In vitro interaction of the carboxy-terminal domain of lamin A with actin

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Abstract The nuclear lamina formed by lamins is localized between the inner nuclear membrane and chromatin. Lamins are thought to be implicated in the higher order chromatin structure. Interactions of lamins with chromatin have been described but the molecular components directly involved in these interactions remain to be identified. Using a GST-C-terminal domain of lamin A fusion protein to probe cellular extracts for interacting proteins, we have found that this domain of lamin A binds to nuclear actin. This result suggests that an actin-based molecular motor linked to the lamina could be involved in the movement of chromatin domains.

Key words: Lamin; Actin; Protein interaction

1. Introduction

The nuclear lamina is a filamentous meshwork of proteins called lamins underlying the nuclear membrane of all eukaryotic cells (for review, see [1]). Like other intermediate filament proteins, lamins possess three distinct domains: a central α-helical rod flanked by a N-terminal head and a C-terminal tail. Lamins are classified as A type and B type according to their sequence, biochemical properties and subcellular distribution in mitotic cells (for a review see [2]). B-type lamins are constitutively expressed while A-type lamin expression is regulated during development and differentiation ([3] and references therein). In mammalian cells, type A lamins consist of two polypeptides derived from a single gene through alternative splicing [4]. These polypeptides, called lamins A and C, share the first 566 amino acids and differ only by the length of their carboxy-termini, which are 98 and 6 amino acids, respectively [5].

Nuclear lamins were originally observed as a ring at the periphery of the nucleus. Structural studies suggested a close association between the lamina and the chromatin [6] and lamins have been proposed to play a role in higher order chromatin organization [7]. More recently, both types of lamins have been found internally within nuclei in a variety of situations, namely during G1 or S phase of the cell cycle ([8] and references therein), in certain pathological states and when mutated or overexpressed ([9] and references therein). Lamin A is synthesized as a precursor [10] that is cleaved proteolytically during the course of its assembly in the lamina to give rise to a mature protein that is 18 amino acids shorter than its precursor [11]. The physiological role of this precursor, which is predominantly localized in intranuclear foci [12,13], is unknown. Lamin A was also shown to be associated with a diffuse skeleton that extends throughout the interior of human nuclei [14]. This nucleoskeleton, when isolated in 'physiological' conditions, retains replication and transcription activity. As lamin foci were shown to colocalize with sites of nuclear activity such as DNA replication [8], it was speculated that these foci represent common nucleoplasmic domains and that lamins might be involved in their organization [3].

Actin, which is the major constituent of microfilaments, plays an important role in cell shape, movement and structure. Recent studies clearly demonstrated the presence of actin, actin-related proteins and actin binding proteins in nuclei but there is still ambiguity concerning the functional significance of these findings. Among the functions suggested for nuclear actin, it has been proposed that it could be an essential component of an intranuclear motor implicated in the dynamic repositioning of the chromatin domains or in the modification of the structural state of chromatin ([15] and references therein). Moreover, as actin was found associated with the nuclear matrix, it could be implicated with lamins in the organization of the nuclear chromatin domains.

The interaction of A-type lamins with chromatin has been suggested by in vivo [16–20] and biochemical in vitro [16,21–24] studies. Some of these studies have shown that the unique C-terminal domain of lamin A could be implicated in these interactions [16] but the molecular component of chromatin involved has not yet been identified. The major limitation of in vitro studies using lamin molecules is the limited solubility of these proteins under physiological conditions. In this study, we have taken advantage of the increased solubility of the carboxy-terminal domain of lamin A relative to full-length lamin A to examine the binding of this domain to cellular proteins and we have found that this domain binds to nuclear actin.

2. Materials and methods

2.1. Cell culture and ^{35}S protein labeling

HEp-2 cells, human epithelioid carcinoma, and A549 cells, human lung carcinoma, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) at 37°C in a humidified 5% CO_2 atmosphere. Labeling was carried out as follows: cells grown in 100 mm plates, after having been washed twice with PBS, were incubated in methionine-free medium and 4% FBS for 12–16 h and then with methionine-free medium with 0.5% FBS and 150 μCi/ml Trans ^{35}S-label (ICN) for 6–8 h.

2.2. Protein expression

The expression vector pET lam A (NΔ562) encoding a lamin A with its N-terminal 562 residues deleted was constructed as described [12]. The carboxy-terminal deletion of 18 amino acids (CΔ18) starting from Leu^{647} was prepared by inserting a stop codon followed by a *Bam*HI site immediately following the codon for Tyr^{646} [25]. The truncated

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Abbreviations: GST, glutathione *S*-transferase; mAb, monoclonal antibody; NP-40, Nonidet P-40

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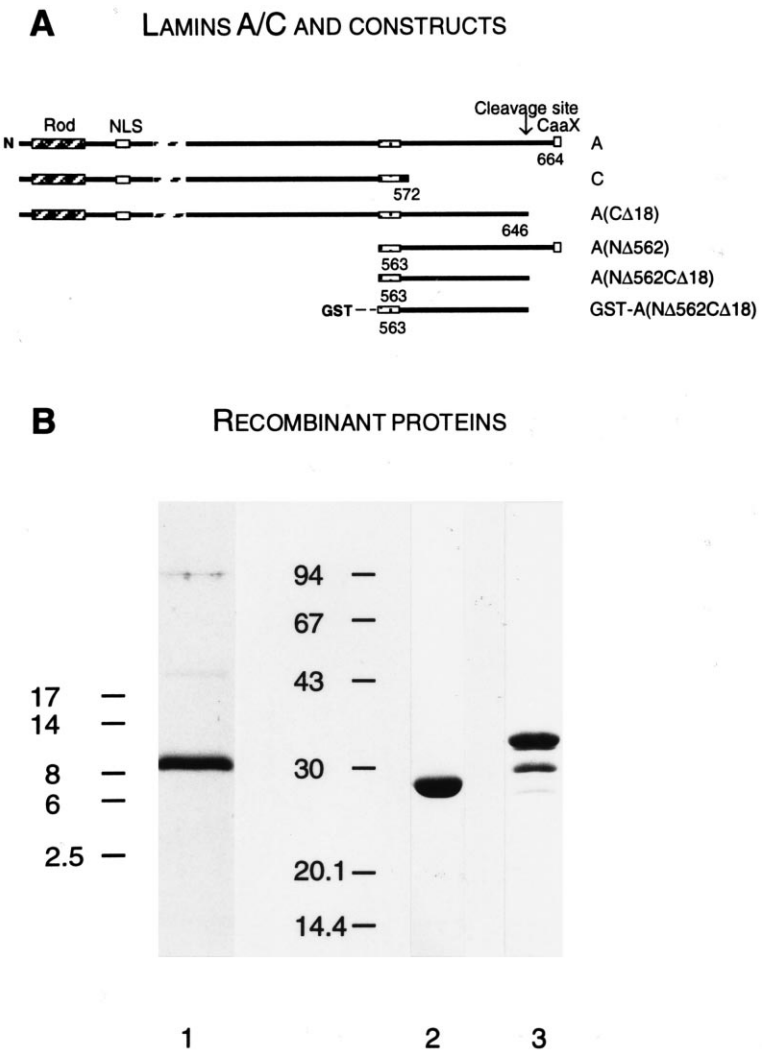

Fig. 1. A: Schematic diagrams of lamin A and C (which is identical to lamin A except for 6 amino acids at the C-terminus) and of lamin A deletion mutants. The diagrams are not drawn to scale, N-termini being shortened to emphasize C-termini. The box at residues 563–566 represents the four-histidine cluster. B: Purified proteins. Lane 1, 5 μg of N-terminal deletion mutant lam A (NΔ562CΔ18) subjected to Tricine SDS-PAGE (10% spacer gel/16.5% polyacrylamide resolving gel) and stained with Coomassie blue. The 19 kDa band reacting in immunoblot with the 133a2 mAb (not shown) probably corresponds to a dimeric form. Lanes 2 and 3, 10 μg of GST and GST-lam A (NΔ562CΔ18) subjected to SDS-PAGE (12% polyacrylamide) and stained with Coomassie blue. The formation of dimers which occurred also with the GST-lam A (NΔ562CΔ18) was abolished by the addition of 1% NP-40. Size (kDa) markers are indicated.

lamin A (NΔ562) and lamin A (NΔ562CΔ18) correspond to the unique carboxy-terminal extension of prelamin A and mature lamin A respectively [25] (see Fig. 1) and overlap the sequence of lamin C only for four amino-terminal histidine residues [5]. Glutathione *S*-transferase (GST) fusion proteins were constructed by the introduction of a *Bam*HI site into the *Nde*I site of the pET vector and the insertion of the *Bam*HI cDNA into the pGEX 2TK vector (Pharmacia). The induction of protein expression in BL21(DE3) *Escherichia coli* (Novagen) was performed by the addition of 0.4–0.7 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to exponentially growing bacteria.

2.3. Protein purification

Lamin A (NΔ562) and lamin A (NΔ562CΔ18) bearing a cluster of six histidine residues at their amino-termini were purified by metal chelate affinity chromatography on pre-packed chelating Sepharose cartridges (Pharmacia) and by electroelution, as described earlier [12]. They were >95% free of bacterial proteins. Fusion proteins were purified by affinity for glutathione Sepharose beads according to Pharmacia Bio-tech's instructions. Proteins were analyzed by SDS-PAGE and Coomassie blue staining. The quantity of proteins produced was determined by comparison to a bovine serum albumin standard using a digital imaging system (IS-1000, α-Innotech). The purity of protein preparations was evaluated by densitometric scanning of a lane containing 10 μg of protein on Coomassie blue-stained gel and by immunoblot analysis to identify degradation products.

2.4. Electrophoresis and immunoblotting

SDS-PAGE was performed as described by Laemmli [26] or using Tricine (Sigma) as the trailing ion for low molecular mass proteins [27]. Conditions for electrophoretic transfer of proteins onto nitrocellulose sheets were as described [12]. An anti-lamin A/C mouse monoclonal antibody (mAb) (clone 1E4 [28]) and an anti-actin mouse mAb (clone C4 from ICN) were used as primary antibodies. Peroxidase-conjugated anti-mouse immunoglobulins were used as secondary antibodies. Detection was performed using chloronaphthol or chemiluminescence (ECL) (Amersham).

2.5. Subcellular fractionation

Cell cultures were trypsinized, washed twice with PBS and resuspended in 10 mM Tris-HCl pH 7.2, 10 mM NaCl, 5 mM KCl, 3 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 5 μg/ml leupeptin (buffer A). When cells were fractionated, the nuclei were

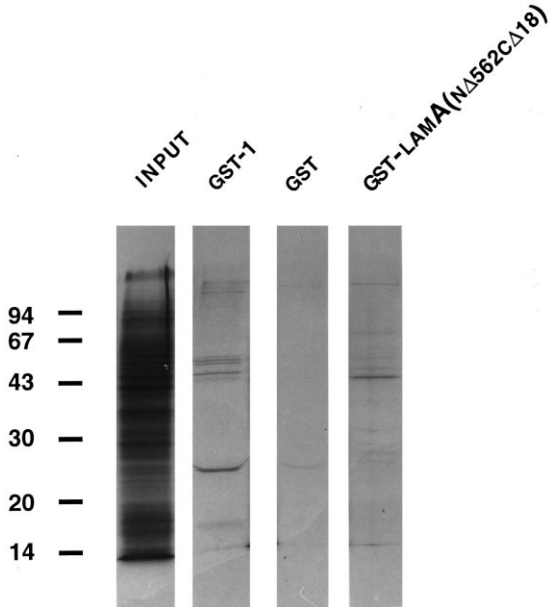

Fig. 2. Interaction of HEp-2 cellular proteins with GST and GST-lam A (NΔ562CΔ18). The ^{35}S-labeled bound proteins were subjected to SDS-PAGE (12% polyacrylamide) and analyzed by autoradiography. GST-1 lane, proteins that were cleared by the first interaction with GST beads; GST lane, proteins that still interacted with GST alone; GST-lam A (NΔ562CΔ18) lane, proteins that interacted with GST-lam A (NΔ562CΔ18).

isolated according to the procedure described by Bridger et al. [29]. Briefly, cells were incubated in buffer A for 10 min on ice and then nuclei were released from the cells by homogenization, using a tight-fitting pestle in a Dounce homogenizer. When more than 90% of

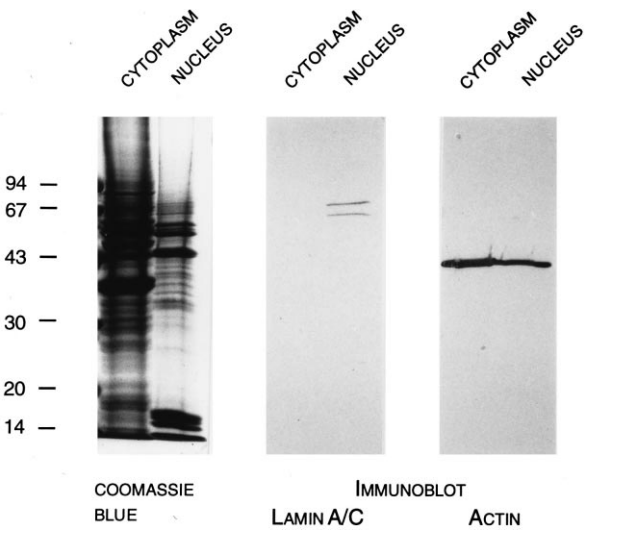

Fig. 3. Subcellular fractionation. Cytoplasm or nucleus of 5×10^5 A549 cells iis shown. Proteins were stained with Coomassie blue (right) or transferred to nitrocellulose (left). Immunoblots were reacted with either anti-lamin A/C antibody or anti-actin antibody and revealed by chloronaphthol.

nuclei were free of cytoplasm, as evaluated by phase contrast microscopy, they were pelleted by centrifugation at $1000\times g$ for 10 min. After removal of supernatant, the nuclei were delicately resuspended in buffer A and centrifuged through a 30% (w/v) sucrose cushion.

2.6. *Detection of proteins interacting with the C-terminal domain of lamin A*

For the blot binding assay, proteins were separated by SDS-PAGE

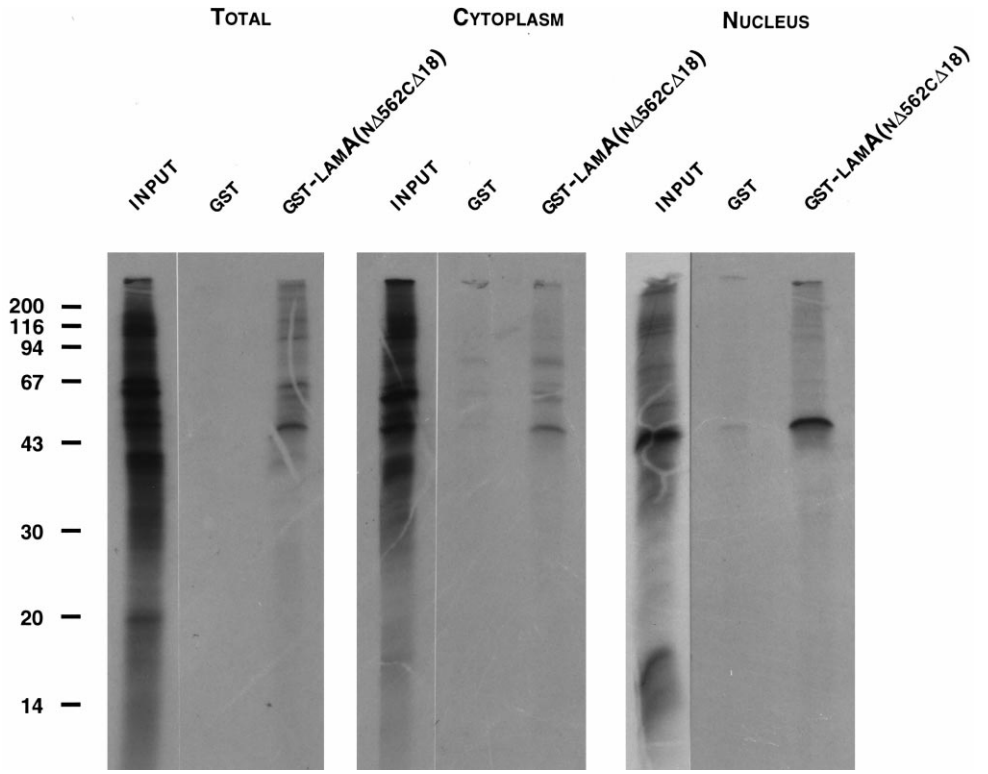

Fig. 4. Interaction of proteins from subcellular fractions of A549 cells with GST and GST-lam A (NΔ562CΔ18). The ^{35}S-labeled proteins bound to GST or GST-lam A (NΔ562CΔ18) were subjected to SDS-PAGE (12% polyacrylamide) and analyzed by autoradiography.

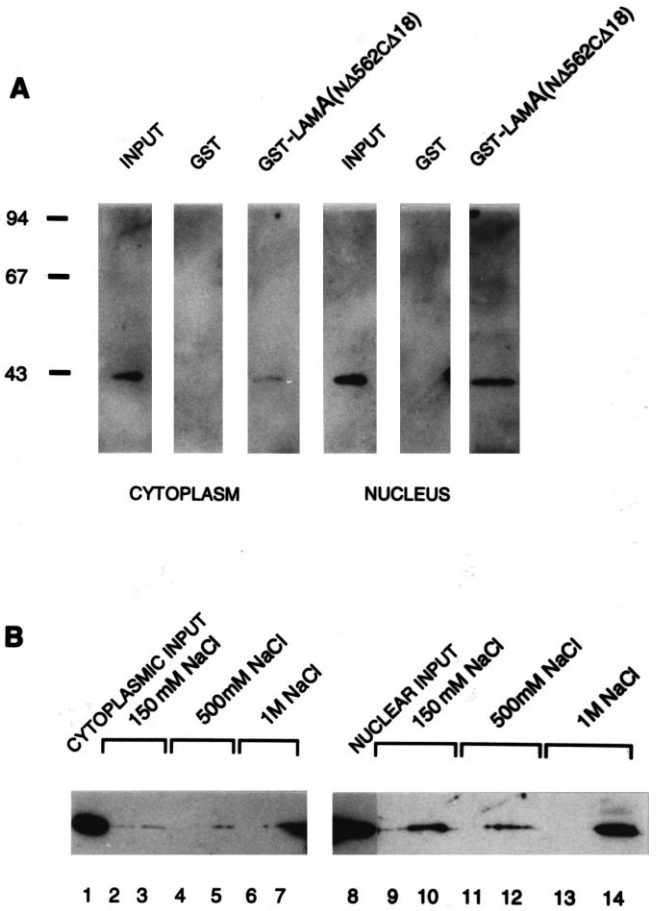

Fig. 5. A: Identification of the 43 kDa protein interacting with GST-lam A (NΔ562CΔ18) as actin. Unlabeled lysates were incubated with either GST or GST-lam A (NΔ562CΔ18) proteins as described in Section 2 and the interacting proteins were transferred to nitrocellulose and then probed with anti-actin mAb. Immunoblots were revealed by chemiluminescence (ECL). B: The actin-GST-lam A (NΔ562CΔ18) interaction is resistant to high ionic strength. Incubations with either GST (lanes 2,4,6,9,11,13) or GST-lam A (NΔ562CΔ18) (lanes 3,5,7,10,12,14) were done with cytoplasmic extract (lanes 1–7) and nuclear extract (lanes 8–14) in the presence of 0.15 M (lanes 2,3,9,10), 0.5 M (4,5,11,12) or 1 M (6,7,13,14) NaCl.

and transferred onto nitrocellulose as described [12]. Membranes were blocked with 2% casein (w/v) in 10 mM Tris-HCl pH 7.4, 150 mM NaCl for 1 h at 37°C and then incubated with lamin A (NΔ562CΔ18) in the same buffer at a concentration of 25 μg/ml for 1 h at 37°C and then at 4°C overnight. The membranes were then washed in Tris-NaCl and the binding of the lamin A (NΔ562CΔ18) was detected with the 133A2 mAb specific for lamin A as described [14]. Interaction of fusion proteins with cellular fractions was based on the method described by Antinore [30]. Total, cytoplasmic or nuclear extracts in buffer A were brought to a final concentration of 150 mM NaCl, 1% Nonidet P-40 (NP-40) and 10 mM dithiothreitol (DTT). All extracts were sonicated and then centrifuged for 10 min at $12\,000\times g$. Standard binding reaction conditions were as follows: 100 μl of supernatant (corresponding to 2.5×10^5 cells for total extract, 5.0×10^5 nuclei or cytoplasmic extract of 3.5×10^5 cells) were incubated with 40 μg of GST alone bound to the Sepharose beads with rotation on a Labquake apparatus at 4°C. After 2 h, beads were pelleted at $500\times g$ and the lysate was cleared again with GST. After this second 2 h clearing, beads were pelleted and the lysate was divided into two parts. One half was added to 20 μg of GST alone with beads and the second half was added to 20 μg of the fusion protein GST-lam A (NΔ562CΔ18) with beads. After overnight incubation with rotation on a Labquake apparatus at 4°C, beads were pelleted and washed three times with Tris-NaCl/NP-40 and three times with Tris-NaCl prior to analysis by SDS-PAGE. When indicated the overnight incubation was performed in a final concentration of 0.5 or 1.0 M NaCl. For immunoblots, unlabeled lysates were incubated with the fusion proteins as described above.

3. Results and discussion

To study interactions between the C-terminal domain of lamin A and chromatin, we took advantage of the increased solubility of this domain of lamin A. As illustrated in Fig. 1A, the N-terminal deletion produces a protein free of the α-helical rod domain which normally allows lamins to form multimeric complexes. The purified truncated protein (NΔ562CΔ18) (Fig. 1B, lane 1) exhibited in physiological buffer (Tris-NaCl) a workable solubility, more than 80% of the protein being found in the supernatant after a 10 min spin in a microfuge (at 1 μg/μl). A blot binding assay was first used to probe extracts from HEp-2 cells with lamin A (NΔ562CΔ18). A number of reactive polypeptides were detected, ranging from 12 to 200 kDa, including a ~43 kDa peptide (data not shown).

After those preliminary studies, we used the GST fusion protein system to detect proteins interacting with the C-terminal domain of lamin A in a more physiological binding assay. As can be seen in Fig. 1B, the purified GST-lam A (NΔ562CΔ18) contained, as identified by immunoblotting (not shown), two degradation products of 30 and 26 kDa (lane 3) whereas the unfused GST protein was undegraded (lane 2). ^{35}S-Labeled HEp-2 cell extracts were prepared,

cleared with GST protein alone to remove non-specific interactions (Fig. 2, lane 2) and then incubated with GST alone (Fig. 2, lane 3) or GST-lam A (NΔ562CΔ18) (Fig. 2, lane 4). This first series of experiments allowed the detection of a number of proteins interacting specifically with GST-lam A (NΔ562CΔ18) (lane 4) and not with GST alone (lane 3). The clearest band was observed at ~43 kDa.

The next question that we addressed was the cellular localization of the interacting proteins. If implicated in the interphase function of lamin A, such proteins should be localized in the nucleus. On the other hand, cytoplasmic proteins interacting with lamin A during mitosis could play a role in nuclear reassembly. Unfortunately, the cellular fractionation described in Section 2 did not give satisfactory results with HEp-2 cells. Among the other cell lines tested, we chose the A549 cells because good fractionation was achieved reproducibly. As seen on the Coomassie blue-stained gel of Fig. 3, we obtained typical cytoplasmic and nuclear fractions. The immunoblot presented in Fig. 3 shows that lamins are not degraded during the cell fractionation and that actin is found in both the cytoplasmic and the nuclear fraction, as other have observed before ([15] and references therein). ^{35}S-Labeled A549 cells were fractionated and used as described above to identify proteins interacting with GST-lam A (NΔ562CΔ18). Typical results of those experiments are shown in Fig. 4. As extracts were first cleared with GST alone, no abundant bands were seen in the control lane with GST in either the total cell extract or the two subcellular fractions. As in the first series of experiments, the clearest interaction of the fusion protein is with a protein of an apparent MW of 43 kDa and the interaction is more prominent in the nuclear fraction. As lamin A and actin have both been suggested to be implicated in higher order chromatin organization, we tested if the 43 kDa interacting protein was actin. Unlabeled cellular fractions were incubated with the fusion proteins and bound proteins were subjected to SDS-PAGE and transferred onto nitrocellulose. Immunoblots were probed with an anti-actin mAb and visualized by chemiluminescence (ECL) (Fig. 5A). This experiment identified actin as a protein interacting with the C-terminal domain of lamin A fused to GST and not with GST alone. In those experiments, nuclear actin seems to bind to lamin A more than the cytoplasmic actin. This could be due to actin isoform differences between these two cellular compartments [31]. However, as these binding assays were not quantitative, this suggestion needs further experimentation. The stability of the lamin-actin interaction was tested by the addition of NaCl up to 1 M for the overnight incubation step. As shown in Fig. 5B lanes 7 and 14, the interaction between these two proteins resists this salt concentration.

The results presented here suggest a tight interaction between lamin A and actin, two important elements of the cytoskeleton. However, we cannot exclude from these experiments that the interaction between lamin and actin is indirect, involving either a third partner or a multiprotein complex. In a recent study, the presence of myosin I and actin in the nucleus was clearly demonstrated and the authors argued positively for the presence of molecular motors in the nucleus [32]. Others have provided evidence that actin [33] and lamin A [29] colocalized with condensed chromatin. An actin-based motor link to the lamina and lamina-associated proteins [34] could form a dynamic network which, by performing structural changes in chromatin, could be involved in the transduction of messages from the cytoskeleton to various genes. In vivo experiments must be done to confirm this hypothesis and to identify the other components of this chromatin/nuclear matrix pathway of signal transmission.

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