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Interphase phosphorylation of the *Drosophila* nuclear lamin: site-mapping using a monoclonal antibody

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SUMMARY

The *Drosophila* nuclear lamin is highly phosphorylated during interphase. Two interphase isoforms, differing in degree of phosphorylation, can be distinguished by one-dimensional SDS-polyacrylamide gel electrophoresis. One migrates with an apparent mass of 74 kDa (lamin Dm_1); the other is more highly phosphorylated and migrates as a 76 kDa protein (lamin Dm_2). We generated a monoclonal antibody, ADL84 which binds to lamin Dm_1 but not lamin Dm_2. Binding of ADL84 to lamin Dm_2 was restored by phosphatase treatment of immunoblots containing lamins. Immunoprecipitation with ADL84 demonstrated that purified *Drosophila* nuclear lamins Dm_1 and Dm_2 are present as a random mixture of homo- and heterodimers. Indirect immunofluorescence experiments suggest that lamin Dm_1 is present in all *Drosophila* cell types. The epitope for ADL84 was mapped by analyzing binding to bacterially expressed lamin deletion mutants and subsequently by screening for point mutants (randomly generated by polymerase chain reaction) which were not recognized by ADL84. The ADL84-epitope encompasses amino acids R^{22}PPSAGP (arginine 22-proline 28). Peptide competition experiments demonstrated directly that phosphorylation of serine 25 impedes lamin binding by ADL84. This suggests that serine 25 is the lamin Dm_2-specific phosphorylation site.

Key words: lamin, nuclear envelope, phosphorylation

INTRODUCTION

Nuclear lamins are major components of the nuclear lamina, a fibrous layer of proteins underlying the inner nuclear membrane and surrounding the nucleus. In *Xenopus* oocytes, lamins form a network composed of 10 nm fibers which cross-over at regular intervals (Aebi et al., 1986; Whytock et al., 1990). The detailed in situ structure of lamins in other cell-types is not well-known (but see Hill and Whytock, 1993). Lamins interact with the inner nuclear membrane via an isoprenylated carboxy-terminal tail (Weber et al., 1989; Kitten and Nigg, 1991) and/or a lamin-receptor in the nuclear membrane (Worman et al., 1988, 1990; Hennekes and Nigg, 1994). In vitro, lamins also bind chromatin (Burke, 1990; Hoger et al., 1991; Yuan et al., 1991; Glass et al., 1993) and DNA of specific sequence (Ludérus et al., 1992). Results from a number of experiments suggest that lamins play a structural role in nuclear formation (Benavente and Krohne, 1986; Ulitzur et al., 1992) and perhaps in nuclear function (Newport et al., 1990; Meier et al., 1991).

Multiple posttranslational modifications occur on lamins (Nigg, 1992). During mitosis, phosphorylation of one or a few specific lamin sites correlates with lamina disassembly (Smith and Fisher, 1989; Heald and McKeon, 1990; Peter et al., 1990, 1991; Ward and Kirschner, 1990; Goss et al., 1994). Rat liver lamin B was shown to be reversibly demethylated during mitosis (Chelsky et al., 1987). During interphase different stimuli can cause specific lamin phosphorylation via protein kinase C (Hornbeck et al., 1988; Tsuda and Alexander, 1990; Kasahara et al., 1991; Martell et al., 1992; Goss et al., 1994) or other kinases (Molloy and Little, 1992; Eggert et al., 1993). In one case where a biological effect of interphase phosphorylation was demonstrated, Hennekes et al. (1993) showed that phosphorylation of chicken lamin B_2 at a site near its nuclear localization signal inhibits lamin import into the nucleus.

In *Drosophila* three lamin cDNAs, coding for two different proteins, were identified (Gruenbaum et al., 1988; Bossie and Sanders, 1993). The protein encoded by one of these cDNAs (lamin C) is developmentally regulated (D. Riemer et al., unpublished). The other two code for the major *Drosophila* embryo lamin, Dm_0. Lamin* Dm_0 has an apparent molecular mass of 76 kDa as determined by SDS-polyacrylamide gel electrophoresis (PAGE) and is rapidly processed in the cytoplasm into a form migrating at 74 kDa (lamin Dm_1). Lamin Dm_1 is imported into the nucleus where about 50% is post-translationally modified resulting in a slower migrating form (76 kDa) called lamin Dm_2. In vivo pulse-chase studies

*Unless indicated otherwise, for *Drosophila*, the term lamin refers solely to protein products encoded by the *Drosophila* lamin Dm_0 gene.

indicate that lamins Dm_1 and Dm_2 are in equilibrium. Treatment of lamins Dm_1 and Dm_2 with phosphatase results in a single form that comigrates with lamin Dm_1 after SDS-PAGE. In conjunction with results of in vivo labeling, this suggests that lamin Dm_2 arises by specific phosphorylation of lamin Dm_1 (Smith et al., 1987; Smith and Fisher, 1989).

Here, we describe the isolation and characterization of a monoclonal antibody, ADL84, which is specific for *Drosophila* lamin Dm_1 and does not bind lamin Dm_2. We used this antibody to show that isolated interphase lamins occur as mixed dimers, indicating that lamins Dm_1 and Dm_2 interact randomly with each other. Mapping of the epitope for ADL84 shows that the lamin Dm_2-specific phosphorylation takes place in the NH_2-terminal 'head'-domain of the lamin, most likely at serine 25.

MATERIALS AND METHODS

Generation of monoclonal antibodies

We employed an immunization protocol that was shown to enhance the recovery of antibodies against relatively less antigenic epitopes (Matthew and Sandrock, 1987; Vermeersch et al., 1992). At day 1 Balb/c mice were injected intraperitoneally with 150 μg of *Drosophila* lamin Dm_0 produced in *Escherichia coli* (see below) mixed with an equal volume of MPL plus TDM emulsion adjuvant (RIB Immunochemical Research Inc., Hamilton, MT). After 10 minutes, 24 hours and 48 hours, mice were injected with a 2 mg/ml solution of cyclophosphamide (Sigma, St Louis, MO) in 0.9% (w/v) NaCl to a concentration of 100 mg/kg body weight. This set of four injections (one of antigen followed by three of cyclophosphamide) was repeated at day 16. At days 32, 49 and 63, mice were immunized with 150 μg each of authentic interphase *Drosophila* lamins Dm_1 and Dm_2, mixed with adjuvant. At day 66, one mouse was killed, and spleen cells were used for monoclonal antibody (mAb) generation according to standard protocols (Harlow and Lane, 1988).

Hybridomas were tested for specific antibody production using an enzyme linked immunosorbent assay (Harlow and Lane, 1988) with *E. coli* expressed or authentic lamin as antigen. Several anti-*Drosophila* lamin antibody secreting hybridomas were selected and cloned by repeated limiting dilution until greater than 90% of the resulting clones produced the specific antibody. Antibody isotype was determined by enzyme linked immunosorbent assay using isotype-specific secondary antibodies (Fisher Scientific, Pittsburgh, PA).

Expression of the full-length *Drosophila* lamin and deletion mutants in *E. coli*

Full-length *Drosophila* lamin and lamin Dm_0 deletion mutants were expressed in *E. coli* and purified as described elsewhere (N. Stuurman et al., unpublished).

Protein purification

Authentic interphase lamin was purified from 6- to 18-hour-old *Drosophila* embryos essentially as previously described (Lin and Fisher, 1990). All steps were performed at 4°C except when indicated. Frozen, dechorionated embryos were thawed in 9 volumes (one volume equals the starting volume of embryos) of buffer A (50 mM sodium phosphate, pH 8.0, 15 mM $MgCl_2$, 1.0 M sucrose, 0.5 mM DTT) supplemented with protease inhibitors (1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml chymostatin, 2 μg/ml pepstatin-A). Embryos were broken using a Dounce homogenizer (5 strokes, tight pestle) and the homogenate was filtered through two layers of 120 μm nylon mesh. Nuclei were collected by centrifugation for 15 minutes at 20,000 ***g*** in a swinging-bucket rotor through a cushion of buffer A supplemented with protease inhibitors. After a single wash in buffer A supplemented with protease inhibitors (by resuspension and centrifugation for 10 minutes at 8,000 ***g***), nuclei were resuspended in one volume buffer B (10 mM sodium phosphate, pH 8.0, 5 mM $MgCl_2$, 0.5 mM DTT, protease inhibitors). DNase I and RNase A were added to final concentrations of 10 μg/ml each and the suspension was incubated for 15 minutes at 23°C. Nuclei were recovered by centrifugation for 10 minutes at 5,000 ***g***, resuspended in 0.9 volumes of buffer C (10 mM sodium phosphate, pH 8.0, 0.1 mM $MgCl_2$, 250 mM sucrose plus protease inhibitors) and Triton X-100 was added to 2% (w/v) from a 20% (w/v) solution in water. After a 10 minute incubation the suspension was centrifuged for 10 minutes at 2,000 ***g***. The pellet was resuspended in 0.5 volumes of buffer D (100 mM Tris-HCl pH 8.1 (4°C), 0.1 mM $MgCl_2$, 250 mM sucrose, 0.1% (w/v) Triton X-100, protease inhibitors) and NaCl was added to a final concentration of 0.5 M from a 1.0 M stock solution. After centrifugation for 10 minutes at 10,000 ***g*** the supernatant was used immediately for immunoaffinity purification of lamins.

To prepare an anti-*Drosophila* lamin affinity resin, antiserum was raised in rabbits. Specific IgG was affinity-purified from this serum using as the immobilized affinity ligand, bacterially expressed lamin Dm_0 fragment L1-522 (containing residues 1 to 522 of full length lamin Dm_0) covalently bound to glutaraldehyde-activated glass beads (Boehringer, Mannheim, FRG). Bound IgG was eluted using 100 mM glycine-HCl, pH 2.3, 500 mM NaCl, 0.1% (w/v) Triton X-100, thus selecting for anti-lamin IgG which does not bind lamin at low pH. The antibody was cross-linked to Protein-A agarose (Schleicher and Schuell, Keene, NH) using dimethylpimelimidate as described (Harlow and Lane, 1988).

The 0.5 M NaCl extract of nuclease treated, Triton X-100 treated nuclei was mixed with anti-*Drosophila* lamin-Protein A agarose for 4 hours at 4°C. The resin was then washed with 40 column volumes of 20 mM Tris-HCl, pH 8.1 (4°C), 0.5 M NaCl, 5 mM EDTA, 0.1% (w/v) Triton X-100, and bound lamin was eluted with 50 mM glycine-HCl, pH 2.3, 0.5 M NaCl, 0.1% (w/v) Triton X-100. Eluted material was immediately neutralized by addition of Na_2HPO_4 to 50 mM; lamin-containing fractions were pooled and stored at −80°C.

Epitope mapping by random mutagenesis

For random mutagenesis by polymerase chain reaction (PCR) (Zhou et al., 1991), pET-DmLFL (N. Stuurman et al., unpublished) was linearized by digestion with *Eco*RI and used at a final concentration of 50 ng/ml in a PCR reaction containing 10 mM Tris-HCl, pH 9.0 (25°C), 50 mM KCl, 0.1% (w/v) Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 22 units/ml Taq DNA polymerase (Boehringer, Mannheim, FRG), 10% (v/v) dimethylsulfoxide, 0.5 pM T7 primer, 0.5 pM primer 5′-TCTTGCAGTCGTTGG. The reaction was performed in multiple 100 μl-aliquots with the following temperature profile: 5 minutes at 94°C followed by 30 cycles (1 minute and 20 seconds at 94°C, 1 minute at 45°C, 4 minutes at 72°C) and a final extension for 10 minutes at 72°C. The product was phenol/chloroform extracted, ethanol precipitated, digested with *Nde*I and *Hin*dIII, and gel-purified. This fragment was ligated into pET-22b (Novagen, Madison, WI) which had been digested with *Nde*I and *Hin*dIII and had been dephosphorylated. The ligation mixture was electrotransformed into HMS174(DE3)pLysS (Novagen). Colonies were duplicated onto nitrocellulose membranes and induced to produce recombinant protein by placing the filter on an agar plate containing 1 mM IPTG. After three hours at 37°C, the filter was removed and cells were lysed by freezing (5 minutes at −80°C) and thawing (5 minutes at 37°C) twice. Lysed cells were labeled in situ using ADL84 followed by anti-mouse IgG conjugated to alkaline phosphatase (Kierkegaard and Perry Laboratories Inc., Gaithersburg, MD) and a one-solution phosphatase substrate (Kierkegaard and Perry Laboratories Inc.). The same filter was subsequently labeled with affinity-purified polyclonal anti-*Drosophila* lamin IgG followed by anti-rabbit IgG conjugated to horseradish peroxidase. Detection was with an enhanced chemiluminescence system (ECL from Amersham, Arlington Heights, IL).

Alkaline phosphatase-conjugated anti-mouse IgG does not bind rabbit IgG; horseradish peroxidase-conjugated anti-rabbit IgG does not react with mouse IgG (not shown). Colonies which reacted with the polyclonal anti-lamin antibody but not with ADL84 were selected. Plasmids were isolated from the colonies, transformed into XL-1 Blue and sequenced using ssDNA (released with helper phage) and the Sequenase 2.0 kit (United States Biochemical, Cleveland, OH).

SDS-PAGE and immunoblotting

Proteins were separated by SDS-PAGE according to the method of Laemmli (1970) and transferred to nitrocellulose using the method described by Towbin et al. (1979). Blots were stained with a 0.2% (w/v) solution of Ponceau-S (Sigma) and destained with 12 mM HCl to visualize transferred proteins. Blots were equilibrated in PBS containing 0.5% (v/v) Tween-20 and incubated with primary antibody (affinity-purified polyclonal rabbit anti-*Drosophila* lamin IgG at 50 ng/ml, or culture supernatants from hybridomas secreting anti-*Drosophila* lamin antibodies at a 10- to 100-fold dilution) for 2-16 hours at room temperature. Bound antibodies were detected using goat anti-rabbit or goat anti-mouse IgG conjugated to alkaline phosphatase (Kierkegaard and Perry Laboratories Inc., Gaithersburg, MD) and a one-solution phosphatase substrate (Kierkegaard and Perry Laboratories Inc.).

In situ alkaline phosphatase treatment of immunoblots

Lamins Dm_1 and Dm_2 were separated by SDS-PAGE and blotted onto nitrocellulose strips. These strips were incubated for 1 hour at room temperature with 10 mg/ml BSA in PBS containing 0.5% (w/v) Tween-20. They were washed three times with 50 mM glycine, pH 9.5, 0.1% (w/v) Tween-20 and incubated for 90 minutes at 37°C with 500 U/ml calf intestine alkaline phosphatase (Boehringer, Mannheim, FRG) diluted in 50 mM glycine, pH 9.5, 0.1% (w/v) Tween-20. Controls were incubated similarly but without added enzyme. Immunoblot strips were incubated with antibodies essentially as described above; detection of immunoreactivity was with goat anti-mouse IgG conjugated to horseradish peroxidase and a one-solution peroxidase substrate (Kierkegaard and Perry Laboratories Inc.).

Immunoprecipitation

For immunoprecipitation monoclonal antibodies were bound to Protein-G Sepharose (Pharmacia, Piscataway, NJ) by incubating 150 μl of culture supernatant of antibody secreting hybridomas supplemented with 100 mM Tris-HCl, pH 8.0, with 50 μl Protein-G Sepharose for two hours at room temperature. The beads were washed three times with 10 mM sodium phosphate (pH 8.0), 0.5 M NaCl, 5 mM EDTA, 0.1% (w/v) Triton X-100; 15 μl of beads were incubated for 16 hours at room temperature with 30 μl of affinity-purified authentic *Drosophila* lamin Dm_1 and Dm_2 diluted to 2 μg/ml in the same buffer. The Sepharose beads were washed five times with the same buffer and boiled in SDS sample buffer.

Immunofluorescence

Indirect immunofluorescence microscopy on third instar larval salivary gland squash preparations was performed as described previously (Fisher et al., 1982).

Peptide competition assays

Peptides were tested in an antibody capture assay with antigen competition (Harlow and Lane, 1988). Peptides L18-32 (T^{18}STPRPP-SAGPQPPP), L18-32;S^{25}P (which has the same primary sequence as L18-32 but is phosphorylated at serine 8), and a control peptide (VGF-PVTPQVPLRPMT) were synthesized and HPLC purified to about 80% purity by Neosystem Laboratoire (Strasbourg, France). Microtiter plates were coated with lamin Dm_0 (0.7 pmol per well) and blocked with BSA. Affinity-purified ADL84 (3.1 nM) in PBS containing 0.1% Tween-20 was preincubated for 75 minutes at 37°C with peptides at the concentrations indicated in Fig. 7. Subsequently, 100 μl of the antibody/peptide mixture was added to each well and incubated for a further 30 minutes at 37°C. After washing with PBS containing 0.1% Tween-20, bound antibody was detected using horseradish peroxidase-conjugated swine anti-mouse Ig (Dako, Rostrup, Denmark) and *o*-phenylenediamine as a substrate. The reaction product was quantified by measuring the absorbance at 495 nm.

RESULTS

ADL84, a monoclonal antibody specific for lamin Dm_1

To study posttranslational modification of the *Drosophila* nuclear lamin, we generated monoclonal antibodies directed against this protein. When immunoblots containing both interphase isoforms (lamins Dm_1 and Dm_2) were probed with these antibodies, one (ADL84) apparently recognized lamin Dm_1 but not lamin Dm_2 (Fig. 1, lane 1). ADL84 also recognized full-length *Drosophila* lamin expressed in bacteria (not shown). Another monoclonal antibody (ADL67) bound both interphase isoforms (lamins Dm_1 and Dm_2) with apparently similar affinity (Fig.1, lane 3). Five other mAbs reacted with both lamins Dm_1 and Dm_2 (not shown).

Previously, it was shown that lamin Dm_2 arises by specific phosphorylation of lamin Dm_1, most likely on one or more serine residues (Smith et al., 1987). To test whether phosphorylation of lamin Dm_2 inhibits binding of ADL84, we treated lamins Dm_1 and Dm_2, separated by SDS-PAGE and immobilized on immunoblots, with alkaline phosphatase before incubation with mAbs. As shown in Fig. 1, lane 2, phosphatase treatment of lamin Dm_2 fixed on nitrocellulose fully restored its reactivity with ADL84. Apparently, the lamin Dm_2-specific phosphorylation decreases substantially the affinity of ADL84 for the *Drosophila* lamin. This suggests that the lamin Dm_2-specific phosphorylation site colocalizes with the epitope for ADL84. Alternatively, specific phosphorylation of lamin Dm_1 to generate lamin Dm_2 causes a structural change in the epitope for ADL84 thus interfering with binding of this antibody; this change would have to survive SDS-PAGE and immunoblot analysis.

Isolated interphase lamins Dm_1 and Dm_2 exist as a random population of mixed dimers

Lamins, like other intermediate filament proteins, form a two-chain (dimeric) coiled-coil by the parallel alignment of

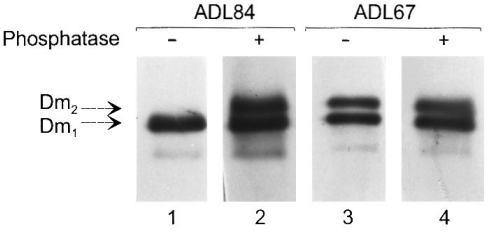

Fig. 1. A monoclonal antibody specific for one of the two *Drosophila* interphase lamin isoforms. Authentic *Drosophila* lamins affinity-purified from 6- to 18-hour-old embryos were separated on an SDS-8% polyacrylamide gel and blotted to nitrocellulose. Separate blot strips were incubated with (lanes 2 and 4) or without (lanes 1 and 3) alkaline phosphatase, followed by ADL84 or ADL67 as indicated. Bound mAbs were detected using a colorimetric assay system. Migration positions of lamin Dm_2 (76 kDa) and lamin Dm_1 (74 kDa) are indicated on the left-hand side of the figure.

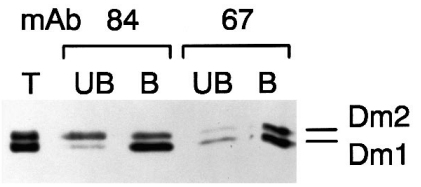

Fig. 2. Immunoprecipitation of isolated authentic *Drosophila* lamins with ADL84. Isolated lamins were immunoprecipitated with the lamin Dm_1-specific mAb ADL84, or with ADL67 which recognizes both lamins Dm_1 and Dm_2. Immunoprecipitation was carried out under conditions that allow lamins to form dimers (see text). Shown is an immunoblot probed with affinity-purified polyclonal anti-*Drosophila* lamin antibodies which recognize both lamins Dm_1 and Dm_2 equally. Lane T, total before immunoprecipitation; lanes UB, unbound material after immunoprecipitation with the indicated mAb; lanes B, material bound after immunoprecipitation with the indicated mAb. Equivalent amounts of total, bound and unbound material were loaded. Migration positions of lamins Dm_1 and Dm_2 are indicated on the right-hand side of the figure.

molecules (Aebi et al., 1986; Parry et al., 1986). This raised the question of whether the two interphase isoforms, lamins Dm_1 and Dm_2, form obligate homodimers, obligate heterodimers, or show no preference for themselves or each other. We addressed this question using ADL84 to immunoprecipitate selectively lamin Dm_1 under conditions where dimer formation takes place but no higher order polymerization is evident (Lin and Fisher, 1990). If the lamin isoforms formed obligate homodimers, only lamin Dm_1 would be found in the fraction bound to ADL84. In the case of obligate heterodimers, lamins Dm_1 and Dm_2 would be found in equimolar amounts in the bound fraction. If the lamin isoforms showed no preference for themselves or each other, one would expect one molecule of lamin Dm_2 for two molecules of lamin Dm_1 (a lamin Dm_2 to Dm_1 ratio of 0.50) in the ADL84 bound fraction.

Affinity-purified interphase lamins were diluted in 10 mM phosphate buffer, pH 8.0 containing 0.5 M NaCl, 5 mM EDTA and 0.1% (w/v) Triton X-100 (a condition which allows dimer formation but prevents higher-order polymerization) and mixed with Protein G-Sepharose beads which had been preadsorbed with either ADL84 or ADL67. ADL67 apparently reacts with both interphase lamin isoforms equally (see Fig. 1). It is evident from Fig. 2 (lane 84, UB) that lamin Dm_1 was efficiently depleted from the unbound fraction by immunoprecipitation with ADL84, while a substantial amount of lamin Dm_2 remained. Comparison with the unbound fraction after immunoprecipitation with ADL67 (which binds both lamin isoforms) demonstrated the specificity of ADL84 for lamin Dm_1 under the conditions used for immunoprecipitation. The fraction bound to ADL84 was enriched in lamin Dm_1 but contained a substantial amount of lamin Dm_2 (Fig. 2, lane 84, B). Densitometry revealed that the ratio of lamins Dm_2:Dm_1 (normalized to the ratio of these isoforms in the starting material) was 0.53±0.07 (n=4). The ratio of lamins Dm_2:Dm_1 bound to mAb ADL67 (normalized to

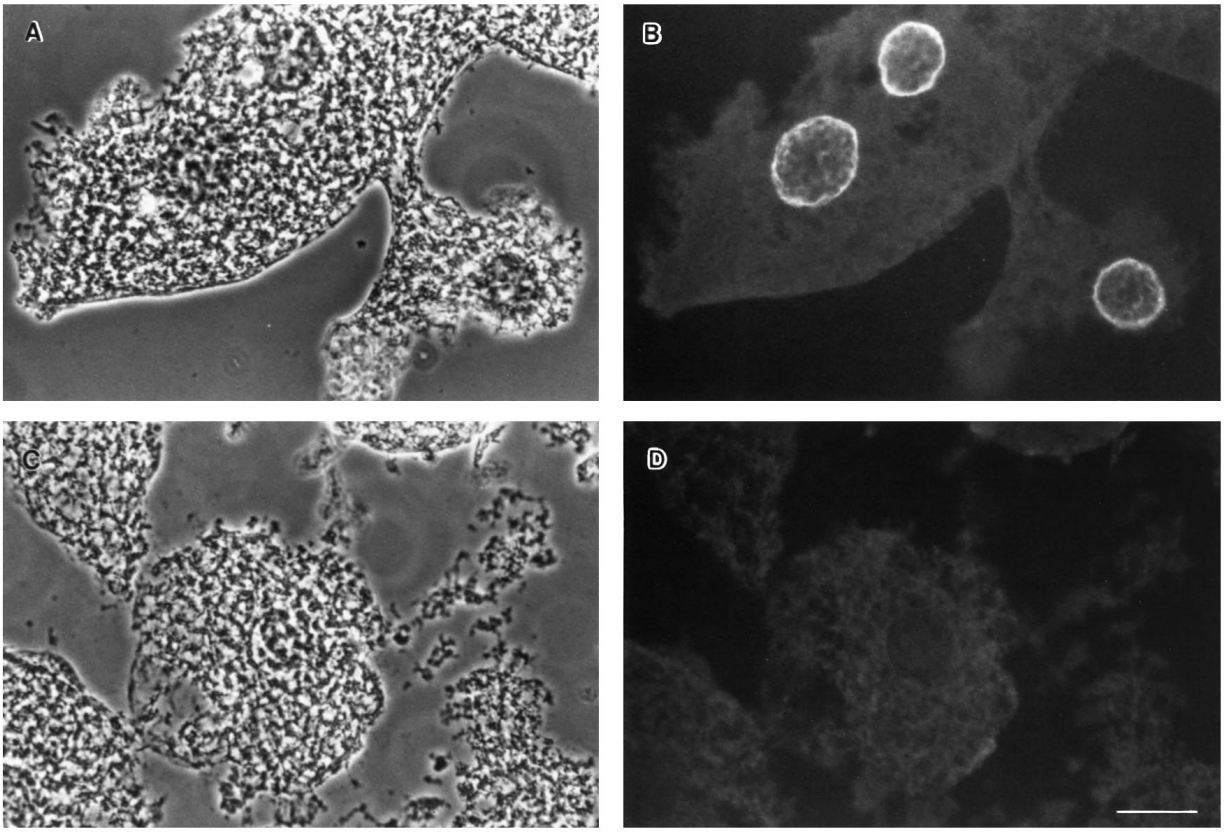

Fig. 3. Indirect immunofluorescence localization of ADL84 reactivity in salivary gland cells. Squashed salivary glands were fixed and incubated with culture supernatant from ADL84 (A and B) or culture supernatant from a hybridoma secreting an unrelated Ig (C and D). Bound antibody was detected with rhodamine conjugated anti-mouse IgG. Phase contrast (A and C) and fluorescence micrographs (B and D) are shown. Bar in D, 20 μm (applies to all panels).

the ratio of the two forms in the starting material) was 0.96±0.03 (n=4). Evidently, isolated lamins Dm_1 and Dm_2 do not show any preference for themselves or each other at the level of dimer formation. Moreover, ADL84 and ADL67 react with nondenatured lamins in solution as well as with SDS-denatured lamins on immunoblots.

Indirect immunofluorescence analysis of *Drosophila* cells and tissues with ADL84

Tissue culture supernatant of ADL84 was used for in situ localization. Results (Fig. 3) show that ADL84 binds to the nuclear periphery of *Drosophila* salivary gland nuclei, suggesting that the antibody also recognizes lamin Dm_1 in situ. Previously, it was shown that most, if not all, cell nuclei of *Drosophila melanogaster* bind a polyclonal antibody specific for lamins Dm_1 and Dm_2 (Whalen et al., 1991). To determine if any cells or tissues lacked lamin Dm_1, ADL84 was used to label cryosections through all stages of the *Drosophila* life cycle. All detectable nuclei were labeled with ADL84 (not shown). To exclude in vitro phosphatase activity, stained cryosections of embryos were analyzed by immunoblotting. No alteration in the ratio of lamins Dm_1 and Dm_2 was seen (not shown) indicating that the observed staining pattern was not caused by an in vitro conversion of lamin Dm_2 into Dm_1. These data suggest that all individual nuclei contain a significant amount of lamin Dm_1.

Mapping the ADL84 epitope by random mutagenesis

To gain insight into the location of the lamin Dm_2-specific phosphorylation site, we mapped the epitope for ADL84. First, we tested binding of ADL84 to two deletion mutants of the *Drosophila* lamin expressed in *E. coli* (schematically represented in Fig. 4A). ADL84 recognized a fragment formed by the 179 NH_2-terminal amino acids of *Drosophila* lamin Dm_0 (Fig. 4B, lanes 3). ADL84 did not bind to the fragment designated Headless which consists of all but the first 56 NH_2-terminal lamin amino acids (Fig. 4B, lanes 2). Evidently, the epitope for ADL84 is located in the first 179 amino acids of the molecule, and is most likely within the NH_2-terminal 56 amino acids.

To map the ADL84 epitope more precisely, random mutations were PCR-generated in the coding region for DmL-179. The mutated fragments were ligated into pET-22b and transformed into a host which expressed the T7 lysozyme gene (HMS174(DE3)pLysS). We chose a host containing the T7 lysozyme gene to ensure cell lysis by simple freezing and thawing. Colonies were transferred to nitrocellulose filters and expression of the mutant protein was induced by transfer of the filters to plates containing IPTG. After induction, cells were lysed and colonies were selected which could be labeled in situ with affinity-purified rabbit anti-lamin antibodies (Fig. 5, lower panel) but not with ADL84 (Fig. 5, upper panel). Of about 2,000 colonies analyzed, 12 satisfied these criteria. Cells from all 12 colonies were grown separately in suspension culture, induced to express the mutant protein with IPTG and total cell lysates were analyzed by immunoblotting using ADL84 (Fig. 6 upper panel) and affinity-purified polyclonal anti-lamin antibody (Fig. 6, lower panel). All showed a highly reduced reactivity of the mutant protein towards ADL84 but maintained full reactivity with the polyclonal anti-lamin antibody. Two representative examples are shown (Fig. 6, lanes 1 and 2).

Sequencing of these 12 clones revealed that all were mutated in an area coding for amino acids 22-28. All mutations resulted in an amino acid change (Table 1). Three mutants were sequenced throughout their coding sequence and were found not to have any additional mutations. These data indicate that

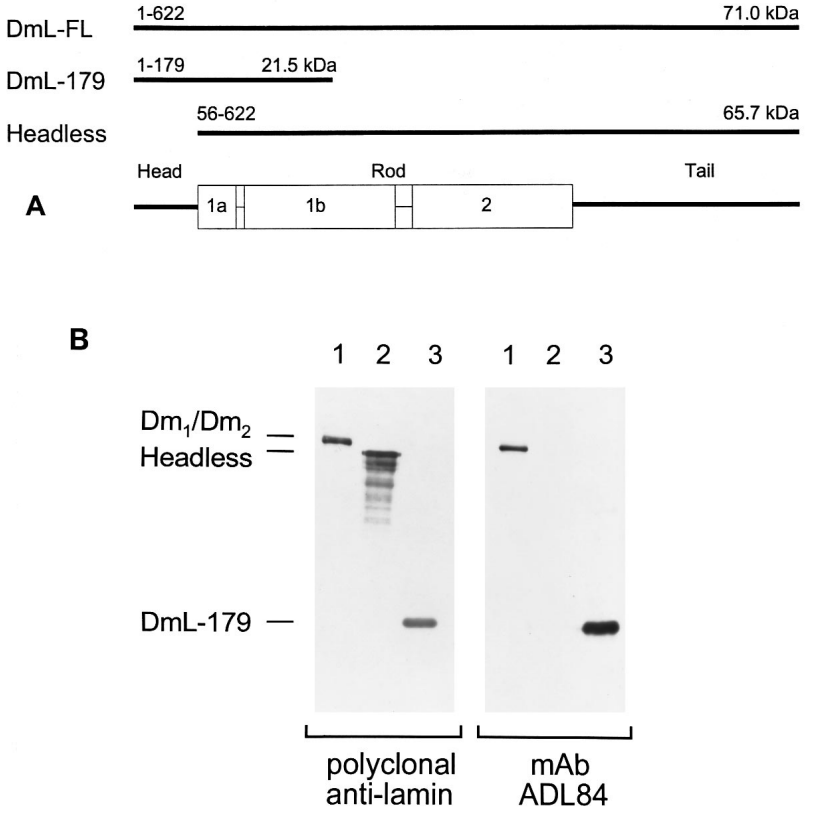

Fig. 4. Binding of ADL84 to full-length *Drosophila* lamin and deletion mutants expressed in *E. coli*. (A) Schematic representation of the proteins used. Numbers at the left-hand side indicate the amino acids of the full-length protein contained in each construct. On the right-hand side the molecular mass of the protein product of each construct is indicated in kDa. The lowest diagram in the panel schematically represents the structure of the lamin molecule. (B) Immunoblots probed with anti-lamin antibodies. Identical blots were probed with affinity-purified polyclonal anti-*Drosophila* lamin IgG or with ADL84, as indicated. Lanes 1, affinity-purified authentic lamins Dm_1 and Dm_2. Lanes 2, product of the Headless mutant. Lanes 3, product of the DmL-179 construct. Approximately the same amount of protein was loaded in each lane. Extra bands in lane 2 are likely to be breakdown products of the Headless mutant, generated within bacteria during production of the protein. Migration positions of authentic lamins (Dm_1 and Dm_2), Headless and DmL-179 are indicated on the left-hand side of the figure.

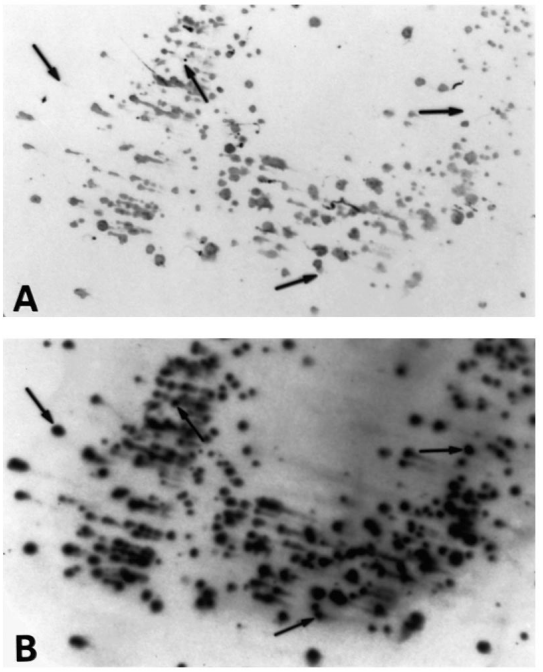

Fig. 5. Epitope mapping of ADL84; screening for point mutants which abolish binding to ADL84. Random mutations were generated in the coding sequence of DmL-179. The plasmid was transformed into *E. coli* strain HMS174(DE3)pLysS. Resulting colonies were transferred to nitrocellulose, protein expression was induced with IPTG and colonies were lysed by freezing and thawing. Filters were double-labeled with ADL84 (using alkaline phosphatase-conjugated anti-mouse IgG secondary antibodies and colorimetric detection; A) and polyclonal affinity-purified rabbit anti-lamin IgG (using peroxidase-conjugated anti-rabbit secondary antibodies and enhanced chemiluminescence; B). Arrows indicate colonies producing mutated DmL-179 that do not bind ADL84.

the epitope for ADL84 is formed by amino acid sequence R^{22}PPSAGP. The location of a serine in the middle of this sequence suggests that this serine is the site specifically phosphorylated in lamin Dm_2.

Peptide competition experiments demonstrate that phosphorylation of serine 25 inhibits binding to ADL84

To corroborate conclusions drawn from epitope mapping by random mutagenesis, we synthesized the peptide representing amino acids 18-32 of lamin Dm_0 (L18-32) and tested its ability to bind to ADL84 in a competition assay. As shown in Fig. 7, this peptide inhibits binding of ADL84 to lamin Dm_0 immobilized on microtiter plates; 50% inhibition is observed at about a 13-fold molar excess of peptide over antibody. An unrelated control peptide did not inhibit binding of ADL84 to lamin Dm_0 (Fig. 7). Similarly, L18-32 did not inhibit binding of another anti-*Drosophila* lamin mAb to lamin Dm_0 (not shown).

To determine whether phosphorylation of serine 25 influenced binding of ADL84 we also tested a variant of the peptide L18-32 that was chemically phosphorylated at the serine residue corresponding to serine 25 in the full length lamin. This phosphorylated peptide inhibited binding of ADL84 to immobilized lamin Dm_0 with an efficiency that was about 19-fold lower than the unphosphorylated peptide (Fig. 7); 50% inhibition occurred at a 250-fold molar excess of the phosphorylated peptide over antibody. Alkaline phosphatase treatment of the chemically phosphorylated peptide increased its ability to compete for binding to ADL84 to levels comparable to the original, unphosphorylated peptide (50% inhibition at an 18-fold molar excess of peptide over antibody). Thus, we conclude that the epitope for ADL84 is contained within amino acids 18-32 of lamin Dm_0 and that phosphorylation of serine 25 inhibits lamin binding to ADL84.

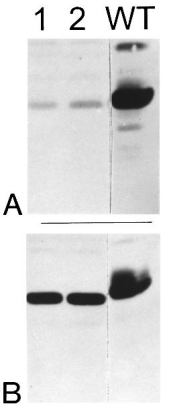

Fig. 6. Epitope mapping of ADL84; analysis of selected mutants by immunoblotting. Selected colonies (as shown in Fig. 5) were grown in liquid culture, induced with IPTG, lysed by boiling in SDS-containing sample loading buffer and separated on SDS-10% polyacrylamide gels. Duplicate gels were transferred to nitrocellulose and probed with ADL84 (A) or with polyclonal affinity-purified rabbit anti-*Drosophila* lamin IgG (B). Lanes 1,2, selected mutants; lane WT, colony containing the unmodified plasmid coding for DmL-179.

DISCUSSION

Interphase *Drosophila* lamins Dm_1 and Dm_2 are highly phosphorylated (2-3 phosphates per molecule). The function of this phosphorylation is unknown. Using a procedure designed to

Table 1. Mutations that disrupt binding of ADL84 to *Drosophila* lamin*

Frequency	Mutation in the DNA	Mutation in the Protein
3	G^{194} A	R^{22} W
1	C^{195} A	R^{22} L
3	A^{203} G	S^{25} P
1	G^{191} T	P^{21} T
	A^{203} G	S^{25} P
1	A^{203} G	S^{25} P
	G^{249} A	A^{40} V
1	G^{204} A	S^{25} L
1	G^{213} A	P^{28} L
1	G^{213} A	P^{28} L
	T^{567} A	K^{146} M

*Mutants with reduced binding to ADL84 as determined by immunoblotting were sequenced throughout the region coding for the head domain (lamin amino acids 1-56). Three mutants (R^{22} W, S^{25} P, and P^{28} L) were sequenced throughout their coding region and found not to contain any additional mutations.

Amino acid sequence of *Drosophila* lamin Dm_0 head; the probable ADL84 epitope is shown in bold: M^1SSKSRRAGT ATPQPGNTST P**R^{22}PPSAGP**QP PPPSTHSQTA SSPLSPTRHS RVAEKV^{56}.

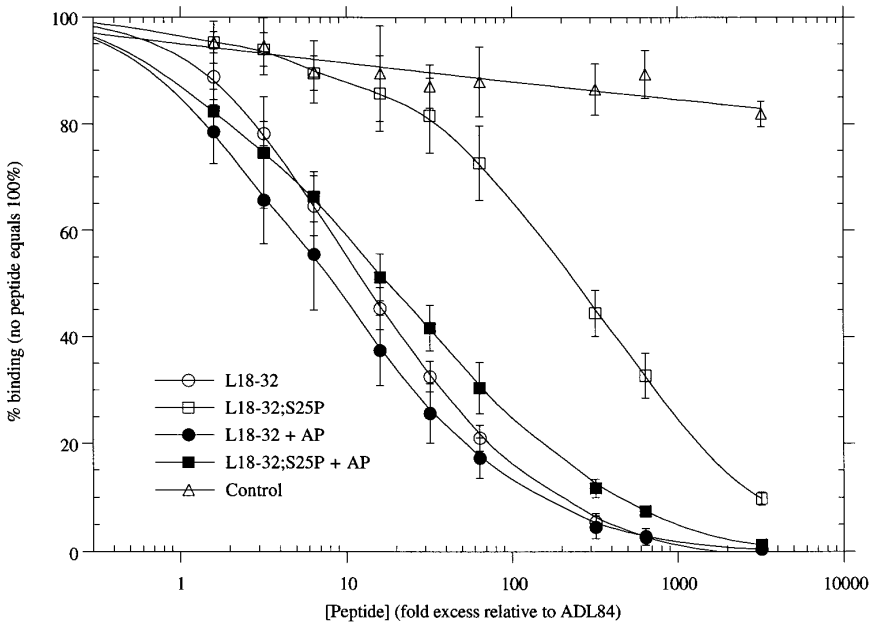

Fig. 7. Phosphorylation of serine 25 decreases the ability of peptide L18-32 to inhibit binding of ADL84 to lamin Dm_0. Purified ADL84 was preincubated with peptide L18-32, a peptide with the same primary sequence but chemically phosphorylated at serine 25 of the full length lamin sequence (L18-32;S25P), either peptide previously incubated with calf intestinal alkaline phosphatase (L18-32 + AP and L18-32;S25P + AP) or a control peptide. Subsequently the antibody/peptide mixtures were incubated with bacterially expressed lamin Dm_0 immobilized on microtiter plates. The amount of ADL84 bound to the microtiter plate was determined using horseradish peroxidase-conjugated secondary antibodies. Signals without added peptide were set at 100%. Peptide L18-32 inhibited binding of ADL84 to lamin Dm_0, whereas peptide L18-32;S25P was much less effective. Alkaline phosphatase treatment of L18-32;S25P largely restored its ability to inhibit binding of ADL84 to lamin Dm_0.

generate mAbs specific for relatively less antigenic sites, we obtained an antibody, ADL84, that reacts with an epitope involved in the posttranslational conversion (via phosphorylation) of lamin Dm_1 to lamin Dm_2. When this site is modified, binding of ADL84 to lamin is largely or completely eliminated. The antibody is specific for both the denatured *Drosophila* lamin Dm_1 presented on immunoblots after SDS-PAGE (Fig. 1), and nondenatured lamin Dm_1 in solution (Fig. 2). Moreover, ADL84 recognizes lamin Dm_2 immobilized on nitrocellulose blots after enzymatic dephosphorylation (Fig. 1) and binds to the nuclear periphery when used for in situ labeling (Fig. 3).

Lamins Dm_1 and Dm_2 have been observed after immunoblot analysis of all *Drosophila* cells and tissues analyzed to date. This includes embryos (Smith et al., 1987), Schneider 2 and Kc tissue culture cells (Smith et al., 1987; L. Lin-Mantell and P. A. Fisher, unpublished) and various larval, pupal and adult tissues (Whalen et al., 1991) including ovaries (Smith and Fisher, 1989). Results of in situ localization studies (Fig. 3) showing uniform distribution of lamin Dm_1 throughout all nuclei examined support the notion that lamins Dm_1 and Dm_2 are present in all interphase cells. This ubiquitous distribution of both lamin isoforms during development and its precise regulation suggest that lamin Dm_2-specific phosphorylation has a general role in lamin function. Based on immunoprecipitation with ADL84, we can now conclude that lamins Dm_1 and Dm_2 interact to form heterodimers in vitro. It seems highly likely that such interactions also take place within the nuclear envelope in vivo.

We used ADL84 to determine the likely phosphorylation site involved in conversion of lamin Dm_1 to lamin Dm_2. Based on phosphoamino acid analysis, it was apparent that this conversion was mediated by serine phosphorylation (Smith et al., 1987). Random mutagenesis followed by screening for selective loss of reactivity with ADL84 (Fig. 5) suggested that the epitope for ADL84 consists of the sequence R^{22}PPSAGP (Fig. 6 and Table 1). We confirmed this by showing that a peptide consisting of amino acids 18-32 of the lamin Dm_0 sequence inhibits binding of ADL84 to lamin Dm_0. In the middle of the epitope is a single serine residue, located at position 25. Phosphorylation of this serine reduced the ability of the peptide to inhibit binding of ADL84 to lamin Dm_0 by about 19-fold. The residual inhibition might be caused by incomplete chemical phosphorylation of the peptide, contaminating phosphatase activity during the assay, or it might reflect a reduced, but measurable affinity of the phosphorylated peptide for ADL84. These data indicate that phosphorylation of serine 25 distinguishes lamin Dm_2 from lamin Dm_1.

The methodology used to define the ADL84-epitope (random mutagenesis followed by screening with both monoclonal and polyclonal antibodies) was both rapid and efficient. A similar approach was used by Ikeda et al. (1992) to map epitopes of two anti-*E. coli* recA protein mAbs. We modified their method in a number of ways. By substituting a T7 RNA polymerase-dependent expression system for λgt11, we were able to both express and sequence mutated proteins without the need for subcloning. Use of an *E. coli* strain expressing the gene for bacteriophage T7 lysozyme facilitated antibody screening in that colonies could be efficiently lysed simply by freezing and thawing. The utility of this approach for antibody screening of expressed protein fragments was demonstrated in this article. This approach may also prove useful for high-resolution mapping of polypeptide domains involved in specific protein-protein interactions.

In conclusion, the most immediate question regarding the conversion of lamin Dm_1 to lamin Dm_2 concerns its physiological/functional significance. The highly dynamic nature of the event as well as the apparently quantitative conversion of lamin Dm_2 to lamin Dm_1 during heat shock (Smith et al., 1987) suggests that it may be involved in such processes as growth of the nuclear envelope during interphase and/or regulation of gene expression. The identification of the site involved in the conversion of lamin Dm_1 to lamin Dm_2 will permit us to address this question directly using genetic analyses of both tissue culture cells and whole organisms.

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REFERENCES

Aebi, U., Cohn, J., Buhle, L. and Gerace, L. (1986). The nuclear lamina is a meshwork of intermediate-type filaments. *Nature* **323**, 560-564.

Benavente, R. and Krohne, G. (1986). Involvement of nuclear lamins in postmitotic reorganization of chromatin as demonstrated by microinjection of lamin antibodies. *J. Cell Biol.* **103**, 1847-1854.

Bossie, C. A. and Sanders, M. M. (1993). A cDNA from *Drosophila melanogaster* encodes a lamin C-like intermediate filament protein. *J. Cell Sci.* **104**, 1263-1272.

Burke, B. (1990). On the cell-free association of lamins A and C with metaphase chromosomes. *Exp. Cell Res.* **186**, 169-176.

Chelsky, D., Olson, J. F. and Koshland, D. J. (1987). Cell cycle-dependent methyl esterification of lamin B. *J. Biol. Chem.* **262**, 4303-4309.

Eggert, M., Radomski, N., Linder, D., Tripier, D., Traub, P. and Jost, E. (1993). Identification of novel phosphorylation sites in murine A-type lamins. *Eur. J. Biochem.* **213**, 659-671.

Fisher, P. A., Berrios, M. and Blobel, G. (1982). Isolation and characterization of a proteinaceous subnuclear fraction composed of nuclear matrix, peripheral lamina, and nuclear pore complexes from embryos of *Drosophila melanogaster*. *J. Cell Biol.* **92**, 674-686.

Glass, C. A., Glass, J. R., Taniura, H., Hasel, K. W., Blevitt, J. M. and Gerace, L. (1993). The alpha-helical rod domain of human lamins A and C contains a chromatin binding site. *EMBO J.* **12**, 4413-4424.

Goss, V. L., Hocevar, B. A., Thompson, L. J., Stratton, C. A., Burns, D. J. and Fields, A. P. (1994). Identification of nuclear $_{II}$ protein kinase C as a mitotic lamin kinase. *J. Biol. Chem.* **269**, 19074-19080.

Gruenbaum, Y., Landesman, Y., Drees, B., Bare, J. W., Saumweber, H., Paddy, M. R., Sedat, J. W., Smith, D. E., Benton, B. M. and Fisher, P. A. (1988). *Drosophila* nuclear lamin precursor Dm_0 is translated from either of two developmentally regulated mRNA species apparently encoded by a single gene. *J. Cell Biol.* **106**, 585-596.

Harlow, E. and Lane, D. (1988). *Antibodies. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Heald, R. and McKeon, F. (1990). Mutations of phosphorylation sites in lamin A that prevent nuclear lamina disassembly in mitosis. *Cell* **61**, 579-589.

Hennekes, H., Peter, M., Weber, K. and Nigg, E. A. (1993). Phosphorylation on protein kinase C sites inhibits nuclear import of lamin B_2. *J. Cell Biol.* **120**, 1293-1304.

Hennekes, H. and Nigg, E. A. (1994). The role of isoprenylation in membrane attachments of nuclear lamins. A single point mutation prevents proteolytic cleavage of the lamin A precursors and confers membrane binding properties. *J. Cell Sci.* **107**, 1019-1029.

Hill, R. J. and Whytock, S. (1993). Cytological structure of the native polytene salivary gland nucleus of *Drosophila melanogaster*: a microsurgical analysis. *Chromosoma* **102**, 446-456.

Hoger, T. H., Krohne, G. and Kleinschmidt, J. A. (1991). Interaction of *Xenopus* lamins A and LII with chromatin in vitro mediated by a sequence element in the carboxyterminal domain. *Exp. Cell Res.* **197**, 280-289.

Hornbeck, P., Huang, K. P. and Paul, W. E. (1988). Lamin B is rapidly phosphorylated in lymphocytes after activation of protein kinase C. *Proc. Nat. Acad. Sci. USA* **85**, 2279-2283.

Ikeda, M., Hamano, K. and Shibata, T. (1992). Epitope mapping of anti-recA protein IgGs by region specified polymerase chain reaction mutagenesis. *J. Biol. Chem.* **267**, 6291-6296.

Kasahara, K., Chida, K., Tsunenaga, M., Kohno, Y., Ikuta, T. and Kuroki, T. (1991). Identification of lamin B_2 as a substrate of protein kinase C in BALB/MK-2 mouse keratinocytes. *J. Biol. Chem.* **266**, 20018-20023.

Kitten, G. T. and Nigg, E. A. (1991). The CaaX motif is required for isoprenylation, carboxyl methylation, and nuclear membrane association of lamin B_2. *J. Cell Biol.* **113**, 13-23.

Laemmli, U. K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lin, L. and Fisher, P. A. (1990). Immunoaffinity purification and functional characterization of interphase and meiotic *Drosophila* nuclear lamin isoforms. *J. Biol. Chem.* **265**, 12596-12601.

Ludérus, M. E. E., de Graaf, A., Mattia, E., den Blaauwen, J. L., Grande, M. A., de Jong, L. and van Driel, R. (1992). Binding of matrix attachment regions to lamin B_1. *Cell* **70**, 949-959.

Martell, R. E., Strahler, J. R. and Simpson, R. U. (1992). Identification of lamin B and histones as 1,25-dihydroxyvitamin D3-regulated nuclear phosphoproteins in HL-60 cells. *J. Biol. Chem.* **267**, 7511-7519.

Matthew, W. D. and Sandrock, A. W. (1987). Cyclophosphamide treatment used to manipulate response for the production of monoclonal antibodies. *J. Immunol. Meth.* **100**, 73-82.

Meier, J., Campbell, K. H., Ford, C. C., Stick, R. and Hutchison, C. J. (1991). The role of lamin LIII in nuclear assembly and DNA replication, in cell-free extracts of *Xenopus* eggs. *J. Cell Sci.* **98**, 271-279.

Molloy, S. and Little, M. (1992). p34cdc2 kinase-mediated release of lamins from nuclear ghosts is inhibited by cAMP-dependent protein kinase. *Exp. Cell Res.* **201**, 494-499.

Newport, J. W., Wilson, K. L. and Dunphy, W. G. (1990). A lamin-independent pathway for nuclear envelope assembly. *J. Cell Biol.* **111**, 2247-2259.

Nigg, E. A. (1992). Assembly and cell cycle dynamics of the nuclear lamina. *Semin. Cell Biol.* **3**, 245-253.

Parry, D. A., Conway, J. F. and Steinert, P. M. (1986). Structural studies on lamin. Similarities and differences between lamin and intermediate-filament proteins. *Biochem. J.* **238**, 305-308.

Peter, M., Nakagawa, J., Doree, M., Labbe, J. C. and Nigg, E. A. (1990). In vitro disassembly of the nuclear lamina and M phase-specific phosphorylation of lamins by cdc2 kinase. *Cell* **61**, 591-602.

Peter, M., Heitlinger, E., Haner, M., Aebi, U. and Nigg, E. A. (1991). Disassembly of in vitro formed lamin head-to-tail polymers by CDC2 kinase. *EMBO J.* **10**, 1535-1544.

Smith, D. E., Gruenbaum, Y., Berrios, M. and Fisher, P. A. (1987). Biosynthesis and interconversion of *Drosophila* nuclear lamin isoforms during normal growth and in response to heat shock. *J. Cell Biol.* **105**, 771-790.

Smith, D. E. and Fisher, P. A. (1989). Interconversion of *Drosophila* nuclear lamin isoforms during oogenesis, early embryogenesis, and upon entry of cultured cells into mitosis. *J. Cell Biol.* **108**, 255-265.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. *Proc. Nat. Acad. Sci. USA* **76**, 4350-4354.

Tsuda, T. and Alexander, R. W. (1990). Angiotensin-II stimulates phosphorylation of nuclear lamins via a Protein kinase-C-dependent mechanism in cultured vascular smooth muscle cells. *J. Biol. Chem.* 1165-1170.

Ulitzur, N., Harel, A., Feinstein, N. and Gruenbaum, Y. (1992). Lamin activity is essential for nuclear envelope assembly in a *Drosophila* embryo cell-free extract. *J. Cell Biol.* **119**, 17-25.

Vermeersch, C. A., Stechmann, C. L. and McLoon, S. C. (1992). Subtractive immunization techniques for the production of monoclonal antibodies to rare antigens. *Biotechniques* **12**, 842-847.

Ward, G. E. and Kirschner, M. W. (1990). Identification of cell cycle-regulated phosphorylation sites on nuclear lamin C. *Cell* **61**, 561-577.

Weber, K., Plessmann, U. and Traub, P. (1989). Maturation of nuclear lamin-A involves a specific carboxy-terminal trimming, which removes the polyisoprenylation site from the precursor - implications for the structure of the nuclear lamina. *FEBS Lett.* **257**, 411-414.

Whalen, A. M., McConnell, M. and Fisher, P. A. (1991). Developmental regulation of *Drosophila* DNA topoisomerase II. *J. Cell Biol.* **112**, 203-213.

Whytock, S., Moir, R. D. and Stewart, M. (1990). Selective digestion of nuclear envelopes from *Xenopus* oocyte germinal vesicles - possible structural role for the nuclear lamina. *J. Cell Sci.* **97**, 571-580.

Worman, H. J., Yuan, J., Blobel, G. and Georgatos, S. D. (1988). A lamin B receptor in the nuclear envelope. *Proc. Nat. Acad. Sci. USA* **85**, 8531-8534.

Worman, H. J., Evans, C. D. and Blobel, G. (1990). The lamin B receptor of the nuclear envelope inner membrane: a polytopic protein with eight potential transmembrane domains. *J. Cell Biol.* **111**, 1535-1542.

Yuan, J., Simos, G., Blobel, G. and Georgatos, S. D. (1991). Binding of lamin A to polynucleosomes. *J. Biol. Chem.* **266**, 9211-9215.

Zhou, Y., Zhang, X. and Ebright, R. H. (1991). Random mutagenesis of gene-sized DNA molecules by use of PCR with Taq polymerase. *Nucl. Acids. Res.* **19**, 6052.

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