Identification of a conserved phosphorylation site modulating nuclear lamin polymerization

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Abstract Mitotic lamin disassembly results from phosphorylation at specific sites. In vitro, lamins can form head-to-tail polymers that disassemble upon phosphorylation by cdc2 kinase. A co-immunoprecipitation assay, employing *Drosophila* nuclear lamin Dm_0 fragments was used to study the effect of phosphorylation on head-to-tail binding. Phosphorylation of serine-50 by cAMP-dependent kinase inhibited head-to-tail binding in the same manner as phosphorylation of serine-42 by cdc2 kinase. Results suggest that multiple pathways may be employed to disassemble nuclear lamins in vivo.

Key words: Nuclear lamin; *Drosophila*; Phosphorylation; cAMP-dependent kinase; cdc2 kinase

1. Introduction

Lamins are specialized intermediate filament proteins that are the principal component of the nuclear lamina, a fibrous layer of proteins underlying the inner nuclear membrane [1–4]. Lamins are thought to play a role in the structural and functional organization of the cell nucleus. The nuclear lamina may provide mechanical stability and, in addition, it may help to organize chromatin [5–9].

During mitosis the nuclear lamina disassembles [2]. Disassembly correlates with phosphorylation of one or a few specific lamin residues [10–13]. One of these sites was identified in chicken lamin B_2 as serine-16. Cdc2 kinase (the $p34^{cdc2}$–cyclin B complex) induces disassembly of the nuclear lamina in vitro [11,14,15] and also phosphorylates serine-16 of chicken lamin B_2 [11]. Mutation of the homologous residue in human lamin A such that phosphorylation was impossible inhibited nuclear lamina disassembly in transient transfection assays [16]. These experiments established that cdc2 kinase regulates lamin assembly in vivo in at least some cell types. On the other hand, it was demonstrated that protein kinase C functions as a mitotic lamin kinase in human leukemic cells and phosphorylates residues in the COOH-terminal part of the protein [13,17].

Like other intermediate filament proteins, lamins consist of a conserved α-helical rod domain flanked by an NH_2-terminal head domain and COOH-terminal tail domain that are variable in secondary structure. The central rod domain allows the molecule to form parallel unstaggered homodimers in a coiled-coil fashion [18–20]. Under non-denaturing conditions lamins remain dimeric at pH 8.5 and 250 mM NaCl [1,21]. At pH 7.0 and 150 mM NaCl, lamin dimers associate longitudinally into head-to-tail polymers as visualized by electron microscopy. These polar head-to-tail polymers can associate laterally to form filament bundles and paracrystals especially in the presence of divalent cations [21–23].

Phosphorylation of lamins with cdc2 kinase before in vitro polymerization inhibits head-to-tail polymerization [21]. Lamin head-to-tail polymers are disassembled in vitro by phosphorylation with cdc2 kinase [24]. Phosphorylation of a single site in the head domain of chicken lamin B_2 (serine-16) is sufficient for disassembly [24]. Recently, a fragment binding assay was developed to study lamin head-to-tail polymerization. Fragments of *Drosophila* nuclear lamin Dm_0 containing either the NH_2-terminal part of the rod domain plus the head domain (L1–179) or the rod and tail domain (L57–622; headless) bind each other in a head-to-tail fashion [25]. Binding is inhibited by phosphorylation of L1–179 with cdc2 kinase. In this paper the effect of phosphorylation by cAMP-dependent kinase (PKA) on fragment binding is described.

2. Materials and methods

2.1. Co-immunoprecipitation assay, protein expression and purification

Expression of *Drosophila* lamin Dm_0 fragments in *Escherichia coli* and their purification were performed exactly as described [25]. Monoclonal antibody ADL67 and its use in the co-immunoprecipitation assay have also been described [25,26].

2.2. In vitro phosphorylation

Purified bacterially expressed lamin fragments were dialyzed into 50 mM Tris-HCl (pH 7.5) at 25°C (for phosphorylation by PKA) or 50 mM Tris-HCl (pH 7.5) at 25°C, 80 mM β-glycerophosphate, 6 mM EGTA (for phosphorylation by cdc2 kinase) for 2 h at room temperature. Samples were centrifuged for 5 min at $12000 \times g$ and protein concentration in the supernatant was measured using the BCA assay (Pierce, Rockford, IL). When phosphorylation reactions were catalyzed by cdc2 kinase they contained 50 mM Tris-HCl (pH 7.5) at 25°C, 80 mM β-glycerophosphate, 6 mM EGTA, 10 mM $MgCl_2$, 250 μM ATP, 0.3 μCi/μl [γ-^{32}P]ATP (NEN, Boston, MA), 50 μM bacterially expressed lamin fragment, cdc2 kinase (UBI, Lake Placid, NY) at a final specific activity of 500 pmol minute^{-1} μl^{-1}. When PKA was used the reactions contained 50 mM Tris-HCl (pH 7.5) at 25°C, 10 mM $MgCl_2$, 125 μM ATP, 0.3 μCi/μl [γ-^{32}P]ATP, 50 μM bacterially expressed lamin fragment, PKA at a final specific activity of 3 pmol min^{-1} μl^{-1} (using casein as substrate). The catalytic subunit of PKA from Sigma (St. Louis, MO), from Promega (Madison, WI), or the bacterially expressed catalytic subunit of PKA (a gift of Dr. Susan S. Taylor, Dept. of Chemistry, University of California, San Diego, CA) all gave similar results. Reactions were for 1 h at 30°C. Phosphorylated lamin fragments were purified batchwise on nickel saturated metal chelation resin [25]. Phosphorylation stoichiometry was determined after dialysis of the purified substrate into polymerization buffer [25] and measurement of protein concentrations and protein bound radioactive phosphate. Kinetic measurements were made using phosphocellulose paper as described [27].

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Abbreviations: PKA, cAMP-dependent kinase

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2.3. Site-directed mutagenesis and sequencing

Site-directed mutagenesis was performed according to Kunkel et al. [28]. The following primers were used (changed bases are in bold typeface): 5′-CCGGGTGGGGGCGAGGGGGGCGGAGGCCGT-C-3′ (for $S^{42} \rightarrow A$, $S^{4} \rightarrow A$), 5′-CGCGCGAGTGATGGGTGGGGC-TG-3′ (for $R^{48} \rightarrow H$), 5′-GCCACGCGCGCGTGCCGGGTG-3′ (for $S^{50} \rightarrow A$). Mutants were identified by sequencing using ssDNA (released with helper phage) and the Sequenase 2.0 kit (United States Biochemical, Cleveland, OH).

3. Results

Drosophila lamin Dm_0 fragments containing either the NH_2-terminal part of the molecule (L1–179) or the COOH-terminal part (L57–622) bind each other specifically. This binding was shown to represent head-to-tail binding of full-length lamin [25]. Co-immunoprecipitation of L1–179, using an antibody specific for L57–622, demonstrates fragment binding (Fig. 1A). Phosphorylation of L1–179 by either cdc2 kinase or PKA inhibited binding to L57–622 (Fig. 1B). In the experiment shown cdc2 kinase phosphorylated L1–179 to a stoichiometry of about 0.88 mol phosphate per mol protein, whereas PKA phosphorylated L1–179 to about 0.17 mol phosphate per mol protein. Accordingly, a substantial fraction of (mainly unphosphorylated) L1–179 in the preparation treated with PKA still bound to L57–622 as evidenced by Western blotting (Fig. 1A). Quantitation of autoradiograms (for phosphorylated L1–179) and Western blots (for unphosphorylated L1–179) showed that the amount of cdc2 kinase phosphorylated L1–179 bound to L57–622 was $19 \pm 8\%$ ($n = 3$) of the unphosphorylated L1–179 bound to L57–622. For PKA phosphorylated L1–179 this was $11 \pm 7\%$ ($n = 4$).

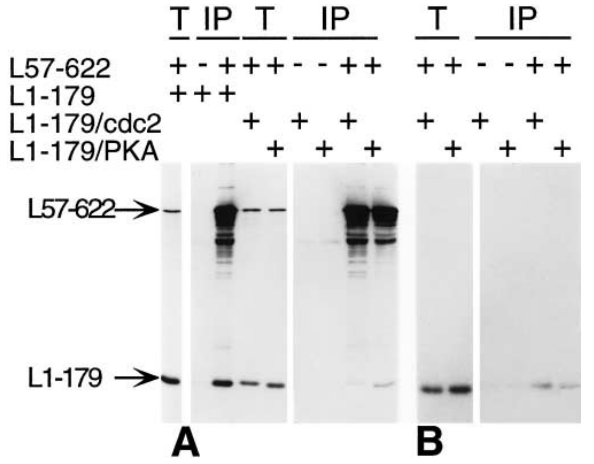

Fig. 1. Phosphorylation of L1–179 with either cdc2 kinase or PKA inhibits binding to L57–622. Shown are a Western blot probed with rabbit IgG affinity purified against lamin fragment L57–179 (A) and an autoradiogram visualizing ^{32}P-labeled protein (B). L1–179 was phosphorylated with cdc2 kinase to 0.88 phosphate/molecule and with PKA to 0.17 phosphate/molecule. Binding reactions were carried out at a concentration of 9 μM L1–179 and 6 μM L57–622. Lanes labeled T contain aliquots of these binding reactions. After incubation, L57–622 was immunoprecipitated with mAb ADL67 (lanes IP), specific for L57–622. The efficiency of immunoprecipitation of L57–622 was identical in all cases as judged by Coomassie blue staining of the gel containing radiolabeled protein (not shown). To compensate for specific activity and to facilitate comparison between cdc2 and PKA phosphorylated L1–179, lanes containing cdc2 kinase treated L1–179 in (B) were loaded with 1/5 of the volume loaded in lanes containing L1–179 phosphorylated by PKA.

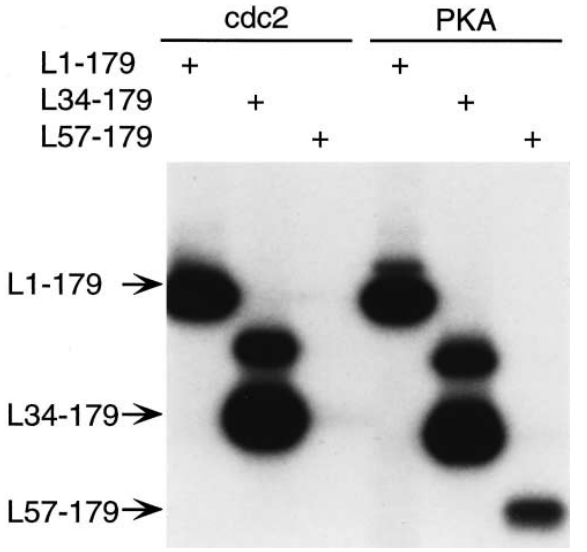

Fig. 2. Phosphorylation of deletion mutants by cdc2 kinase and PKA. L1–179, L34–179, or L57–179 were phosphorylated by either cdc2 kinase or PKA (Section 2). Equal amounts of protein were loaded in each lane and subjected to SDS-PAGE. Positions of the three peptides are indicated with arrows. The autoradiogram was exposed without a screen.

The K_m of starfish cdc2 kinase for L1–179 was measured to be 49 ± 5 μM. For comparison, the K_m of cdc2 kinase for calf thymus histone is 2 μM [29]. The K_m of PKA for L1–179 was 260 ± 34 μM. Full-length *Drosophila* lamin Dm_0 has a second cAMP-dependent phosphorylation site in its tail domain (most likely formed by the sequence R^{592}RRS). The K_m of PKA for that site was found to be lower than 1 μM.

To start mapping which site is phosphorylated, the abilities of PKA and cdc2 kinase to phosphorylate two deletion mutants, L34–179 and L57–179, were compared. Cdc2 kinase phosphorylated L1–179 and L34–179 to a similar extent but was unable to phosphorylate L57–179 (Fig. 2). This was expected since the sites predicted to be phosphorylated by cdc2 kinase, serine-42 and serine-45, are not present in L57–179 [24]. PKA also phosphorylated L1–179 and L34–179 to a similar extent; however, L57–179 was phosphorylated to a level about 9% of that of L1–179 (Fig. 2). Accordingly, the major target site for PKA phosphorylation is contained within amino acids 34–179, and is likely between amino acids 34–57.

The PKA consensus recognition sequence is K/R–K/R–X–

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                          42 45   50
                          |  |    |
Human lamin A             TPLSPTRITRLQEKEDL
Xenopus lamin B1          TPLSPTRITRLQEKVDL
Xenopus lamin B2          SPGSPTRISRMQEKEDL
Drosophila lamin C        SPTSPTRTSRQQEKEEL
Drosophila lamin Dm0      SPLSPTRHSRVAEKVEL
C. elegans lamin          FGSTLLETSRLQEKDHL
S. purpuratus lamin       SLLSPAKISRHEEKEEL
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Fig. 3. A phosphorylation site homologous to *Drosophila* lamin Dm_0 serine-50 is conserved among lamins. Lamin amino acid sequences were aligned in the region between the conserved NH_2-terminal cdc2 phosphorylation sites and the beginning of coil 1A. Other vertebrate lamin sequences (chicken lamins A, B_1, B_2, human lamins B_1, B_2, C, mouse lamins B_1, B_2, C, and *Xenopus* lamin B_2) are identical to the human lamin A sequence shown in this alignment. Numbers on top refer to *Drosophila* lamin Dm_0 sequence.

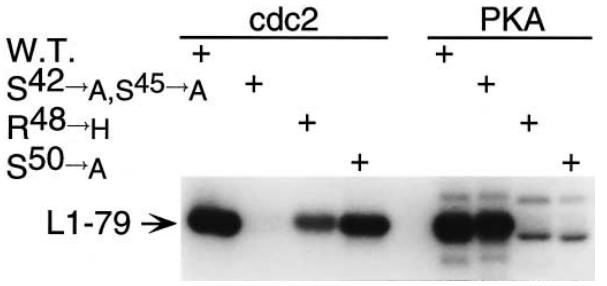

Fig. 4. *Drosophila* lamin Dm_0 serine-50 is the major phosphorylation site for PKA in L1–179. Point mutants in L1–179 as indicated were phosphorylated under identical conditions with either cdc2 kinase or PKA. Aliquots of each reaction containing identical amounts of protein were separated by SDS-PAGE. The dried gel was exposed to film without a screen. The position of L1–179 is indicated on the left hand side.

S/T. PKA phosphorylates either serine or threonine [30]. Inspection of the lamin Dm_0 amino acid sequence between positions 34 and 179 revealed a loose match (T^{47}RHS) with this consensus sequence at serine-50. Remarkably, the amino acid at position 50 is a serine or threonine in all lamins sequenced to date (Fig. 3). A synthetic peptide (SPLSPTRHS^{50}RV) encompassing this site was phosphorylated by PKA in vitro (data not shown). To examine whether serine-50 is indeed phosphorylated by PKA, three point mutants were constructed. One mutant contained the substitution serine-50 to alanine, another one the substitution arginine-48 to histidine (a lysine or arginine at the position of arginine-48 is predicted to be essential for phosphorylation of serine-50 by PKA [30]. The third mutant was a control in which both possible cdc2 kinase sites were modified: serine-42 and serine-45 were converted to alanine. All three mutants bound to L57–622 exactly as wild-type L1–179 (data not shown).

As expected, cdc2 kinase did not phosphorylate the double mutant ($S^{42}\rightarrow A$; $S^{45}\rightarrow A$) (Fig. 4). The mutants in the putative PKA site ($R^{48}\rightarrow H$ and $S^{50}\rightarrow A$), however, were phosphorylated by cdc2 kinase to appreciable levels (Fig. 4). The reduced level of phosphorylation of the mutant $R^{48}\rightarrow H$ was expected since basic amino acids COOH-terminal from the target site stimulate cdc2 kinase phosphorylation [30]. PKA, on the other hand, phosphorylated the double mutant in the putative cdc2 sites ($S^{42}\rightarrow A$; $S^{45}\rightarrow A$) to the same extent as wild-type L1–179 (Fig. 4). Both mutants in the putative PKA site ($R^{48}\rightarrow H$ and $S^{50}\rightarrow A$) were phosphorylated by PKA to reduced levels (13% of that of wild-type L1–179), similar to those found with L57–179 (Fig. 4, compare with Fig. 2). It was also found that mutation of threonine-47 into arginine (which is predicted to render serine-50 a better PKA site) lowered the K_m of PKA at least 10-fold. These data demonstrate that phosphorylation of *Drosophila* lamin Dm_0 serine-50 inhibits binding between fragments L1–179 and L57–622.

4. Discussion

Phosphorylation of *Drosophila* nuclear lamin Dm_0 serine-50 by PKA inhibits binding between L1–179 and L57–622, evidencing that phosphorylation of this serine inhibits lamin head-to-tail polymerization. Therefore, phosphorylation/dephosphorylation of this residue can potentially control the polymerization state of nuclear lamins in a manner similar to phosphorylation/dephosphorylation of serine-45 (homologous to serine-16 in chicken lamin B_1) by cdc2 kinase.

The demonstration that phosphorylation of lamin fragments by PKA interferes with head-to-tail binding is in apparent contrast to the reported inability of PKA to cause lamina depolymerization in vertebrate systems [11,15]. However, the K_m of PKA for serine-50 of *Drosophila* nuclear lamin Dm_0 is 0.26 mM. To obtain phosphorylation of a substantial fraction of lamin molecules a high amount of kinase and/or long incubation periods are necessary. It is likely that PKA has a similar low affinity for the analogous site in vertebrate lamins. Therefore, it is probable that in the experiments with vertebrate lamins [11,15] PKA did not phosphorylate a significant fraction of lamin at the site homologous to *Drosophila* lamin Dm_0 serine-50.

Is this site phosphorylated in vivo? So far, no evidence for mitotic in vivo phosphorylation of the vertebrate nuclear lamin serine or threonine residue homologous to *Drosophila* lamin Dm_0 serine-50 has been reported [11–13,31]. Likewise, an extract from *Drosophila* embryos did not phosphorylate lamin fragment L1–179 at serine 50 (N.S., unpublished observation). It is possible that the site is used in other cell types and/or phases of the cell cycle than the ones that were tested (nocodazole arrested chicken hepatoma cells, *Xenopus laevis* egg extracts, synchronized murine A9 cells, aphidicolin/nocodazole arrested human erythroleukemia K562 cells, 0–3 h *Drosophila* embryos). Alternatively, phosphorylation of *Drosophila* lamin Dm_0 serine-50 might not occur in vivo.

If serine-50 is being phosphorylated in vivo, then is PKA the responsible kinase? The K_m for L1–179 (0.26 mM) is 50-fold higher than the K_m for the peptide Kemptide (5 mM) which corresponds to the phosphorylation site in liver pyruvate kinase. A second site for cAMP-dependent kinase in the lamin tail domain has a K_m smaller than 1 mM. Thus, if PKA phosphorylates lamin Dm_0 serine-50 in vivo, high local concentrations of active kinase and lamin are needed to achieve phosphorylation of a substantial fraction of lamin molecules. Also, although the residue at position 50 of the *Drosophila* lamin is serine or threonine in all lamins (Fig. 3), the surrounding amino acids are less well conserved. The *C. elegans* lamin and *S. purpuratus* lamin (Fig. 3) are predicted not to be phosphorylated by PKA. Thus, phosphorylation of the site in vivo might well be executed by a kinase other than PKA.

Mitotic disassembly of the nuclear lamina proceeds via the cdc2 phosphoacceptor site in the lamin head domain in human Hela [16] and chicken hepatoma cells [11], whereas it occurs via protein kinase C sites in the lamin tail domain in human K562 cells [13]. The demonstration that phosphorylation of yet another serine residue (serine-50 in *Drosophila* lamin Dm_0) in the lamin head domain interferes with lamin assembly suggests that multiple, cell type-specific mechanisms may exist to disassemble the nuclear lamina in vivo. In addition, the site may be used during interphase to locally disassemble the lamina, thereby allowing lamina growth.

That *Drosophila* lamin Dm_0 residue 50 is serine or threonine in all lamins sequenced to date, including the *C. elegans* lamin which lacks a cdc2 kinase phosphoacceptor site in its NH_2-terminal head domain [32], suggests a conserved function. Mutation of this residue to alanine does not interfere with head-to-tail binding (this communication), indicating that it is not directly involved in lamin head-to-tail polymerization. Thus, conservation of this amino acid might be dic-

tated by its involvement in regulating the lamin polymerization state.

The prediction that phosphorylation of this site interferes with lamin polymerization agrees with the idea that phosphorylation of multiple sites just NH_2-terminal and COOH-terminal from the central coiled-coil rod domain affect lamin polymerization [11,12,16,17]. To fully understand which of these sites are functionally used in vivo it is essential to monitor the phosphorylation state of these multiple sites throughout the cell cycle in all cell types of the organism. Antibodies specific for the phosphorylated sites as well as expression throughout the organism of lamin proteins containing point mutations in each phosphorylation site could enable such a mapping.

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