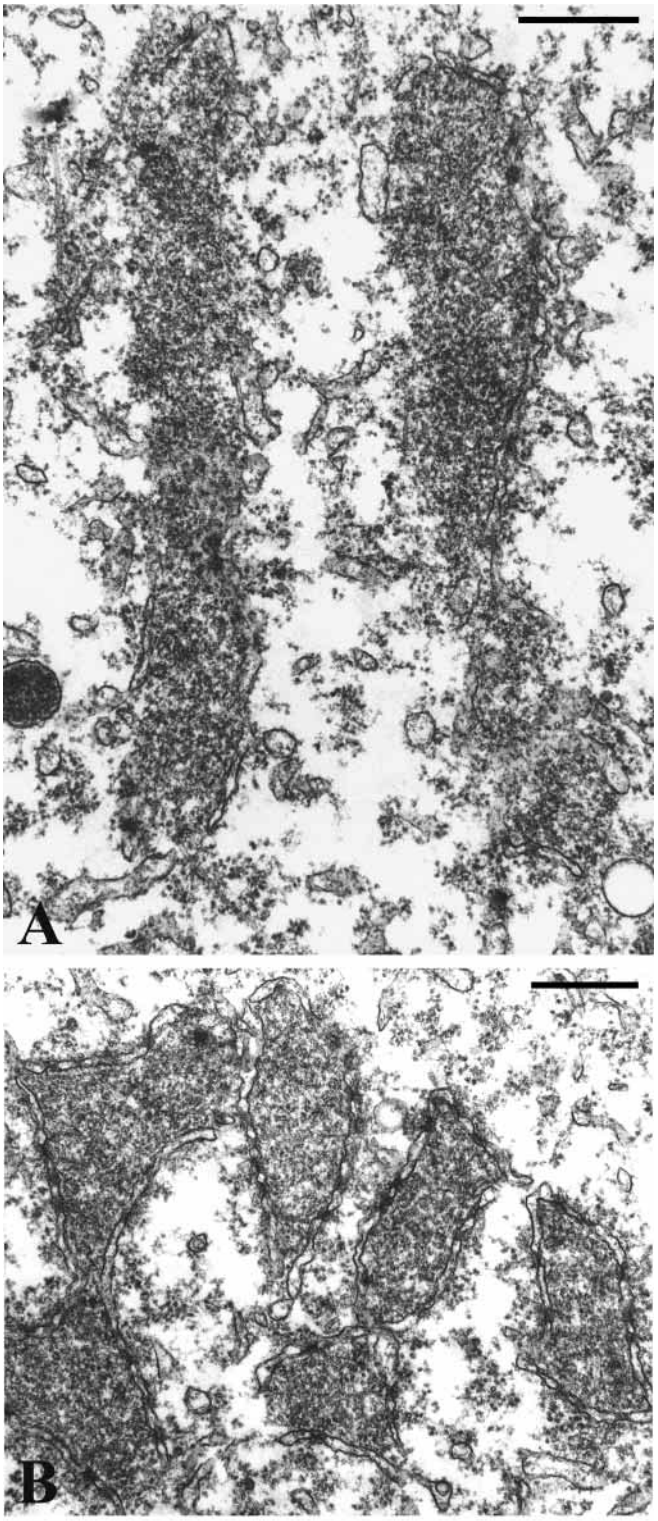


Fig. 6. Morphology of chromosomes during cell division of zebrafish blastomeres at the early blastula stage (2.5 hpf). Electron micrographs showing longitudinal sectioned mitotic chromosomes (A, compare with Fig. 4B) and chromosomes at an early karyomere stage (B, earlier than that shown in Fig. 4C). Numerous flattened vesicles are attached to the surface of mitotic chromosomes (A), and nuclear envelope fragments containing pore complexes are attached to the surface of each chromosome at the early karyomere stage (B). Bars, 0.5 μm.

Table 2. Behavior of zebrafish LAP2-GFP fusion proteins in transfected *Xenopus* A6 cells during mitosis

ZLAP2-GFP fusion protein	Association with mitotic chromosomes
1-214GFP (common domain)	+/–
1-360GFP (β)	+++
1-502GFP (ω)	+++
214-314GFP (ω)	–
315-460GFP (ω)*	–
214-502GFP (ω)	–

*Identical to amino acids 215-360 of zebrafish LAP2β.
Numbers denote amino acids of the zebrafish LAP2 isoforms listed in brackets (see also Fig. 1A). In the fusion proteins used GFP was always localized at the C-terminus of ZLAP2.
+/–, weak staining of chromosomes.
+++, intense staining of chromosomes.
–, no chromosome staining.

formation of a common nuclear envelope enclosing all chromosomes in later interphase (Fig. 5D). Further electron microscopic analysis revealed that during telophase each chromosome assembles its own nuclear envelope (Fig. 6B). In older embryos (11-13 hpf = 5-8 somite stage; see Fig. 4A) that contained beside LAP2ω considerable amounts of the somatic isoforms, only minute amounts of membranes were found in contact with anaphase chromosomes, and no karyomere formation could be seen in telophase. In the embryos aged 12 hours, only very faint, if any, staining of anaphase chromosome was detectable with ZLAP2-specific antibodies.

When we analyzed cultured zebrafish ZF4 and AB9 cells that expressed exclusively the ZLAP2β and γ (Fig. 7C), we noted that γ was the predominant isoform, whereas only minor amounts of the β-isoform were detectable. In these cultured cells, we observed no labeling of metaphase chromosomes during mitosis (Fig. 7A,A') and, in anaphase cells, only areas on the chromosome surface were stained where the reassembly of the nuclear envelope has begun (Fig. 7B,B'). Our data indicate that, during early embryogenesis, a significant amount of ZLAP2ω associates with chromosomes much earlier during the progression of mitosis than LAP2 isoforms in somatic zebrafish cells.

Distinct behavior of GFP-ZLAP2 fusion proteins during mitosis in transfected A6 cells

Our immunofluorescence data suggest that ZLAP2ω possesses properties distinct from those of the β and γ isoforms. To verify our observations by a methodology independent of antibodies, we generated eukaryotic expression vectors allowing the in vivo analysis of the three isoforms as GFP fusion proteins in transfected *Xenopus* A6 cells. We selected *Xenopus* A6 cells for transfection because they are cultured at a temperature that is optimal for the growth of zebrafish embryos and adults. A further reason is that the folding and assembly of at least some zebrafish and *Xenopus* proteins is not optimal at temperatures above their body temperature (Cerda et al., 1998).

The analysis of total proteins from transfected cells by SDS-PAGE and immunoblotting with GFP antibodies revealed that each of the three GFP-LAP2 fusion proteins possessed the expected apparent molecular weight (data not shown). During interphase in A6 cells, all three GFP-ZLAP2 fusion proteins

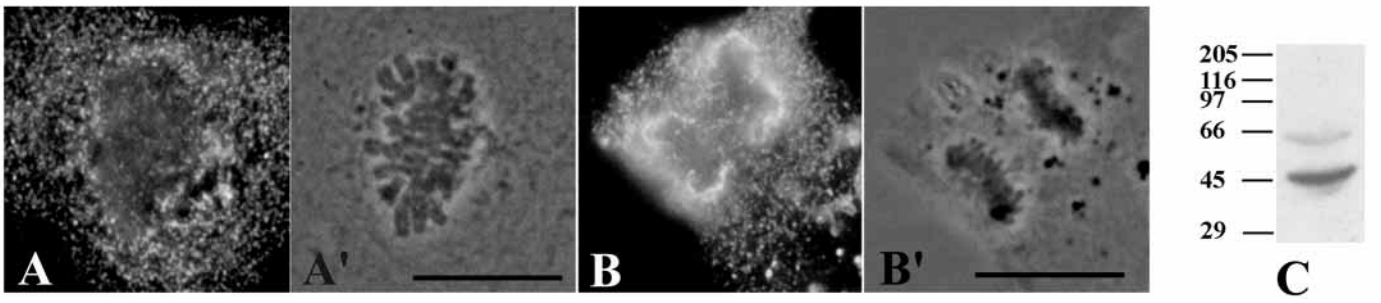
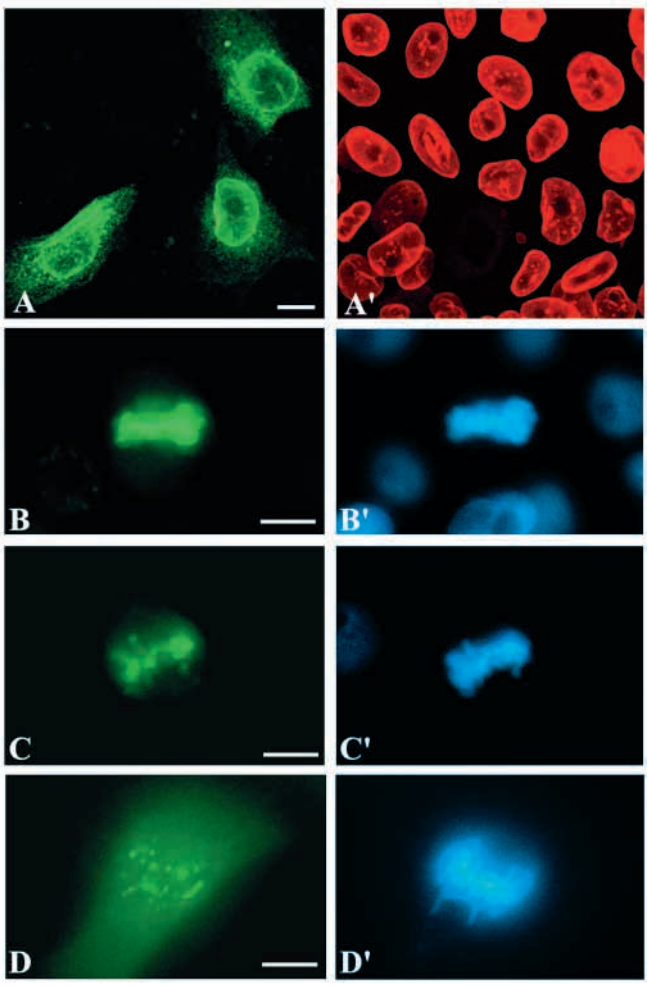


Fig. 7. The behaviour of LAP2 during mitosis in zebrafish AB9 cells. Immunofluorescence microscopy of an AB9 cell at metaphase (A; A', phase contrast) and anaphase (B; B', phase contrast), after staining with ZLAP2-specific antibodies. Note that metaphase chromosomes are not stained by LAP2 antibodies, and that anaphase chromosomes are stained only in regions where the nuclear envelope has begun to reassemble. Bars, 10 μm. (C) LAP2 isoforms of zebrafish AB9 cells. Total proteins of AB9 cells were separated by SDS-PAGE (11% acrylamide) and immunoblotted with ZLAP2-serum1. ZLAP2γ is the major isoform in AB9 cells, and only minor amounts of ZLAP2β were detectable. Identical results were obtained with zebrafish ZF4 cells (data not shown). Molecular masses of reference proteins (in kDa) are marked.

were localized in the nuclear envelope (Fig. 8A) and variable amounts in the endoplasmic reticulum. In mitotic cells, ZLAP2ω-GFP (Fig. 8B) and ZLAP2β-GFP (Fig. 8C) were found in association with chromosomes. By contrast, ZLAP2γ-GFP exhibited a diffuse and, in addition, dot-like staining in mitotic cells but did not colocalize with chromosomes (Fig. 8D). The comparative analysis of the three ZLAP2-GFP fusion proteins suggest that the domains common to all three isoforms do not mediate binding to mitotic chromosomes. To verify our hypothesis we generated a number of deletion mutants (see Table 2). A GFP-ZLAP2 fusion protein containing exclusively the N-terminal domain (amino acids 1-214 of ZLAP2; 1-214GFP) common to all three isoforms was homogeneously distributed in the cytoplasm of A6 cells during mitosis and also detectable in regions containing the mitotic chromosomes (Fig. 9A). Two fusion proteins containing the common aminoterminus in conjunction with β-specific sequences (Fig. 9B; amino acids 1-360 of ZLAP2β; 1-360GFP) or β-sequences together with the ω-sequences (amino acids 1-502 of ZLAP2ω; 1-502GFP; data not shown) were predominantly localized on chromosomes in mitotic cells. By contrast, GFP fusion proteins containing exclusively β- and/or ω-specific sequences were excluded from cellular regions containing chromosomes during mitosis in transfected *Xenopus* A6 cells (Fig. 9C; shown for mutant 214-502GFP) (see Table 2). Our results indicate that not a single domain but the common N-terminal domain in conjunction with β- and/or ω-specific sequences mediates binding of the β- and ω-isoforms to mitotic chromosomes.



Ectopically expressed ZLAP2β and ZLAP2ω target vesicles to mitotic chromosomes

We have shown that the nucleoplasmic domain of ectopically expressed ZLAP2β and ω bind to chromatin and that ZLAP2ω isolated from zebrafish ovaries possesses properties characteristic for integral membrane proteins (see Fig. 3B). To verify whether these two isoforms – in contrast to ZLAP2γ – could be involved in the early targeting of vesicles to mitotic

Fig. 8. Intracellular distribution of ZLAP2-GFP fusion proteins in transfected *Xenopus* A6 cells during interphase and cell division. In all constructs GFP was fused to the C-terminus of full-length LAP2 isoforms. Transfected cells expressing ZLAP2ω-GFP (A,B), ZLAP2β-GFP (C) and ZLAP2γ-GFP (D) are shown during interphase (A) and mitosis (B-D). The GFP fluorescence (A-D) and the same cells after staining with lamin B2 antibodies (A') or the DNA dye Hoechst (B'-D') are shown. ZLAP2γ-GFP is distributed in mitotic cells throughout the cytoplasm and forms local aggregates (D), whereas ZLAP2ω-GFP (B) and ZLAP2β-GFP (C) predominantly colocalized with chromatin. Digital images were taken with a Leica CLSM (A,A') and a Zeiss Axiophot (B-D'). Bars, 10 μm.

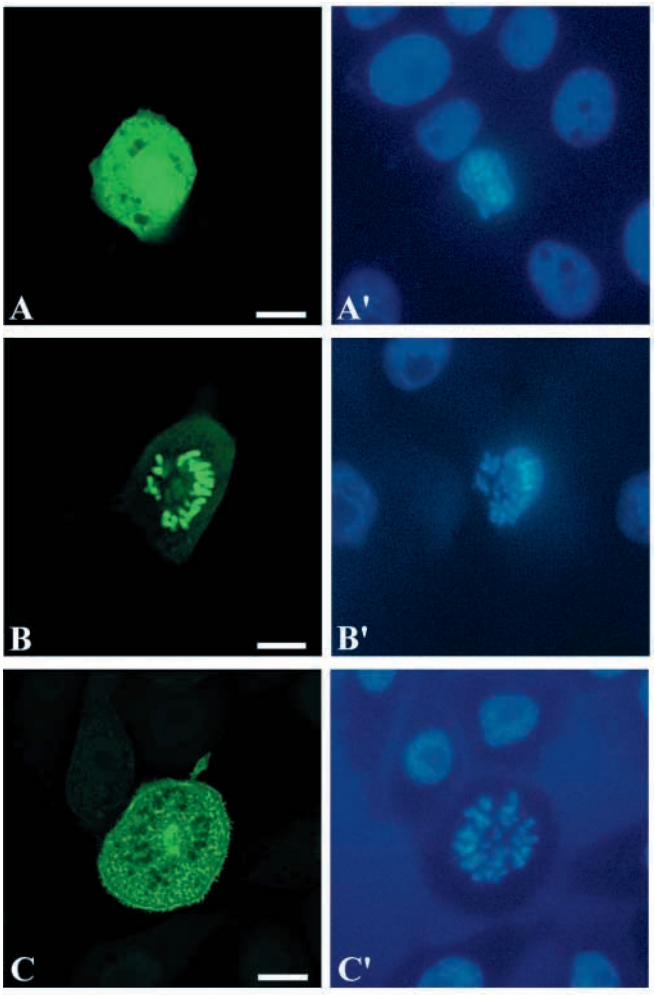


Fig. 9. Intracellular distribution of deletion mutants of ZLAP2 expressed as GFP fusion proteins in transfected *Xenopus* A6 cells during cell division. In all constructs, GFP was fused to the carboxyterminus of LAP2 deletion mutants. Transfected cells expressing mutant 1-214GFP (A; amino acids 1-214 common to all ZLAP2 isoforms), mutant 1-360GFP (B, amino acids 1-360 of ZLAP2β) and mutant 214-502GFP (C; amino acids 214-502 of ZLAP2ω) are shown during mitosis. The GFP fluorescence (A-C) and the same cells after staining with the DNA dye Hoechst (A'-C') are shown. Note the association of mutant 1-360GFP with mitotic chromosomes and the exclusion of 214-502GFP from regions containing chromosomes. Digital images were taken with a Leica CLSM (A-C) and a Zeiss Axiophot (A'-C'). Bars, 10 μm.

chromosomes, we expressed the three full-length polypeptides as GFP-fusion proteins in *Xenopus* A6 cells. When cells comparable to those shown in Fig. 8B-D were processed for electron microscopy, we noted that mitotic chromosomes of cells expressing ZLAP2ω (Fig. 10A,B,D) or ZLAP2β (data not shown) were associated with numerous vesicles, giving the chromosomes a 'sponge-like' morphology. Vesicles of comparable morphology could not be detected on mitotic chromosomes of cells expressing ZLAP2γ (Fig. 10C,E). The membrane stacks (Fig. 10C,E) visible in the vicinity of the mitotic chromosomes corresponded to the dot-like structures seen by fluorescence microscopy in mitotic (Fig. 8D) and interphase cells (Fig. 8A).

Discussion

We have described with the zebrafish LAP2ω a novel polypeptide that is expressed in oocytes and during early embryonic development. ZLAP2ω possesses properties distinct from that of ZLAP2γ, the major isoform in zebrafish somatic cells.

What are the putative functions of ZLAP2ω during early embryonic development?

ZLAP2ω is a type II membrane protein and the only LAP2 isoform expressed during a developmental stage that is characterized by very rapid cell divisions (cell-cycle length 15-20 minutes) (Kimmel et al., 1995). Further important features of this stage include the association of vesicles with mitotic chromosomes, and the nuclear envelope assembly around individual chromosomes (karyomere formation) at the end of mitosis. By the expression of GFP-LAP2ω fusion proteins in cultured cells, we have provided clear evidence that the ω isoform binds to chromatin and associates with mitotic chromosomes. The mitotic chromosomes of transfected cells ectopically expressing full-length LAP2ω-GFP have similarities with the mitotic chromosomes of rapidly dividing zebrafish early blastomeres. First, many vesicles are associated with the chromosomes, and second, the chromatin of these chromosomes is less condensed than that of chromosomes free of membranes. Our data therefore indicate that ZLAP2ω is involved in the very early binding of nuclear envelope forming vesicles to the surface of individual chromosomes during early embryonic development, thus facilitating the reformation of the nuclear envelope around each chromosome at the end of the very short embryonic cell cycles (see Fig. 5A-C). A protein with comparable properties has so far not been described in mammals.

Karyomere formation is also characteristic of the very short cell cycles before the midblastula transition in *Xenopus* (Montag et al., 1988; Lemaitre et al., 1998) (see also therein for karyomere formation in other organisms). *Xenopus* embryos contain a maternally expressed LAP2-related integral membrane protein that has several properties in common with ZLAP2ω (Lang et al., 1999). The characterization of a *Xenopus* LAP2 cDNA clone (Accession No. AJ514937) that had been isolated in the course of the characterization of *Xenopus* LAP2β (Lang et al., 1999) revealed that the in vitro translated polypeptide encoded by this cDNA is comigrating with the *Xenopus* LAP2 protein of Mr 84,000 expressed in oocytes and during early embryonic development (Lang et al., unpublished). This protein exhibited a sequence identity of 97% with a previously published *Xenopus* LAP2 isoform (Gant et al., 1999) (Accession No. AF048815). The *Xenopus* LAP2ω is 99 amino acids shorter than the zebrafish isoform but much more acidic (XLAP2ω: pI 6.16; ZLAP2ω: pI 9.17). This is most probably the reason for its unusual slow mobility on SDS-PAGE (Lang et al., unpublished). The low sequence identities of the *Xenopus* and zebrafish LAP2ω in the central part of both molecules suggests that these isoforms are adaptations to the different cell cycle length of *Xenopus* and zebrafish blastomeres before the midblastula transition.

Binding of ZLAP2β to mitotic chromosomes

In the cultured zebrafish cells tested, ZLAP2β was present only in minor amounts and could not be distinguished from the

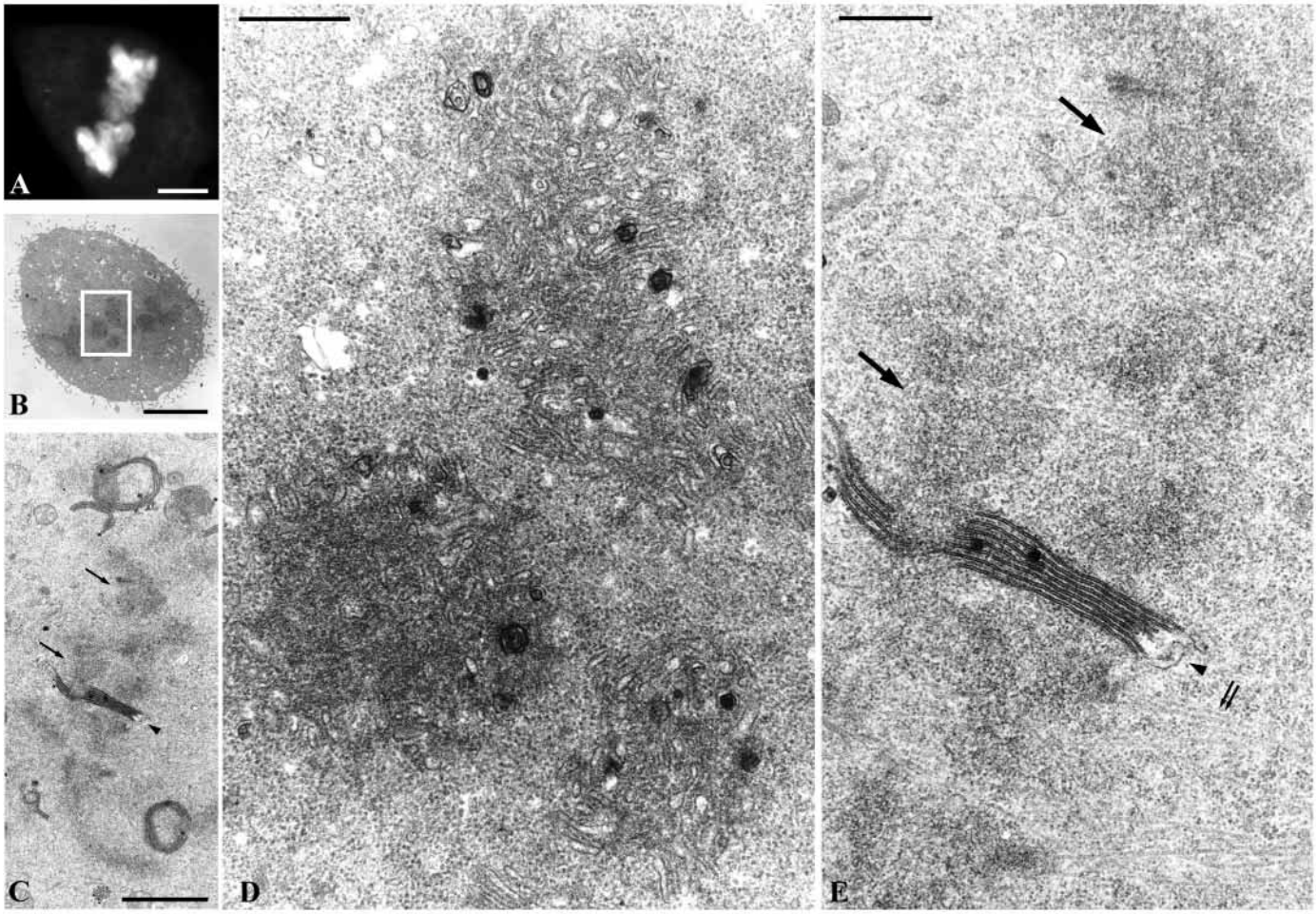


Fig. 10. Mitotic chromosomes of transfected *Xenopus* A6 cells expressing full-length ZLAP2ω-GFP (A,B,D) or ZLAP2γ-GFP (C,E) analyzed by light (A) and electron microscopy (B-E). (A,B,D) The same cell at metaphase is shown by fluorescence microscopy (A) and on electron micrographs of ultrathin sections through this cell (B,D). The metaphase chromosomes are clearly visible at low magnification (B). (D) Higher magnification of the boxed area in (B) shows that chromosomes are associated with vesicles. (C,E) Electron micrographs of an ultrathin section through a transfected cell at metaphase expressing ZLAP2γ-GFP. The chromosomes are shown in an overview (C) and higher magnification (E, central region of C). A membrane stack (arrowheads in C and E) containing ZLAP2γ-GFP, chromosomes (single arrows) and spindle microtubules (double arrows) are marked. Metaphase chromosomes of cells expressing ZLAP2γ-GFP are not associated with vesicles. Bars, 0.5 μm (D,E), 2 μm (C) and 5 μm (A,B).

abundant γ-isoform due to the lack of isoform-specific antibodies. Therefore the staining of mitotic cells with LAP2 antibodies (see Fig. 7) is most likely to reflect the distribution ZLAP2γ and is in agreement with the behavior of ZLAP2γ-GFP in transfected cells. It is questionable whether chromosome-bound endogenous ZLAP2β could be detected in cultured zebrafish cells with the presently available tools.

The comparison of the behavior of our ZLAP2 fusion proteins during mitosis indicates that the GFP moiety of the fusion protein does not mediate chromosome binding, and that the common carboxyterminus of the three isoforms is not involved in this process. The intracellular distribution of the ZLAP2 deletion mutants tested (see Fig. 9 and Table 2) suggests that the common N-terminal domain alone does possess a weak affinity for mitotic chromatin in transfected *Xenopus* A6 cells. This affinity is enhanced when β- and/or ω-specific sequences are present in the proteins. An observation of Vlcek and coworkers (Vlcek et al., 1999) could help to explain why the common aminoterminus in conjunction with β- and/or ω-specific sequences enhances the binding of ZLAP2 to mitotic chromosomes. Vlcek found that a GST fusion protein containing the common N-terminal domain (amino acids 1-187) of the mammalian LAP2 binds in vitro to mitotic chromosomes (Vlcek et al., 1999). It is known that GST forms dimers and oligomers. It is worth speculating that β- and ω-specific sequences do modulate oligomerization of ZLAP2 thus facilitating the binding of the common aminoterminus to chromatin.

Ectopically expressed LAP2 fusion proteins are often present in the transfected cells in a higher concentration than the endogenous LAP2 isoforms. If the oligomerization of ZLAP2β was a prerequisite for its binding to mitotic chromosomes, binding to mitotic chromosomes in cells expressing ZLAP2β-GFP fusion proteins would be expected to occur much earlier during the cell cycle than in non-transfected cells because the ectopically expressed protein is present in higher cellular concentrations than the endogenous LAP2. This notion is

supported by the observation that ectopically expressed ZLAP2β and ω are found in association with metaphase chromosomes (see Fig. 8B,C). Other than for ZLAP2β, the behavior of ZLAP2ω-GFP in transfected cells is in agreement with the distribution of endogenous ZLAP2ω in early embryonic cells during mitosis. In the cleavage period the ω-isoform is associated with chromosomes throughout all mitotic stages (Fig. 5). We conclude from our data that the observed differences in the mitotic behavior of ZLAP2γ compared with the β- and ω-isoforms reflect intrinsic properties of the polypeptides.

How many LAP2 isoforms are expressed in zebrafish?

We have identified by cDNA cloning three different splice products of the ZLAP2 gene: one β, one γ and one ω isoform. To our surprise, several mRNAs of embryos aged 48 hours hybridized to the LAP2-specific probe. Therefore, we cannot rule out that some of the mRNAs detected are coding for additional minor LAP2 isoforms with mobilities on SDS-PAGE close to that of ZLAP2β, γ or ω.

For the following reasons it is unlikely that *D. rerio* is expressing a homologue to the mammalian LAP2α. Antibodies specific for the common N-terminal domain of all so far identified vertebrate LAP2 isoforms (MAN antibodies) (Paulin-Levasseur et al., 1996; Lang et al., 1999) (ZLAP2-serum1 and ZLAP2-serum2) (this manuscript) did not detect a polypeptide with the size of mammalian LAP2α in protein samples of somatic tissues and cultured cells but only two significant smaller polypeptides that represent zebrafish LAP2β and γ. The only immunoreactive polypeptide of similar size to the mammalian LAP2α is expressed in zebrafish oocytes and during early embryonic development. We have shown that this protein represents ZLAP2ω. In this respect it is interesting that a homologue to the mammalian LAP2α is apparently also absent from somatic cells of *Xenopus* (Lang et al., 1999), and other amphibia and fishes (del Pino et al., 2002). Our present study will now enable us to analyze zebrafish LAP2 functions at the genetic level in this model vertebrate.

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