Specific and conserved sequences in *D. melanogaster* and *C. elegans* lamins and histone H2A mediate the attachment of lamins to chromosomes

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Summary

The intimate association between nuclear lamins and chromatin is thought to regulate higher order chromatin organization. Previous studies have mapped a region between the rod domain and the Ig fold in the tail domain of *Drosophila melanogaster* lamin Dm_0, which binds chromatin in vitro via the histone H2A/H2B dimer. This region contains an evolutionarily conserved nuclear localization signal (NLS) KRKR, and a sequence composed of the amino acids TRAT. Here we show that binding of lamin Dm_0 to chromatin requires both NLS and TRAT sequences. Substituting either of the threonine residues in the TRAT sequence with negatively charged residues decreases the binding of lamin Dm_0 to chromatin, indicating that this binding could be regulated by phosphorylation. Both lamin Dm_0 and *C. elegans* Ce-lamin bind directly to histone H2A in vitro and this binding requires the NLS. The amino and carboxyl tail domains of histone H2A are each essential, but not sufficient, for binding to lamin Dm_0; only a polypeptide containing both histone H2A tail domains binds efficiently to lamin Dm_0. Taken together, these results suggest that specific residues in lamin Dm_0 and histone H2A mediate the attachment of the nuclear lamina to chromosomes in vivo, which could have implications on the understanding of laminopathic diseases.

Key words: Chromatin, Histone, Nuclear envelope, NLS, Tail domain

Introduction

The nuclear lamina is composed of lamins and lamin-associated proteins. It is positioned underneath the inner nuclear membrane and is closely associated with the peripheral chromatin (Franke, 1974; Goldman et al., 2002). In several types of cells, the centrosomes, telomeres and intercalary heterochromatin are always in close proximity to the nuclear lamina (Gruenbaum et al., 2003; Marshall et al., 1996). Lamins are type V intermediate filament (IF) proteins and are the major structural constituents of the metazoan nuclear lamina (Cohen et al., 2001; Stuurman et al., 1998). Lamins are also present in the nucleoplasm (Foisner, 2001). They are classified as either A- or B-type, according to their primary sequence, expression pattern and biochemical properties (Stuurman et al., 1998). Like other IF proteins, lamins contain an α-helical 'rod' domain, composed of coiled-coils, flanked by amino 'head' and carboxyl 'tail' domains (Fig. 1A). At their first level of structural organization, lamins make dimers that later associate as polar head-to-tail polymers. These polymers further associate laterally to form the lamin filaments (Herrmann and Foisner, 2003).

The number and complexity of nuclear lamin genes and proteins increased during metazoan evolution (Cohen et al., 2001). For example, *Caenorhabditis elegans* has only a single B-type lamin gene (Riemer et al., 1993), *Drosophila melanogaster* has one B-type and one A-type lamin gene (Bossie and Sanders, 1993; Gruenbaum et al., 1988), whereas mammals have two B-type and one A-type lamin genes that give rise to seven known isoforms (Fisher et al., 1986; Furukawa et al., 1994; Hoger et al., 1988; McKeon et al., 1986).

Lamin complexes are involved in many nuclear activities including chromatin organization, maintaining nuclear shape, DNA replication, gene transcription, nuclear positioning, spacing of nuclear pore complexes, apoptosis, differentiation and signaling (Gruenbaum et al., 2005). B-type lamins are ubiquitously expressed and are essential for cell viability (Harborth et al., 2001). By contrast, A-type lamins are expressed in differentiated cells and are not essential for cell viability. The functional importance of the nuclear lamins has been emphasized by the recent discovery of many different heritable diseases that are caused by mutations in the human *LMNA* gene, encoding lamins A/C, which are collectively termed 'laminopathies' (Worman and Courvalin, 2005).

Lamins bind chromatin both directly and indirectly via lamin-associated proteins (Mattout-Drubezki and Gruenbaum, 2003). In *Drosophila* nuclei, the distribution of the B-type lamin (Dm_0) is highly correlated to the underlying chromatin distribution, where it covers the surface of nuclear envelope-associated large-scale chromatin domains (Belmont et al., 1993). Photo-crosslinking experiments showed that *Drosophila* lamin Dm_0 associates with nucleic acids in vivo during interphase, but not during mitosis (Rzepecki et al., 1998). In vitro, lamins bind matrix attachment/scaffold associated regions (MARs/SARs) (Luderus et al., 1994; Zhao et al., 1993), as well as centromeric and telomeric sequences

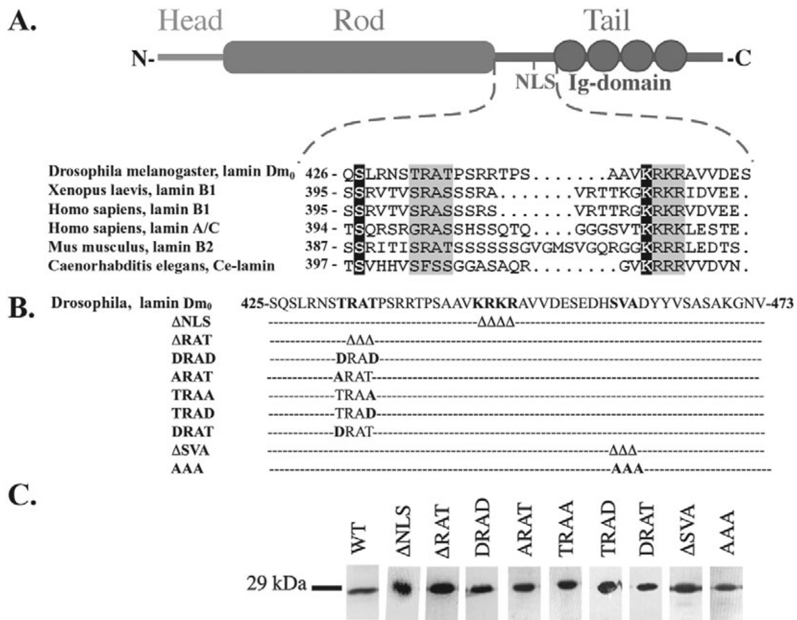

Fig. 1. Expression of wild-type and mutant lamin Dm_0 constructs. (A) Schematic view of the lamin monomer, which is composed of a short N-terminal head, α-helical rod and carboxyl terminal tail domains. The major region required for binding of lamin Dm_0 to chromosomes is enlarged (below) and compared with those of other species. ClustalW (http://www.ebi.ac.uk/clustalw) was used to align the sequences. Conserved residues in that region are highlighted in black (homology) or in gray (similarity). (B) The QuickChange site-directed mutagenesis method was used to produce different mutant constructs of the lamin Dm_0 tail domain. These include deletions (Δ) and substitutions with alanine or aspartate residues. (C) Wild-type and mutant proteins were expressed, purified to near homogeneity and subjected to 12% SDS-PAGE analysis. Proteins were stained with Coomassie Brilliant Blue.

(Baricheva et al., 1996; Shoeman and Traub, 1990). Binding of *Drosophila* lamin Dm_0 to DNA is mediated through the rod domain and requires lamin polymerization (Zhao et al., 1993). Mammalian lamins A/C can also bind DNA via their tail domain (Stierle et al., 2003).

In vitro, lamins from different species interact with chromatin (Glass and Gerace, 1990; Goldberg et al., 1999; Hoger et al., 1991; Yuan et al., 1991). This interaction requires the lamin tail domain (Goldberg et al., 1999; Hoger et al., 1991; Taniura et al., 1995). Two separate regions are required for *Drosophila* lamin Dm_0 binding to mitotic chromosomes in vitro: a stronger binding region including residues 425-473, which are immediately adjacent to the carboxyl part of the rod domain, and a weaker binding region including the C-terminal residues 572-622 (Goldberg et al., 1999). The binding of human lamins A/C to chromosomes involves residues 396-430, also located immediately adjacent to the rod domain (Taniura et al., 1995). Similarly, the binding of *Xenopus* lamin B2 to chromosomes involves residues 404-419 located eleven residues downstream of the rod domain, and residues 432-467 (Hoger et al., 1991). The above sequences are just outside the Ig fold in an unstructured region of the lamin tail domain (Krimm et al., 2002). Lamins from *Drosophila*, turkey and human interact in vitro with polynucleosomes. The binding affinities of the human lamin A tail domain to both polynucleosomes and purified core histones are similar and in the range of 0.12-0.3 μM (Taniura et al., 1995). The tail domain of *Drosophila* lamin Dm_0 binds polynucleosomes with an affinity of ~1 μM. The binding of the *Drosophila* lamin Dm_0 tail domain to chromatin could be displaced with histone H2A or H2B but not by histones H1, H3 or H4 (Goldberg et al., 1999).

In this study, we further characterize the interaction between lamins and histones. We show that the interaction with histone H2A requires specific lamin residues in *Drosophila* and *C. elegans* and both N- and C-terminal tail domains of histone H2A. We also demonstrate that the role of the lamin nuclear localization signal (NLS) sequence in lamin-histone interaction is conserved in *C. elegans* and suggest that interphase lamin phosphorylation could regulate that binding.

Results

The interaction of the lamin Dm_0 tail domain with chromosomes in vitro requires both the NLS and the RAT sequences

Previous studies have mapped a region between the end of the rod domain and the Ig fold in the tail domain, which is required for lamin Dm_0 binding to chromatin (Fig. 1A) (Goldberg et al., 1999). A similar region is also required for the binding of human lamin A or *Xenopus* lamin B2 to chromatin (Hoger et al., 1991; Taniura et al., 1995). There are two stretches of evolutionarily conserved residues in that region, present in both A- and B-type lamins (Fig. 1A): the nuclear localization signal (NLS) and the T/SRAT/S sequences (amino acid single letter code). We prepared a series of expression constructs with wild-type and mutant lamin Dm_0 tail domains and used them to analyze whether the binding of the lamin Dm_0 tail domain to chromosomes requires these conserved sequences (Fig. 1B). The constructs were bacterially expressed and purified to near homogeneity (Fig. 1C). We used a chromosome-binding assay (Goldberg et al., 1999) to test the ability of wild-type and mutant lamin peptides to bind the mitotic chromosomes. Negative controls included reaction mixtures without lamin peptides, primary or secondary antibodies. As expected (Goldberg et al., 1999), the purified wild-type lamin Dm_0 tail domain bound mitotic chromosomes (Fig. 2A and Table 1). By contrast, lamin Dm_0 tail domains in which either the KRKR (ΔNLS) or the RAT sequence (ΔRAT) were deleted showed an ~88% or ~80% reduction in the intensity of binding to chromosomes, respectively (Fig. 2A and Table 1), resembling the binding of the lamin tail domain in which the entire strong binding region (residues 423-473) was deleted (Goldberg et al., 1999). A deletion that removes the non-conserved residues SVA (ΔSVA, Fig. 1B) did not significantly affect the intensity

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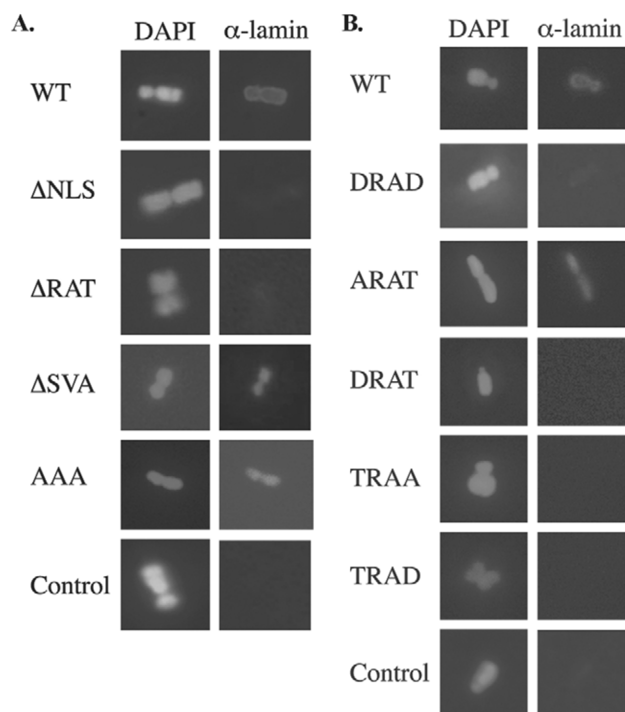

Fig. 2. The binding of lamin Dm_0 to chromosomes requires both the KRKR (NLS) and the RAT sequences. (A,B) The lamin Dm_0 tail domains (residues 425-622), wild-type and containing the indicated mutations, were used to bind mitotic CHO chromosomes in the presence of 10% BSA. The control did not include the lamin Dm_0 protein. Chromosomes were co-stained with DAPI (left) and with 611A3A6 monoclonal anti-lamin Dm_0 antibody as primary antibody and Cy3-conjugated anti-mouse antibodies as secondary antibodies (right). Quantification of these experiments is shown in Table 1.

of the binding of the lamin Dm_0 tail domain to chromosomes (Fig. 2A and Table 1). Similarly, substituting SVA residues with alanine residues (AAA; Fig. 1B) only slightly affected the intensity of the lamin Dm_0 tail binding to chromosomes (Table 1). We concluded that lamin Dm_0 binding to chromatin requires both the KRKR and RAT sequences.

Interphase phosphorylation of the TRAT sequence could regulate the binding of lamin Dm_0 to chromatin

During interphase, lamin Dm_0 is phosphorylated at three positions: one in the head domain (S_{25}) and two in the tail domain (S_{595} and T_{432} or T_{435}) (Schneider et al., 1999). Only ~20% of either T_{432} or T_{435}, which are the first and last threonine residues in the TRAT sequence (Fig. 1B), are phosphorylated in vivo (Schneider et al., 1999). We therefore used the chromosome-binding assay to analyze whether a change in the charge of one or both of these threonine residues affects the binding of lamin Dm_0 to chromosomes. Mutating T_{432}, T_{435} or both threonine residues into aspartate residues (DRAT, TRAD or DRAD), which mimics the negative charge caused by phosphorylation, decreased the binding intensity of the lamin tail domain to chromosomes (Fig. 2B and Table 1). Replacing the first threonine in the TRAT sequence, T_{432}, with alanine (A), which is a common substitution for a non-charged amino acid (ARAT), did not significantly change the intensity

Table 1. Results from the chromosome-binding assay using wild-type and different mutant lamin Dm_0 tail domain proteins

Construct	Mutation(s)	Binding to mitotic chromosome, AU/px^2 (±s.d.)
WT	None	62.8 (15.9)
ΔNLS	$KRKR_{446\text{-}449}$→ΔΔΔΔ	7.6 (3.9)
ΔRAT	$RAT_{433\text{-}435}$→ΔΔΔ	12.6 (3.0)
DRAD	T_{432}→D; T_{435}→D	8.4 (4.3)
ARAT	T_{432}→A	37.8 (16.7)
TRAA	T_{435}→A	1.9 (1.6)
TRAD	T_{435}→D	2.1 (2.4)
DRAT	T_{432}→D	1.0 (0.7)
ΔSVA	$SVA_{459\text{-}461}$→ΔΔΔ	55.0 (14.0)
AAA	$SVA_{459\text{-}461}$→AAA	33.8 (11.9)
Control	No protein	1.6 (2.2)

Constructs used to express the proteins are described in Fig. 1B. Examples of the experiments are shown in Fig. 2. Quantification of the fluorescent signal was performed using the Science Laboratory 99 Image Gauge software and is presented as the average fluorescence signal of binding to mitotic chromosomes in arbitrary units/sq. pixel. Data was taken from at least three independent experiments. s.d., standard deviation.

of lamin tail binding to chromatin, whereas replacing T_{435} with alanine (TRAA) reduced the binding to mitotic chromosomes, almost to that observed with the ΔRAT deletion (Fig. 2B and Table 1). We concluded that a negative charge on residues 432 or 435 reduces lamin interaction with chromatin. It is, therefore, tempting to suggest that phosphorylation of T_{432} or T_{435} affects lamin binding to chromatin.

Lamin interaction with chromosomes in vitro is conserved in *C. elegans* and requires the Ce-lamin tail domain and its NLS motif

The *C. elegans* lamin, Ce-lamin, tail domain or complete ORF have ~30% homology to lamins from insects or vertebrates (Mattout et al., 2006). However, the NLS motif (KRRR) and its position in the tail domain are evolutionarily conserved (Riemer et al., 1993) (Fig. 1A). We bacterially expressed and purified the Ce-lamin complete open reading frame, as well as its isolated tail domain (residues 399-566), both either as wild type or with the KRRR residues substituted with AAAA residues (mNLS). We then used the chromosome-binding assay to test the ability of these proteins to bind mitotic chromosomes. The purified wild-type Ce-lamin, as well as its isolated tail domain, specifically bound mitotic chromosomes with roughly similar intensity, as determined by the fluorescent signal (Fig. 3). In contrast, substituting the KRRR sequence with AAAA in the Ce-lamin tail domain caused ~80% reduction in its binding to mitotic chromosomes (Fig. 3). We concluded that the tail domain of Ce-lamin binds chromosomes in vitro and this binding requires the NLS, similarly to the binding of the tail domain of lamin Dm_0 to chromosomes.

The tail domains of both Ce-lamin and lamin Dm_0 bind histone H2A

Our previous studies have shown that histones H2A or H2B displaced lamin Dm_0 binding to mitotic chromosomes in vitro (Goldberg et al., 1999). We tested whether lamin Dm_0 can bind directly to the isolated histone H2A in vitro, using the blot overlay assay. Wild-type lamin Dm_0 tail domain bound specifically to purified histone H2A. The ΔNLS protein

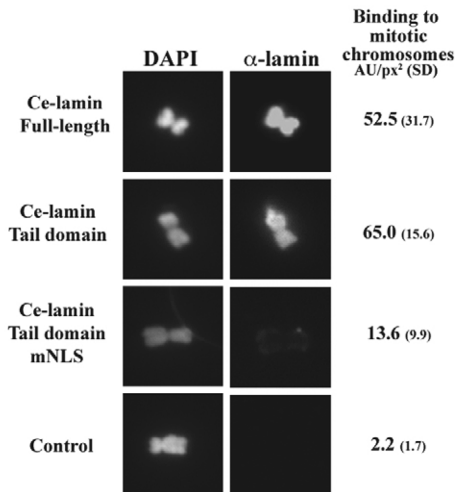

Fig. 3. The Ce-lamin tail domain binds to mitotic chromosomes and the NLS motif is important for this binding. Binding of full-length Ce-lamin, Ce-lamin tail domain and tail domain mutated in its NLS (mNLS) to mitotic CHO chromosomes was performed in the presence of 10% BSA. The control did not include the Ce-lamin protein. Chromosomes were co-stained with DAPI (left) and with affinity purified polyclonal Ce-lamin antibody as primary antibody and Cy3-conjugated anti-rabbit antibodies as secondary antibodies (right). Quantification of the fluorescent signal (far right) was performed using the Science Laboratory 99 Image Gauge software and is given as the average fluorescence signal of binding to mitotic chromosomes in arbitrary units/sq. pixel. Data was taken from at least three independent experiments. SD, standard deviation.

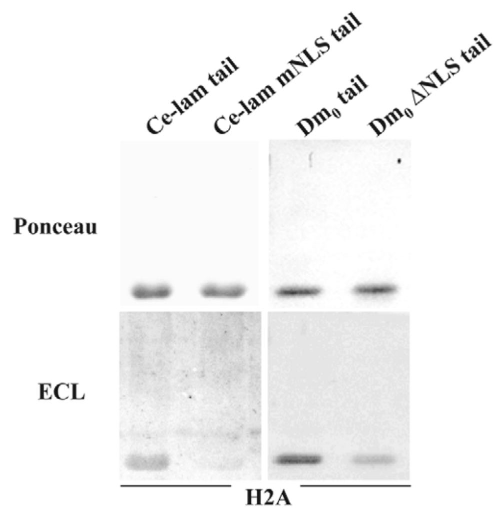

Fig. 4. The tail domains of lamin Dm_0 and Ce-lamin bind directly to histone H2A in vitro and the NLS motif is important for this binding. Blot overlay analysis of binding interactions between histone H2A and the lamin Dm_0 tail domain; wild-type and ΔNLS and the Ce-lamin tail domain; wild-type and mNLS. Equal amounts of protein (5 μg) were resolved using SDS-PAGE and then transferred to a nitrocellulose membrane. Relative protein content was confirmed by Ponceau S staining of the membrane before incubation with the overlay solution (Ponceau, upper panel). Immunostaining was performed with anti-histone H2A antibody after incubation with overlay buffer containing 12 μg /ml histone H2A (ECL, lower panel). BSA was used as a negative control (not shown). Purified proteins and their corresponding overlay signals are indicated above the lanes.

exhibited a significant decrease in binding (Fig. 4), showing again the importance of the NLS in lamin Dm_0 binding to chromatin. Similarly, the isolated tail domain of Ce-lamin also bound specifically to histone H2A, whereas the mNLS protein did it very inefficiently (Fig. 4), suggesting that lamin tail domain binding to histone H2A is evolutionarily conserved.

The N- and C-terminal tail domains of histone H2A are each required for binding to lamin Dm_0

We next identified the regions in histone H2A that are required for the interaction with lamin Dm_0. Wild-type and deletion constructs of histone H2A were either bacterially expressed and purified to near homogeneity or chemically synthesized (Fig. 5A). The ability of the lamin Dm_0 tail domain to bind the purified H2A-derived proteins was then analyzed in both the blot overlay assay and in displacement experiments using the chromosome-binding assay. Blot overlay experiments showed that lamin Dm_0 bound directly to the full-length histone H2A (Fig. 5B and Fig. 6). About 60% of the binding signal was lost when the histone H2A lacked the first 15 amino acids (N-tailless) (Fig. 5B and Fig. 6) and so was ~70% of the binding signal with a histone H2A peptide containing only the first 50 amino acids (N50aa) (Fig. 5B and Fig. 6). There was essentially no binding of lamin Dm_0 to a synthetic peptide containing the first 15 residues (N-peptide) (Fig. 5C and Fig. 6). Also, about 75% of the binding signal with lamin Dm_0 tail domain was lost when the histone H2A lacked its C-terminal 38 amino acids (C-tailless) (Fig. 5D and Fig. 6). These results showed that both the N- and C-tails of histone H2A contribute to the binding to lamin Dm_0, but each one of the tails is not sufficient for that binding. We next tested whether having both isolated tails is sufficient for the binding of lamin Dm_0 by expressing a fusion protein containing the first 32 and the last 38 residues of histone H2A (N+C). Indeed, lamin Dm_0 tail domain bound this protein with efficiency similar to that of the full-length histone H2A (Fig. 5E and Fig. 6).

We also tested the ability of the histone H2A-derived proteins to displace lamin Dm_0 tail domain binding to mitotic chromosomes. As expected (Goldberg et al., 1999), a 100-fold molar excess of the full-length histone H2A efficiently displaced lamin Dm_0 tail domain binding to chromosomes (Fig. 7). Likewise, a fusion protein containing the histone H2A N- and C-tails (N+C) efficiently displaced that binding. A 100-fold molar excess of a peptide containing the first 50 amino acids of histone H2A (N50aa) displaced ~60% of the binding of the lamin Dm_0 tail domain to chromosomes (Fig. 7), whereas a 100-fold molar excess of the synthetic peptide containing the first 15 amino-acids of histone H2A (N-peptide) had only a slight effect on that binding (Fig. 7). We concluded that the presence of both N-terminal and C-terminal tail domains of histone H2A are required for its binding to lamin Dm_0.

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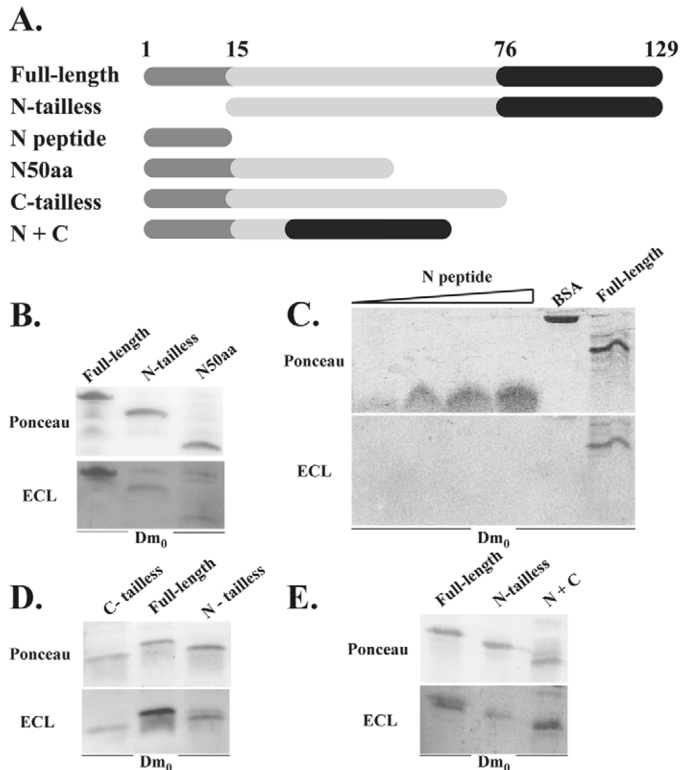

Fig. 5. Blot overlay assay showing that lamin Dm_0 binding to histone H2A requires both the N- and C-tail domains of histone H2A. (A) Schematic view of the different histone H2A-derived polypeptides used in the blot overlay and chromosome binding experiments. (B,D,E) Equal amounts of protein (6 μg) were resolved using SDS-PAGE and transferred to a nitrocellulose membrane. Relative protein content was confirmed by staining the membrane with Ponceau S before incubation with the overlay solution (Ponceau, top panels). Immunostaining was performed with 611A3A6 monoclonal lamin Dm_0 antibody after incubation with overlay buffer containing 12 μg/ml lamin Dm_0 tail domain protein (ECL, bottom panels). Purified proteins and their corresponding overlay signals are indicated above the lanes. (C) Increasing amounts of the synthetic H2A N-tail peptide (N-tail) were used in the blot overlay experiments. BSA was used as a negative control (shown only in C).

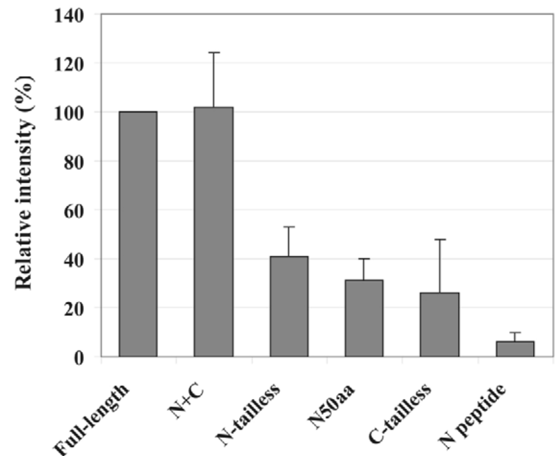

Fig. 6. Relative binding intensity of histone H2A-derived polypeptides to the lamin Dm_0 tail domain. The relative average intensity was calculated from three to six independent blot overlay experiments and was compared to that of the full-length histone H2A. The blot overlays reaction products were visualized with an ECL western blotting detection system and a luminoimage analyzer (LAS-1000 plus). Densitometric quantification was performed using the Science Laboratory 99 Image Gauge software. Bars indicate the standard deviation.

Discussion

The nuclear localization signal and the T/SRAT/S sequences in lamin are required for its binding to chromatin

We mapped two evolutionarily conserved sequences in the lamin Dm_0 tail, the nuclear localization signal (KRKR) and TRAT that are each required for binding to chromosomes. These sequences are included in the previously identified strong chromatin-binding site of the lamin Dm_0 tail domain (Goldberg et al., 1999). Deleting or substituting these sequences had a similar effect on binding as deleting the entire strong binding region in the tail domain of lamin Dm_0. The evolutionarily conserved NLS motif and T/SRAT/S sequence probably play a role in the binding of other lamins to chromatin as well, since, (i) both sequences are present in similar positions in the tail domains of human and *Xenopus* lamins in the region that is required for binding to chromatin (Hoger et al., 1991; Taniura et al., 1995), (ii) the binding of the lamin tail domain to chromosomes is conserved in Ce-lamin and requires the NLS (this study), and (iii) the NLS is required for efficient binding of mammalian lamin C to chromatin (Taniura et al., 1995). We hypothesize that the NLS has a dual role in vivo where it is initially required for lamin entry into the nucleus and later for lamin interaction with chromatin. A dual role for the NLS is not specific for lamins. About 90% of the NLS motifs of DNA-binding proteins overlap with their DNA-binding regions (Cokol et al., 2000). In addition, the NLS of the tumor suppressor menin protein is also required for its binding to the IGFBP-2 promoter in vivo (La et al., 2006), and the NLS of LEDGF/p75 protein is also required for mediating the association of this growth factor with chromatin in vivo (Turlure et al., 2006).

Phosphorylation probably regulates the dynamic interaction between lamin and chromatin

Drosophila interphase lamins are phosphorylated at three sites (Schneider et al., 1999). Ser_{25} is positioned in the head domain, which is not required for the interaction between lamin Dm_0 and chromatin, whereas Ser_{595} is positioned in the weaker binding site (Goldberg et al., 1999). The third site is located between residues 430 and 438 within the strong binding region of lamin Dm_0 to chromosomes (Schneider et al., 1999). It includes both threonine residues of TRAT and is ~20% phosphorylated (Schneider et al., 1999). Our analysis suggests that change in charge of the TRAT sequence reduces the interaction between lamin Dm_0 and chromatin, since substitution of each or both threonine residues to aspartate residues, which mimics the negative charge of phosphorylated threonine residues, significantly decreased the binding. However, since substituting T_{435} with alanine also reduced the binding to chromatin, it is possible that the effect of the

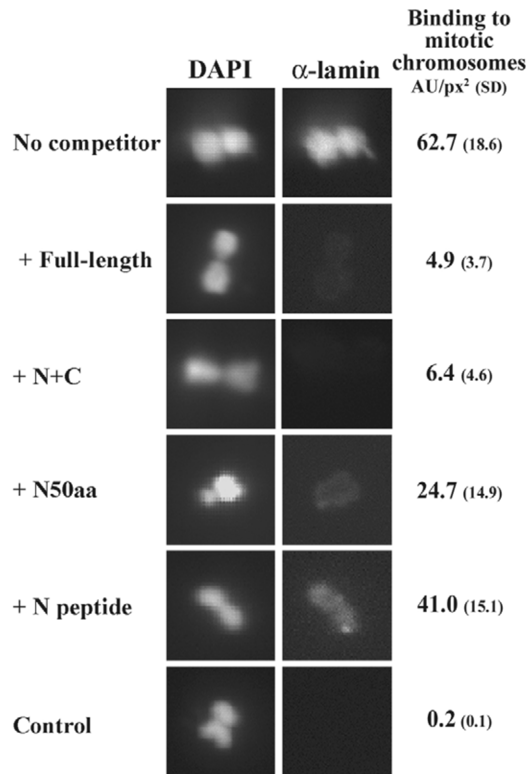

Fig. 7. Chromosome-binding assay showing that lamin Dm_0 binding to histone H2A requires both the N- and C-tail domains of histone H2A. The lamin Dm_0 tail domain (residues 425-622) was used to bind mitotic CHO chromosomes in the presence of 10% BSA with or without 100-fold molar excess of competitor histone H2A-derived proteins. Chromosomes were co-stained with DAPI (left) and with 611A3A6 monoclonal anti lamin Dm_0 antibody as primary antibody and Cy3-conjugated anti-mouse antibodies as secondary antibodies (right). The control did not include the lamin Dm_0 protein. Quantification of the fluorescent signal was performed using the Science Laboratory 99 Image Gauge software and is given as the average fluorescence signal of binding to mitotic chromosomes in arbitrary units/sq. pixel. Data was taken from at least three independent experiments. SD, standard deviation.

aspartate substitution was not specific to change in charge, but rather to the essentiality of this residue. Based on these data, we hypothesize that phosphorylation of either residue 432 or 435 decreases the binding of lamin Dm_0 to chromatin. We further suggest that one role of interphase phosphorylation of the T/SRAT/S motif in lamins is to help regulate the dynamic interaction between lamins and chromatin.

Lamins from *Drosophila* and *C. elegans* bind directly to histone H2A

Our previous studies showed that histones H2A and H2B, but not histones H3 or H4, displaced lamin Dm_0 binding to chromosomes in vitro (Goldberg et al., 1999). Here we show a direct binding of histone H2A to B-type lamins from *Drosophila* and *C. elegans*. The sequences required for that binding are both highly restricted and located in regions plausibly involved in such interactions in vivo. Thus, the lamin sequences are in a linker region between two structured domains, which is likely to be available for interaction in vivo, and this direct binding requires the NLS of lamins, similarly to their binding to chromosomes.

The amino and carboxyl tail domains of histone H2A are each necessary, but not sufficient, for histone H2A binding to lamin Dm_0. Histone H2A is unique among core histones in having its C-terminal tail, in addition to its N-terminal tail, exposed at the nucleosomal surface, thus being more accessible to posttranslational modifications and for binding protein partners, such as lamins (Luger et al., 1997). The histone H2A posttranslational modifications include methylation, acetylation, phosphorylation, ubiquitination, biotinylation and deimination (Aihara et al., 2004; Chew et al., 2006; Foster and Downs, 2005; Hagiwara et al., 2005; Wang et al., 2004; Wyatt et al., 2003; Zhang et al., 2003). Although the functions of most histone H2A modifications are not known, they were suggested to play roles in the epigenetic control of chromatin structure, genomic stability and gene expression (Peterson and Laniel, 2004). It would be interesting to investigate the effect these modifications have on the lamin-histone H2A interactions. Since simultaneous acetylation on the first two lysines of H2A, as well as phosphorylation of the last threonine were found in *Saccharomyces cerivisiae* to play a role in telomere position effect (Wyatt et al., 2003), and since a recent study found that Dm_0 regulates position effect variegation (PEV), which is phenotypically similar to telomere position effect (Bao et al., 2006), it would be particularly interesting to determine whether these histone H2A modifications regulate binding to lamins.

Histone H2A is also unique because of its large number of protein variants, including H2A1, H2A2, H2A-Bdb, H2A.X, H2A.Z, macroH2A1 and macroH2A2. These variants can substitute histone H2A in the histone octamer where they modulate chromatin organization and activity including DNA repair and transcriptional regulation (Jin et al., 2005). The binding efficiency of these histone H2A variants to lamins is currently unknown. Determining the binding affinities of the different H2A variants with lamins should enable better understanding of the role(s) of lamin binding to chromatin.

It is also interesting to note that in addition to their direct binding to histone H2A, lamins can bind core histones indirectly. Young Arrest (YA) binds directly to both lamin Dm_0 and core histone H2B. The mammalian lamin B receptor (LBR) binds the histone H3/H4 tetramer (Polioudaki et al., 2001), and its *Drosophila* homolog binds lamin Dm_0 (Wagner et al., 2004). Additionally, lamins make complexes with many other proteins, which in turn bind chromatin (Gruenbaum et al., 2005).

Possible roles for the lamin-chromatin interactions in chromatin organization, gene regulation and laminopathies

The higher-order chromatin organization maintains the differentiation state and epigenetic inheritance and affects gene expression. Several studies have shown evolutionarily conserved roles for lamins in chromatin condensation and distribution of the heterochromatin at the nuclear periphery. For example, peripheral heterochromatin attachment is abnormal in mouse cells lacking lamins A/C (Sullivan et al., 1999), human cells expressing mutant lamins A/C (Goldman et al., 2004; Mattout et al., 2006), *Drosophila* cells lacking lamin Dm_0 (Guillemin et

al., 2001; Lenz-Bohme et al., 1997) and *C. elegans* cells lacking Ce-lamin (Cohen et al., 2002). The lamin-chromatin interactions are intriguing because of the many ways by which lamins can bind chromatin, either directly or indirectly (Mattout-Drubezki and Gruenbaum, 2003; Pickersgill et al., 2006). They also suggest that lamin-based regulation of higher order chromatin organization is involved in DNA replication, transcription and aging (Mattout et al., 2006). It is tempting to hypothesize that direct interactions between lamins and histones help organize the peripheral heterochromatin.

The presence of histones in chromatin affects chromatin condensation state, binding of transcription factors and promotes basal repression of transcription (Boeger et al., 2003; Boeger et al., 2004; Lenfant et al., 1996; Recht et al., 1996). Thus, lamin-histone H2A interactions could regulate gene expression by interfering with the ability of the nucleosomes to detach from DNA, and thus interfere with chromatin decondensation. Also, the lamin-histone H2A interactions could disturb the equilibrium of histone H2A in and out of nucleosomes, thus stabilizing the nucleosomes at certain chromatin domains and maintaining gene silencing.

Different mutations in the *LMNA* gene cause a wide range of human disorders, collectively termed laminopathies, including progeria syndromes, lipodystrophies, muscular dystrophies, cardiomyopathies, diabetes and neuropathies (Broers et al., 2004; Broers et al., 2006; Worman and Courvalin, 2005). Regulation of gene expression by lamins A/C, either specifically or at the epigenetic level, was suggested as one of the molecular mechanisms leading to laminopathies (Cohen et al., 2001; Gotzmann and Foisner, 2005). Disease-causing mutations were also found in human *LMNB1* (Padiath et al., 2006) and *LMNB2* (Hegele et al., 2006) genes. According to the gene expression model, lamins regulate expression of specific genes and the diseases arise through altered gene expression in specific tissues. Better understanding of lamin functions and why mutations in lamins lead to specific phenotypes including laminopathic diseases, should require further characterization of the interactions between lamins and histones.

Materials and Methods

Antibodies, constructs, and bacterial expression of polypeptides derived from lamin Dm_0, Ce-lamin and histone H2A

Monoclonal antibody against *Drosophila* lamin Dm_0 (611A3A6) and polyclonal antibodies against *C. elegans* lamin (3932) have been described previously (Harel et al., 1989; Tzur et al., 2002). Cy3-conjugated goat anti-mouse and goat anti-rabbit, peroxidase-conjugated goat anti-mouse and goat anti-rabbit antibodies were purchased from Jackson Laboratories. Monoclonal anti-histone H2A antibody was purchased from Upstate (07-146). A synthetic peptide containing residues 1-15 of histone H2A was purchased from Boston Biomolecules, Inc. (Boston, MA) bovine serum albumin (BSA) was purchased from USB (Cleveland, OH).

All lamin Dm_0 tail domain constructs were derived from the T425–622 construct in pET20b(+) (Goldberg et al., 1999). All mutant constructs of lamin Dm_0 were prepared by either PCR mutagenesis followed by insertion, first in the pEGM-T vector (Promega) and then in the pET20b(+) vector (Novagen) between the *Nde*I and *Sal*I sites, or by the QuickChange site-directed mutagenesis method (Stratagene). The Ce-lamin full-length construct in pET20b(+) has been described previously (Fridkin et al., 2004). Ce-lamin tail (residues 399-566) was cloned into the pET28a vector (Novagen). This construct was also used to substitute the amino acids KRRR with AAAA (mNLS). A cDNA clone encoding the full-length *Xenopus* histone H2A (residues 1-129) Has been described previously (Goldberg et al., 1999). This cDNA was used to prepare a series of histone H2A deletion constructs in pET20b(+), including the H2A N-tailless (residues 15-129), H2A N 50 aa (residues 1-50), H2A C-tailless (residues 1-91), and H2A N+C tails (residues 1-32; 91-129) constructs. All constructs were verified by DNA-sequencing. *Escherichia coli* BL21(DE3) pLysS cells were used to express the different lamin Dm_0 and *Xenopus* histone H2A constructs. All constructs were purified to near homogeneity by a one-step affinity chromatography on a His-bind resin column (Qiagen). When used in chromosome binding assay, the concentration of proteins was between 0.5 and 5 mg/ml, and they were dialyzed against TK buffer (50 mM Tris-HCl, pH 7.5, 70 mM KCl, 1 mM β-mercaptoethanol, 2.5 mM benzamidine). Ce-lamin and its derived polypeptides were expressed and purified to near homogeneity as described previously (Fridkin et al., 2004). Bradford Protein Assay (Sigma B6916) was used to determine protein concentration.

Binding of lamin-derived polypeptides to mitotic chromosomes in vitro

Chromosomes were isolated from mitotic Chinese Hamster Ovary (CHO) cells and were used for binding reactions as described previously (Goldberg et al., 1999). Binding reactions were in a total volume of 10 μl of TK buffer containing 0.15 μM or 0.25 μM isolated lamin-derived polypeptides with or without competitor substrates, 5-10% BSA and CHO mitotic chromosomes (0.1–0.3 OD260 units/ml). When different histone H2A and H2A-derived polypeptides were used as competitors with the lamin Dm_0 tail domain, they were added in 100-fold molar excess and incubated for 1 hour at 22°C with lamin Dm_0 tail in the reaction mixture before adding the mitotic chromosomes. After 45 minutes of incubation at 22°C, paraformaldehyde (0.05%) was added to the complete reaction mixture, which was immediately transferred to poly-L-lysine-coated slides and placed in a humidity chamber. After 15 minutes of incubation, the slides were washed three times with PBS followed by fixation for 20 minutes at 22°C with PBS containing 2% paraformaldehyde. The slides were washed twice with PBS and once with PBS containing 0.2% Tween 20 (PBST), each time for 10 minutes, incubated for 50 minutes with PBST containing 10% low fat milk and 1% BSA (PBSTB), and washed twice with PBST, 5 minutes each time. Slides were then incubated for 40 minutes at 30°C or overnight at 4°C with anti-lamin monoclonal 611A3A6 for lamin Dm_0 (Gruenbaum et al., 1988) or with anti-Ce-lamin polyclonal antibodies 3932 in PBST, washed three times with PBSTB, and washed twice for 5 minutes at 22°C with PBST. Slides were then incubated with PBST containing 1 μg/ml Cy3-conjugated anti-rabbit (for polyclonal antibodies) or anti-mouse (for monoclonal antibodies) (Jackson ImmunoResearch) and 1 μg/ml 4′,6′-diamidino-2-phenylindole (DAPI), and incubation proceeded for an additional 30 minutes at 30°C. After two washes with PBST and one wash with PBS, each for 10 minutes, the slides were mounted with coverslips in PBS containing 50% glycerol and 2% *n*-propyl gallate and viewed under a Zeiss Axioplan microscope equipped with an Axiocam CCD camera. Each experiment was repeated at least three times. The data of each experiment were taken using the same gain and exposure values. Quantification of the fluorescent signal was performed using the Science Laboratory 99 Image Gauge software (Fuji Photo Film). The fluorescence intensity of the binding was calculated in arbitrary units/sq. pixel with respect to background levels. Averages of fluorescence intensity derived from at least three independent experiments and the standard deviation were both calculated using the Excel program.

Blot overlay assays

Purified proteins and peptides were resolved on Tris-glycine SDS-PAGE (12% or 15%). The protein concentration was calculated using the Bradford Reagent (Sigma, B6916). Each gel contained equal amounts of proteins as indicated in the figure legends. Tris-Tricine SDS-PAGE was used for low molecular mass proteins or small peptides (Schagger and Von Jagow, 1987). The proteins were transferred to a nitrocellulose membrane (Schleicher and Shuell) and stained with Ponceau S (Sigma). After rinsing the Ponceau S from the membrane, with PBST, the membrane was blocked for 2 hours at 22°C, or overnight at 4°C, in PBST containing 5% low fat milk and washed twice for 2 minutes in PBST. The membrane was then incubated with the desired protein at a concentration of 12 μg/ml in 1% milk in PBST for 2 hours at 22°C, or overnight at 4°C. The membrane was rinsed twice for 2 minutes in PBST, incubated with anti-lamin Dm_0 or anti-Ce-lamin antibodies at a 1:1000 dilution, or with the anti-H2A antibody at 1:3000 dilution for 2 hours at 22°C and rinsed in PBST three times, 5 minutes each time. The membrane was then incubated with a peroxidase-conjugated-anti-mouse or rabbit antibody, respectively, at a 1:10,000 dilution for 1 hour at 22°C. This was followed by three PBST washes, 5 minutes each. Antibody binding was detected with the ECL chemiluminescence system (Amersham Biosciences) and a LuminoImage analyzer (LAS-1000 plus, Fuji Photo Film). The binding intensity was always compared to that of the full-length histone H2A. Each experiment contained BSA as a negative control. Densitometric quantification was performed using the Science Laboratory 99 Image Gauge software (Fuji Photo Film). The area of a single band was selected and the immunoreactivity per area was determined; histone H2A full-length was used in each assay as standard (100%), and the immunoreactivity background was deduced from the obtained values. The average intensity is shown as a percentage and is calculated from at least three independent blot overlay experiments.

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