The highly conserved nuclear lamin Ig-fold binds to PCNA: its role in DNA replication

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Introduction

Nuclear lamins are type V intermediate filament (IF) proteins. Like all IF proteins, lamins have a tripartite structure consisting of non-α-helical N-terminal head and C-terminal tail domains flanking an α-helical central rod domain (Goldman et al., 2002; Gruenbaum et al., 2005). However, unique among IF proteins, lamins contain a C-terminal tail motif, the Ig-fold, known to be involved in protein–protein interactions (Dechat et al., 2000; Zastrow et al., 2006). Although present throughout the nucleus, the nuclear lamins are concentrated in the lamina located at the nucleoplasmic surface of the inner nuclear envelope membrane (Shumaker et al., 2003; Gruenbaum et al., 2005). The lamins play important roles in determining the shape, size, and mechanical properties of the nucleus (Goldman et al., 2004). They are also involved in the disassembly (Dessev et al., 1989) and assembly (Nigg, 1992) of the nucleus during cell division, the assembly of the mitotic spindle matrix (Tsai et al., 2006), transcription, and DNA replication (Spann et al., 1997, 2002; Moir et al., 2000a).

Evidence supporting a role for lamins in DNA replication comes from the finding that lamin B1 (LB1) colocalizes with proliferating cell nuclear antigen (PCNA) at sites of DNA synthesis in late S phase (Moir et al., 1994), and that lamin A (LA)/lamin C (LC) is associated with replication sites in early S phase (Kennedy et al., 2000; Johnson et al., 2004). Furthermore, fibroblasts derived from LA knockout mouse embryos replicate their DNA at a slower rate relative to controls as assayed by nucleotide (BrdU) incorporation. When these embryonic fibroblasts are transiently transfected to express GFP-LA, the cells incorporate BrdU at wild-type levels (Johnson et al., 2004).

Biochemical evidence in support of a role for nuclear lamins in DNA replication has been derived from nuclei assembled in Xenopus laevis egg interphase extracts (Blow and Laskey, 1986). DNA replication is initiated in these extracts ~40 min after the introduction of demembranated sperm chromatin, the time when nuclear envelope assembly is completed (Newport et al., 1990; Goldberg et al., 1995). Importantly, the assembly of nuclei from sperm chromatin requires polymerization of lamin B3 (LB3), the major lamin present in X. laevis eggs (Lourim et al., 1996). This polymerization process involves the head-to-tail...
interaction of lamin dimers that form the higher order structures located in the lamina and nucleoplasm (Heitlinger et al., 1991). Inhibition of LB3 polymerization by an excess of its non-α-helical C terminus (LB3T) prevents both nuclear envelope assembly and DNA replication (Lopez-Soler et al., 2001). Furthermore, deletion analyses of LB3T have demonstrated that the Ig-fold motif within LB3T (LB3T-Ig) is sufficient to block both lamin polymerization and nuclear envelope assembly in these extracts (Shumaker et al., 2005). These results suggest that lamin polymerization, nuclear assembly, and DNA replication are linked in both a temporal and spatial fashion.

Treatment of replicating X. laevis nuclei with either of the dominant-negative mutants, N-terminally deleted human LA or X. laevis LB3, induces a redistribution of LB3 from the lamina into nucleoplasmic foci. Under these conditions, >90% of DNA synthesis is blocked exclusively at the chain elongation phase of replication (Spann et al., 1997; Moir et al., 2000a). Interestingly, these LB3 foci contain the replication elongation factors PCNA and replication factor C (RFC), which are displaced from their normal association with chromatin (Moir et al., 2000a). In contrast, DNA replication initiation factors such as MCM3 and ORC2 remain associated with chromatin, and DNA polymerase α appears to function normally (Spann et al., 1997; Moir et al., 2000a). These results suggest that there may be a specific interaction between PCNA and/or RFC and the nuclear lamins.

PCNA increases the processivity of DNA polymerase δ during the chain elongation phase of DNA replication by >100-fold. In the absence of PCNA, DNA polymerase δ is readily released from DNA and has a reduced affinity for nucleotides during replication (Lee and Hurwitz, 1990). PCNA forms a homotrimeric (Kong et al., 1992) sliding clamp ring structure, which is loaded onto DNA through its interactions with the AAA+ pentameric clamp loading protein RFC (Yao et al., 2003). The ATP-dependent release of RFC from PCNA on DNA allows DNA polymerase δ to associate with the same face of PCNA formerly occupied by RFC. This face contains the inter-domain connecting loop, a sequence of ~12 amino acids (L121–E132) that is the main binding site for proteins that have been shown to interact with PCNA (Tsurimoto, 1999). Two other regions on PCNA are also involved in protein–protein interactions: the central loop (D41–H44) and the C-terminal tail (K254–E256; Tsurimoto, 1999). These sites are situated close to each other and are localized to the same face of PCNA. All known PCNA binding partners have the conserved binding motif, the PCNA-interacting protein (PIP) box (QQX[h]XX[a][a]; where X is any amino acid, [h] is a residue with a moderately hydrophobic sidechain, and [a] is a residue with an aromatic sidechain; Warbrick et al., 1998).

In this study, the role of lamins in DNA replication is more precisely defined. The results demonstrate that the C-terminal non-α-helical domains of all lamins bind directly to PCNA. The binding site lies within the Ig-fold and an excess of either the C-terminal domain containing this motif or the Ig-fold alone inhibits DNA synthesis. Studies of the steps in the assembly of X. laevis nuclei suggest that lamins are involved in the proper positioning of PCNA on chromatin during the early stages of nuclear assembly. Using their known crystal structures, the most likely binding sites between the Ig-fold and PCNA have been determined by molecular docking simulations. Overall, these results reveal that lamins are essential for the steps in assembly and function of the complex required for the chain elongation phase of DNA replication.

Results

The lamin C-terminal domain containing the Ig-fold inhibits DNA replication

We reasoned that the non-α-helical C-terminal domain of nuclear lamins contained the binding site for PCNA, as previous studies showed that N-terminal–deleted LA and X. laevis LB3 retained their association with PCNA in situ (Spann et al., 1997; Moir et al., 2000a) and that the binding sites for several known lamin-associated proteins are located in this domain (Zastrow et al., 2004). To test this possibility, we added excess LB3T to X. laevis nuclei engaged in DNA replication. Nuclei were assembled for 60 min and LB3T was added for an additional 30 min. Subsequently, biotin-11-2′-deoxyuridine-5′-triphosphate (bio-11-dUTP) was added for 10 min and the nuclei were processed for immunofluorescence. Nuclei treated with ~19 μM LB3T showed a reduction in nucleotide incorporation as measured by the fluorescence intensity of Texas red streptavidin (Fig. 1, D–F) relative to controls (Fig. 1, A–C). When nuclei were assembled as above but exposed to bio-11-dUTP for 30 min instead of 10 min, there appeared to be a recovery of DNA replication (unpublished data). This recovery of replication is likely caused by the excess of PCNA and LB3 in the X. laevis extract. Nuclei incubated with LB3T containing the mutation R454W (LB3T RW) displayed a bio-11-dUTP pattern similar to controls (Fig. 1, G–I). This mutation in human LA causes Emery–Dreifuss muscular dystrophy (EDMD; Bonne et al., 1999).

Using a more quantitative assay, we determined the effect of LB3T on DNA synthesis by pulse labeling with [3H]dip-dCTP for 10 min under identical conditions (see the previous paragraph). The addition of 11 μM LB3T decreased DNA replication by ~50 ± 1.0% (n = 3), whereas ~19 μM LB3T caused a decrease of ~92 ± 0.5% (n = 3; Fig. 1 J). The LB3T RW mutant was less efficient in inhibiting DNA replication in X. laevis nuclei showing a reduction of ~50 ± 8.6% relative to controls at a concentration of ~19 μM (Fig. 1 K).

The localization of LB3T and its effect on the normal association of PCNA with chromatin was also examined in X. laevis nuclei 60 min after assembly was initiated. After the addition of His-tagged LB3T for 30 min, the nuclei were prepared for immunofluorescence with antibodies directed against PCNA and His. In controls, most of the PCNA was closely associated with chromatin, which is typical for X. laevis nuclei (Fig. 2, A–H; Spann et al., 1997). When LB3T was added to identical preparations of nuclei, it was incorporated into the lamina region and became associated with chromatin (Fig. 2, I, J, L–N, and P). In these nuclei, there was a substantial decrease in the amount of PCNA associated with chromatin compared with controls (compare Fig. 2, K, L, O, and P with Fig. 2, C, D, G, and H). Overall, the anti-His fluorescence intensity was decreased.
in nuclei treated with LB3T RW appeared to be reduced in comparison with nuclei treated with LB3T (Fig. 2, J, N, R, and V). Furthermore, the nuclei exposed to LB3T RW appeared to have more PCNA associated with chromatin than nuclei treated with LB3T (Fig. 2, K, O, S, and W). These findings suggest that an excess of the lamin C-terminal domain inhibits PCNA binding to chromatin.

Clues regarding the likely lamin binding site for PCNA come from comparing the lamin C-terminal domains (Fig. 3 A). The lamins contain two highly conserved motifs: a classical nuclear localization sequence (NLS; e.g., residues 413–416 in X. laevis LB3, residues 417–420 in human LA/C, and residues 415–418 in human LB1) and the Ig-fold (residues 437–545 in X. laevis LB3, residues 436–544 in human LA/C, and residues 438–545 in human LB1). Because the Ig-fold motif of all lamins is highly conserved and known to be involved in protein–protein interactions (Dhe-Paganon et al., 2002), we reasoned that this domain might be involved in the binding of lamins to PCNA (Fig. 3 A). To test this possibility, X. laevis egg extracts were used to determine whether X. laevis LB3 Ig-fold (LB3T-Ig) could bind PCNA. Bacterially expressed and purified His-tagged LB3T-Ig (Fig. 3 B) was bound to an Ni-agarose column, and X. laevis high-speed ultracentrifuged supernatant (us) was passed over the column for 18 h at 4°C. Bound proteins were eluted with a linear gradient from 0.1 to 1.5 M NaCl. PCNA eluted with a peak at 600 mM NaCl and is shown by SDS-PAGE. This peak fraction contains a band of the appropriate size (37 kD) as detected by Coomassie staining. The presence of PCNA was confirmed by immunoblotting with anti-PCNA (Fig. 3 C). These results suggest that the Ig-fold motif could either directly bind to PCNA or may bind to a component of a complex containing PCNA.

PCNA binds directly to the C-terminal Ig-fold motif of nuclear lamins

It is obvious from Fig. 3 C that the binding of PCNA to the Ig-fold motif could involve other proteins, as multiple bands are seen on the Coomassie-stained gel. To determine whether the Ig-fold could directly bind to PCNA, we performed a solution binding assay. Purified recombinant lamin C-terminal domains were attached to protein S–agarose beads and incubated with purified recombinant human PCNA (Fig. 4, A and B). The bound proteins were subjected to SDS-PAGE and the gels were stained with Coomassie blue (Fig. 4, A and B). Full-length LA was used as a positive control and the Ran-binding domain of importin β (Imp βR) was used as the negative control for the solution binding assay. The results showed that full-length LA binds directly to PCNA (Fig. 4 B, LA and +; note the additional band in the + lane indicated by the arrow). Furthermore, the tail domains of LA (Fig. 4 B, LAT − and +), LCT (LCT − and +), LB1 (LB1T − and +), and X. laevis LB3 (LB3T − and +) also bind to PCNA (Fig. 4 B). The low level of human PCNA binding to X. laevis LB3T in this solution binding assay may be caused by species differences. Immunoblotting using the PCNA antibody demonstrates that the light band (Fig. 4, B and C) is PCNA (not depicted). The S-tagged Imp βR did not

Figure 1. Addition of LB3T to assembled X. laevis nuclei inhibits DNA replication. Nuclei were assembled for 60 min followed by the addition of control buffer (A–C), ~19 μM LB3T (D–F), or ~19 μM LB3TRW (G–I) for 30 min followed by 5 μM bio-11-dUTP for 10 min. Nuclei were fixed, pelleted onto poly-L-lysine–coated coverslips, and prepared for immunofluorescence using Hoechst 33342, rhodamine avidin, and anti-LB3. Control nuclei continued to grow after addition of the buffer (A–C) and incorporated bio-11-dUTP (B). Using the same image capture settings, there was less incorporation of bio-11-dUTP after the addition of LB3T (E), and the nuclei appeared to be the same size as those in buffer controls at 60 min. In contrast, LB3T RW had a lesser effect on incorporation of bio-11-dUTP (H). Bars, 5 μm. (J) X. laevis nuclei were assembled for 60 min and different amounts of LB3T were added for 30 min followed by a 10-min incubation in 2 μC [32P]α-dCTP. The reactions were stopped by the addition of replication sample buffer for 10 min. The samples were run on 0.8% agarose gels and dried under vacuum, and radioactivity was measured. Under these conditions, there was a concentration-dependent inhibition of DNA replication attributable to LB3T. When ~11 μM LB3T was added, there was an ~50% decrease, and with ~19 μM LB3T, replication decreased by ~92%. (K) We also tested the effect of LB3T RW and determined that at a concentration of ~19 μM, DNA replication was reduced by ~30%. Each experiment was repeated three times and the results were averaged.
beads containing S-tagged Imp/H9252R (Fig. 4 F). Under these conditions, PCNA binding to LA-Ig RW was reduced by 28% (n = 5) of the amount bound to wild-type LA-Ig (n = 6) as determined densitometrically (Fig. 4 F, right). To make certain that equivalent amounts of Imp/H9252R, LA-Ig, and LA-Ig RW were transferred to nitrocellulose, the proteins were stained with India ink (Fig. 4 E). This assay demonstrates that LA-Ig binds to PCNA. This interaction is decreased by an EDMD mutation known to destabilize the Ig-fold, which suggests that its structure is important in binding to PCNA.

Because we have shown that the human lamin Ig-fold binds to PCNA, it was of interest to determine the effects of an excess of this domain on DNA replication in human cells. Therefore, HeLa cells were transfected to express a GFP-tagged lamin Ig-fold (GFP-NLS-Ig). After 24 h, BrdU was added to the culture medium for 30 min and the cells were fixed and processed for immunofluorescence. Transfected cells were identified microscopically
by GFP fluorescence, which was detected exclusively in the nucleus. Approximately 36% of the cells expressing GFP-NLS-Ig incorporated BrdU (n = 525) compared with ~51% of those transfected with GFP-LA (n = 352). We observed that the nuclei showing the brightest GFP fluorescence did not incorporate BrdU (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200708155/DC1). These experiments demonstrate that the expression of the lamin Ig-fold domain reduces DNA replication by ~29% (P = 0.0021) and extend our findings in X. laevis nuclei to mammalian cells.

Lamins are associated with chromatin before PCNA during nuclear assembly

To gain further insights into the spatial and temporal interactions between lamins and PCNA, we examined their organizational states during nuclear assembly and the initiation of replication in X. laevis interphase extracts. Demembranated X. laevis sperm chromatin contains LB3 but not PCNA (Fig. 5, A–D). The LB3 present on sperm head chromatin frequently appears as a filamentous structure at the surface of the condensed chromatin (Fig. 5, B, D, and E). The presence of LB3 and the absence of PCNA in these preparations was confirmed by immunoblotting analyses of different numbers of sperm heads (50,000–200,000; Fig. 5 K).

Within 5 min after the initiation of nuclear assembly, the filamentous pattern of LB3 was transformed into small foci and large patches on the surface of decondensing chromatin and throughout the forming nucleoplasm (Fig. 5 G). LB3 was present in the lamina region throughout the ~40-min process of nuclear envelope assembly and as a relatively diffuse “veil” punctuated with LB3 foci in the nucleoplasm (unpublished data; Moir et al., 2000b). Coincident with these changes in lamin distribution and organization, PCNA became associated with both chromatin and PCNA, further supporting a role for lamins in the elongation phase of DNA replication.

After the completion of nuclear envelope assembly, LB3 and PCNA retained their close associations with chromatin as replication was initiated and the nucleus increased in size (Fig. 6, A–I). By 120 min after the initiation of nuclear assembly, the chromatin appeared more threadlike (Fig. 6 J), and its association with LB3 and PCNA was retained throughout the nucleoplasm (Fig. 6, J–M) and in the lamina region (Fig. 6, N–Q). These morphological observations show that lamins are closely associated with both chromatin and PCNA, further supporting a role for lamins in the elongation phase of DNA replication.

Modeling the PCNA-lamin Ig-fold interaction

In an attempt to predict the mode of binding between the LA-Ig domain and PCNA in three dimensions, we have performed molecular docking simulations with the known crystal structures of the LA Ig-fold motif (PDB code 1IFR) and the PCNA trimer (PDB code 1VYM) using two different algorithms available through the web-based services GRAMM-X and ClusPro (Comeau et al., 2004; Tovchigrechko and Vakser, 2006). These simulations suggest two potential binding “modes” (Fig. 7). Using the GRAMM-X software, several top-scoring solutions place the Ig-fold inside the “hole” made by the PCNA homotrimer (Fig. 7 A). In each of these solutions, the center of gravity for LA-Ig remains almost the same, even though the orientation of the Ig-fold motif varies. Using the ClusPro software, LA-Ig is docked at the periphery of the homotrimeric PCNA ring (Fig. 7 B). Importantly, the majority of docking solutions suggested by ClusPro show that the binding site involves portions of the PCNA interdomain connecting loop (residues L121–E132), which is known to serve as a binding site for other proteins involved in DNA replication (Tsurimoto, 1999).

PCNA’s role in DNA replication is thought to begin after RFC binds to and opens the homotrimeric PCNA ring. This complex is then loaded onto primed DNA (Bowman et al., 2004; Yao et al., 2006). The possibility that the PCNA ring is opened is supported by molecular modeling studies (Kazmirski et al., 2005). The “open” ring structure of a sliding clamp appears to have a gap of 4 nm resulting from the opening of one of the three intersubunit interfaces (Millar et al., 2004). This opening...
Discussion

In this study, we demonstrate that the DNA replication elongation factor PCNA binds directly to nuclear lamins in their highly conserved Ig-fold motifs. Our results also show that a structurally stable Ig-fold is necessary for PCNA binding. In previous studies, it has been shown that an excess of the Ig-fold inhibits, in a concentration-dependent fashion, lamin polymerization, sperm chromatin decondensation, and nuclear envelope assembly in X. laevis interphase extracts (Lopez-Soler et al., 2001; Shumaker et al., 2005). The inhibition of nuclear assembly by excess Ig-fold could either be attributable to alterations in the

should be large enough to accommodate the lamin Ig-fold.

Based upon these considerations, we have examined the open ring situation by repeating the docking calculations with PCNA taken as a monomer. In this case, the top docking solution shows the Ig-fold interacting with a side of PCNA that is normally blocked by intersubunit contacts within the homotrimer (Fig. 7 C). This simulation predicts the alignment of two β strands, one derived from the Ig-fold and the other from the PCNA monomer. This results in a continuous β sheet similar to the one present in the intersubunit contact region of the PCNA trimer. This latter model suggests a potential novel regulation of homotrimeric PCNA loading onto primed DNA.
to the length of the non-α-helical lamin tail domains. For example, LAT appears to bind more PCNA than either LCT or LA-Ig (Fig. 4). These differences may be attributable to the variability in the regions downstream of the lamin Ig-fold that may be involved in stabilizing their interactions with PCNA. Our docking analysis suggests several models for lamin Ig-fold interactions with PCNA. To better understand which residues on the Ig-fold motif and PCNA interact, mutational analysis and high-resolution structural studies are clearly needed. The models based on the GRAMM-X software suggest that lamins could bind homo-trimeric PCNA, thereby facilitating RFC assembly onto a PCNA ring. The ClusPro software indicates that a portion of the PCNA interdomain connecting loop (residues 121-132) could bind LA-Ig, which is unexpected for proteins lacking a PIP domain. The proposed interactions between the lamin Ig-fold and PCNA could spatially and temporally regulate the binding of lamin–lamin interactions required for their polymerization (Lopez-Soler et al., 2001; Shumaker et al., 2005) or the competitive inhibition of lamin binding partners such as PCNA (Lopez-Soler et al., 2001; Shumaker et al., 2005). Interestingly, it has been shown that the depletion of PCNA from X. laevis extracts results in the formation of small nuclei with abnormal envelopes that are unable to replicate their DNA (Mattock et al., 2001). Other studies have shown that nuclei assembled in extracts “depleted” of LB3 accumulate PCNA (Jenkins et al., 1993) but are also unable to replicate DNA (Newport et al., 1990; Goldberg et al., 1995). These findings and the biochemical and morphological results of this study suggest that the interaction between lamins and PCNA is necessary early in nuclear assembly.

Unlike other binding partners of PCNA, nuclear lamins do not contain the characteristic PIP motif (see Introduction). Interestingly, the amount of PCNA bound is likely to be related to the length of the non-α-helical lamin tail domains. For example, LAT appears to bind more PCNA than either LCT or LA-Ig (Fig. 4). These differences may be attributable to the variability in the regions downstream of the lamin Ig-fold that may be involved in stabilizing their interactions with PCNA. Our docking analysis suggests several models for lamin Ig-fold interactions with PCNA. To better understand which residues on the Ig-fold motif and PCNA interact, mutational analysis and high-resolution structural studies are clearly needed. The models based on the GRAMM-X software suggest that lamins could bind homo-trimeric PCNA, thereby facilitating RFC assembly onto a PCNA ring. The ClusPro software indicates that a portion of the PCNA interdomain connecting loop (residues 121-132) could bind LA-Ig, which is unexpected for proteins lacking a PIP domain. The proposed interactions between the lamin Ig-fold and PCNA could spatially and temporally regulate the binding of lamin–lamin interactions required for their polymerization (Lopez-Soler et al., 2001; Shumaker et al., 2005) or the competitive inhibition of lamin binding partners such as PCNA (Lopez-Soler et al., 2001; Shumaker et al., 2005). Interestingly, it has been shown that the depletion of PCNA from X. laevis extracts results in the formation of small nuclei with abnormal envelopes that are unable to replicate their DNA (Mattock et al., 2001). Other studies have shown that nuclei assembled in extracts “depleted” of LB3 accumulate PCNA (Jenkins et al., 1993) but are also unable to replicate DNA (Newport et al., 1990; Goldberg et al., 1995). These findings and the biochemical and morphological results of this study suggest that the interaction between lamins and PCNA is necessary early in nuclear assembly.

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During S phase, PCNA foci form, disassemble, and reassemble in the nucleus. The sequence of the organization of these foci has been well documented and the patterns can be used to distinguish early, middle, and late S phase (Kennedy et al., 2000; Leonhardt et al., 2000). Using fluorescence recovery after photobleaching, GFP-PCNA is stably bound within replication foci, as demonstrated by the slow exchange between the nucleoplasm and these foci (Leonhardt et al., 2000; Sporbert et al., 2002). This indicates that homotrimeric PCNA functions as a stable core within a replication complex and is subsequently disassembled and then reassembled at another replicon (Sporbert et al., 2002). However, the mechanisms regulating the disassembly, reassembly, and repositioning of PCNA remain unknown. We believe that nuclear lamin networks are involved in these dynamic properties of replicons by acting as a scaffold or platform upon which PCNA and other components of the replication–elongation complex are assembled and disassembled.

It is conceivable that the Ig-fold motif could inhibit PCNA activity if it binds to PCNA after RFC opens the PCNA homotrimer. This possibility is supported by evidence from studies of gp45, a homologue of PCNA in the bacteriophage T4. In solution, gp45 has been shown to form an open ring structure when one of the three intersubunit interfaces is separated by ~4 nm (Millar et al., 2004). This opening is comparable with the size of the Ig fold, which suggests that the Ig-fold could bind to the open interface.

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Figure 6. LB3 is closely associated with PCNA and chromatin after nuclear envelope assembly. Nuclei were fixed and prepared for immunofluorescence with Hoechst 33342 and antibodies directed against LB3 and PCNA 60 min after assembly was initiated in X. laevis extracts (A–I). There appears to be substantial overlap of DNA, LB3, and PCNA throughout the nucleus and at the nuclear periphery (A–E). The overlay in D shows LB3 and PCNA, whereas E displays DNA, LB3, and PCNA. The area in the box in E was enlarged (6.3x) to show the details of DNA, LB3, PCNA, and the overlay (F–I). We have also examined nuclei after 120 min of assembly both in the nuclear interior (J–M) and at the nuclear periphery (N–Q). These images are approximately the same magnification as in F–I. Frequently, these nuclei have filamentous chromatin (J), which is closely associated with LB3 and PCNA (K–M). The association between these three components is also observed at the nuclear periphery (N–Q). We have used grayscale images of chromatin in A, F, J, and N and blue in the overlays (E, I, M, and Q) for purposes of improved contrast. Bar, 5 μm.
and LB3T RW is most likely attributable to its solubility after the fixation and permeabilization protocols used for immunofluorescence observations.

At the present time, we can only speculate on the precise mechanisms linking the replication machinery to the lamins. The observation that DNA replication is inhibited after the addition of excess lamin tail domain and/or its Ig-fold suggests that these lamin fragments act as competitive inhibitors by preventing the normal interactions between PCNA and chromatin. This idea is supported by our finding that DNA synthesis recovers in in vitro assembled X. laevis nuclei exposed to a known concentration of LB3T for longer periods (~19 μM LB3T up to 60 rather than 30 min). Under these conditions, it is likely that PCNA and wild-type LB3 continue to become imported into assembled nuclei, thereby diluting the amount of LB3T relative to the LB3 present within each nucleus. In this fashion, the inhibitory effect of LB3T on DNA replication could be reversed. The apparent loss of PCNA in X. laevis nuclei exposed to LB3T and LB3T RW is most likely attributable to its solubility after the fixation and permeabilization protocols used for immunofluorescence observations.

It has also been shown that an excess of either LB3T or the Ig-fold inhibits lamin polymerization into the higher-order structures required for normal nuclear assembly (see Fig. 5; Shumaker et al., 2005). When LB3T is added to assembled X. laevis nuclei, it prevents further growth of these nuclei (Shumaker et al., 2005). Here, we show that the addition of LB3T also prevents PCNA from associating with chromatin, thereby inhibiting replication. These findings point to a complex relationship between lamin assembly, nuclear growth, the assembly of PCNA, and the formation of replicons, as well as DNA replication. In addition, we have shown that HeLa cells expressing GFP-NLS-Ig show an ~29% decrease in the number of cells incorporating BrdU. This result extends the findings regarding a role for nuclear lamins in DNA replication from
X. laevis nuclei to mammalian cells. The difference in the level of inhibition of DNA synthesis between X. laevis nuclei exposed to LB3T and mammalian cells expressing GFP-NLS-Ig may be related to the expression levels of GFP-NLS-Ig in individual cells in asynchrony cultures. In support of this, HeLa cells with the highest expression of GFP-NLS-Ig did not incorporate BrdU.

There are >250 allelic variants of the LA gene (LMNA) comprised of ~170 different mutations causing >15 human diseases known as the “laminopathies” (Gruenbaum et al., 2005; Shumaker et al., 2005). The laminopathies display a broad range of phenotypes including muscular dystrophies, cardiomyopathies, lipodystrophies, and premature aging. Of the ~170 LA mutations, ~45 are located in the Ig-fold. Therefore, ~26% of the mutations are located in only 16% of the protein chain. This suggests that the Ig-fold motif is necessary for lamin function and that alterations in either the structure or stability of the lamin Ig-fold motif can lead to disease. In this study, we have examined a point mutation in the Ig-fold that causes EDMD when present in LA. The addition of LB3T containing this mutation, which is located at a highly conserved site, results in significantly less inhibition of DNA replication. Furthermore, the mutant LA Ig-fold binds ~29% less PCNA than the wild type, implying that EDMD as well as numerous other laminopathies could be at least in part explained by alterations in the normal process of DNA replication.

In addition to PCNA, nuclear titin has also been shown to bind to the Ig-fold of lamins (Zastrow et al., 2006). Nuclear titin is required for chromosome condensation and separation during mitosis in Drosophila melanogaster (Machado and Andrew, 2000). Because Ig-folds frequently serve as protein–protein interacting motifs (Dhe-Paganon et al., 2002), a logical conclusion is that other many of the proteins known to bind to the C terminus of the lamins (Zastrow et al., 2004) may interact with the Ig-fold.

In conclusion, nuclear lamins most likely regulate the spatial and temporal arrangement of PCNA during DNA replication. Although details of the structure of lamins within the nucleus are yet to be determined, they are known to polymerize into a variety of higher order structures including short and long head-to-tail arrays of dimers and lattice works of 10-nm filaments (Aebi et al., 1986; Sasse et al., 1997). These structures could provide a nuclear scaffold or platform (Goldman et al., 2002) for organizing PCNA and its other binding partners into DNA replication factories. A logical extension of this hypothesis is that lamins are also involved in the regulation of other processes that require PCNA including DNA repair (Toschi and Bravo, 1988), DNA ligation (Mortusewicz et al., 2006), removal of Okazaki fragments (Li et al., 1995), DNA ligation (Mortusewicz et al., 2000), and that alterations in either the structure or stability of the lamin Ig-fold (Zastrow et al., 2004) may interact with the Ig-fold.

Materials and methods

Nuclear assembly in vitro

X. laevis egg interphase extracts, 200,000 g supernatants [μS], 30,000 g membrane fractions [Sm], and sperm chromatin were prepared essentially as described previously (Newmeyer and Wilson, 1991). Sperm were suspended in 1% vol/vol Triton X-100 in spermin buffer (SB; 80 mM KC1, 15 mM NaCl, 5 mM EDTA, 15 mM Pipes, pH 7.4, 0.2 M sucrose, and 7 mM MgCl₂ instead of 0.05% lysolcellin. The chromatin was pelleted at 2,500 g for 10 min through 20% sucrose in SB, resuspended in 1 ml SB containing 1% BSA wt/vol, and pelleted for 10 min at 2,000 g. In some preparations, the chromatin was preswelled with nucleoplasmin for 5 min with heated cytosol [μS] boiled for 5 min then centrifuged at 16,000 g for 15 min; Newmeyer et al., 1986).

Cell culture

HeLa cells were maintained in DME high glucose, 10% FBS, and penicillin/streptomycin and grown at 37°C in 5% CO₂.

Immunofluorescence and immunoblotting

X. laevis nuclei and sperm chromatin were prepared as previously described (Spann et al., 1997; Shumaker et al., 2005). HeLa cells were also prepared as described previously (Shumaker et al., 2006) and observed at 22°C. Images were taken with either an Axio-Imager Z1 or Axioplan 2 using AxioVision 4.6 (both from Carl Zeiss, Inc.) with multichannel fluorescence and z stack modules. The camera used with the Axio-Imager was the AxioCAM MRm r3 (Carl Zeiss, Inc.). For the Axioplan 2, we used the AxioCam MRm r1.1 camera (Carl Zeiss, Inc.). Some images were also taken with a confocal microscope (LSM 510 Meta) using LSM 3.2 SP2 software (both from Carl Zeiss, Inc.). The objectives used were Plan Achromat 100X NA 1.4 (Carl Zeiss, Inc.). DNA deconvolution was performed using the AxioVision 4.5 deconvolution module (Carl Zeiss, Inc.). Total fluorescence was determined by multiplying the mean nuclear fluorescence intensity by nuclear area. Statistical differences were determined using the Student’s t test or χ² test. Sperm chromatin was suspended in SDS sample buffer (Laemmli, 1970) and immunoblotting was performed as described previously (Shumaker et al., 2006) and quantified by chemiluminescence detection using an ImageStation 440CF and 1D software (Kodak). PCNA binding to LA-Ig and LA-Ig RW was quantified after subtracting the background binding of PCNA to protein S-agarose beads. Figures for plates were prepared in CorelDRAW (Corel Corp.) and image gamma and contrast were adjusted in PhotoPaint (Corel Corp.) and Photoshop (Adobe).

Antibodies

For immunofluorescence, primary antibodies included a mouse mAb directed against X. laevis LB3 (1:1,000, immunofluorescence and immunoblotting; L6-SDS, provided by R. Stick, University of Bremen, Bremen, Germany; Stick, 1988), a rabbit pAb against LB3 (1:1,000; 299), a goat pAb against PCNA (1:50; C-20; Santa Cruz Biotechnology, Inc.), a rat pAb anti-BrdU (1:50; Abcam) and a mouse mAb directed against Hisα (1:200; QIAGEN).

For immunoblotting, primary antibodies were used a mouse mAb (1:1,000; PC10; Santa Cruz Biotechnology, Inc.) and a goat pAb (1:1,000; sc-9857; Santa Cruz Biotechnology, Inc.) directed against PCNA and the rabbit anti-LB3 (1:3,000, 299).

Secondary antibodies used for immunofluorescence were goat anti-rabbit IgG–Alexa Fluor 488 (1:400; Invitrogen), donkey anti–mouse IgG–Alexa Fluor 488 (1:400; Invitrogen), donkey anti–goat IgG–Alexa Fluor 568 (1:400; Invitrogen), goat anti–mouse IgG–Alexa Fluor 568 (1:400; Invitrogen), goat anti–rat Alexa Fluor 568 (1:400; Invitrogen), and rhodamine avidin (1:400; GE Healthcare). For immunoblotting, the secondary antibodies were goat anti–mouse IgG, goat anti–rabbit IgG, and donkey anti–goat IgG conjugated to horseradish peroxidase (1:5,000; Invitrogen).

Protein expression and purification

Expression and purification of His-tagged LB3 Ig-fold motif (LB3Ig, residues 433–540) was described previously (Shumaker et al., 2005). The LA Ig-fold motif (LA-Ig, residues 436–544) was prepared by PCR and inserted into the pET30a vector (EMD). The C-terminal tail domains of LA (LAT, residues 243–664), LC (LCT, residues 243–572), LB1 (LB1T, residues 440–544), and LB3 (LB3T, residues 438–583) were prepared by PCR and inserted into pET30a. The LB3T clone was also mutagenized using the Quick-Change Mutagenesis kit (Stratagene) to convert Arg 453 to Trp (LA-Ig RW). The LB3T and LB3T RW proteins were expressed and purified as described previously (Spann et al., 1997; Lopez-Soler et al., 2001). In some preparations, the chromatin was prepared and inserted into the Ig-fold containing vector. The LB3T was also mutagenized using the Quick-Change Mutagenesis kit (Stratagene) to convert Arg 453 to Trp (LA-Ig RW). The LB3T and LB3T RW proteins were expressed and purified as described previously (Spann et al., 1997; Lopez-Soler et al., 2001). The constructs in the pET 30 vectors were expressed and purified as described previously (Spann et al., 1997; Lopez-Soler et al., 2001). The Ig-fold containing vector was used for the other proteins.
EDTA, 5% glycerol, and 2 mM DTT), and sonicated. The samples were centrifuged at 16,000 g, the expressed proteins were purified on a His-trap column with fast protein liquid chromatography (Äkta), and the fractions containing the protein of interest were further purified on a mono Q column (GE Biosciences) using a linear gradient of 0–750 mM NaCl in 50 mM Tris, pH 8, and 2 mM EDTA.

Affinity chromatography
A Hitrap chelating HP 1-ml column (GE Biosciences) was loaded with NISO, according to the manufacturer’s instructions. Approximately 1.5 mg of purified His-tagged LB3T, LB3Tig protein was added to the column and washed with column buffer (20 mM NaPO₄, pH 7.4, and 0.5 mM NaCl). 15 μg US was diluted to 4 ml in 20 mM NaPO₄, pH 7.4, and 100 mM NaCl and repeatedly passed over the column for 18 h at 4°C using a peristaltic pump (GE Biosciences). Bound proteins were eluted with a fast protein liquid chromatography column with a gradient of 0–1–5 mM NaCl in 20 mM NaPO₄, pH 7.4.

Solution binding assay
Purified LA, LAT, LCT, LA-Ig, LA-Ig RW, and Imp βr (5 μg) were bound to protein S-agarose beads (Emdogene) for 2 h at 22°C in binding buffer (1X PBS, 10% glycerol, 0.1% Tween-20, 50 μM NaPO₄, 50 mM BSA, and 1 mg/mL gelatin) and washed three times with binding buffer followed by the addition of 20 μg of purified PCNA in binding buffer. The samples were rotated for 18 h at 4°C, washed three times in binding buffer, suspended in SDS sample buffer, and separated on an SDS-PAGE gel. The gels were then stained with Coomassie blue G250. PCNA binding to LA-Ig, LA-Ig RW, and Imp βr was determined by densitometry. The amount of PCNA bound to Imp βr was similar to the amount of PCNA bound to protein S-agarose beads alone and was subtracted as background.

DNA replication assay
Nuclei were assembled in X. laevis interphase extracts for 60 min and, in some experiments, lamin protein fragments were added at a concentration of ~10–20 μM for 30 min followed by the addition of 2 μCi [32P]deCTP for 10 min and analyzed as described previously (Spann et al., 1997). The amount of DNA synthesis in each lane was analyzed by fluororescence microscopy as described previously (Spann et al., 1997). Hela cells were transfected with pEGFP-UMNA (Moir et al., 2000b) or pEFG-NLS-MNB1 Ig (SV40 NLS and IB1 residues 434–537) vectors in a Gene Pulser II (Bio-Rad Laboratories) at 160 V and 500 μA, and 24 h later, DNA replication was examined as described previously (Solovei et al., 2004).

Molecular docking analysis
Crystal structures of the human LA-Ig (PDB code 1FR) and human PCNA trimer (1VYM) were downloaded from the Protein Data Bank (http://www.rcsb.org/pdb/home/home.do) and analyzed using two online servers for molecular docking: GRAMMIX (http://vakser.bioinformatics .ku.edu/resources/gramm/grammx) and ClusPro (http://nrc.bu.edu/cluster/) with the DOT docking algorithm. LA-Ig was docked as a rigid body onto the PCNA trimer using default settings. Resulting molecular models were examined using Deepview (GleasonSmithKline and the Swiss Institute of Bioinformatics; Guex and Peitsch, 1997) and the images were prepared using POV-Ray 3.6c (Persistence of Vision Raytracer Pty. Ltd.).

Online supplemental material
Fig. S1 shows that Hela cells expressing GFP-NLS-Ig show a decrease in the incorporation of BrdU. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200708155/DC1.

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