The nucleoplasmic protein, Lamina-associated polypeptide (LAP) 2α, is one of six alternatively spliced products of the LAP2gene, which share a common N-terminal region. In contrast to the other isoforms, which also share most of their C termini, LAP2α has a large unique C-terminal region that contains binding sites for chromatin, A-type lamins, and retinoblastoma protein. By immunoprecipitation analyses of LAP2α complexes from cells expressing differently tagged LAP2α proteins and fragments, we demonstrate that LAP2α forms higher order structures containing multiple LAP2α molecules in vivo and that complex formation is mediated by the C terminus. Solid phase binding assays using recombinant and in vitro translated LAP2α fragments showed direct interactions of LAP2α C termini. Cross-linking of LAP2α complexes and multangle light scattering of purified LAP2α revealed the existence of stable homo-trimers in vivo and in vitro. Finally, we show that, in contrast to the LAP2α-lamin A interaction, its self-association is not affected by a disease-linked single point mutation in the LAP2α C terminus.

The nuclear envelope comprises the inner and outer nuclear membranes, the nucleo pore complexes, and the nuclear lamina, which underlies the inner nuclear membrane (1, 2). The nuclear lamina is the major structural framework in the nucleus of multicellular eukaryotes and is composed of a filamentous meshwork of type V intermediate filament proteins, the lamins. B-type lamins, encoded by two human genes (LMNB1 and LMNB2), are essential for cell viability. In contrast, the four A-type lamins (A, C, C2, and Δ10), representing splicing isoforms of the LMNA gene, are dispensable for viability of individual cells but have crucial functions in tissue organization after birth (3, 4).

In addition to the lamins, the nuclear lamina contains a number of integral membrane proteins of the inner nuclear membrane, the best characterized of which are the Lamin B receptor, Lamina-associated polypeptide (LAP), and the three LEM domain-containing proteins LAP2β, emerin and MAN1 (5, 6). All these proteins interact with lamin A/C and/or B and contribute to anchorage of the nuclear membrane to the lamina. The LEM domain, a conserved 40-amino-acid motif located near the N terminus of the LEM family proteins, interacts with the DNA-binding protein barrier-to-autointegration factor (BAF) and mediates the binding of these proteins to chromatin (7). In LAP2 proteins, a LEM-like segment at the very N terminus has been shown to interact with DNA directly (8).

The family of LAP2 proteins includes six alternatively spliced isoforms derived from the same gene (9). Most LAP2 isoforms are closely related structurally and functionally and are localized to the inner nuclear membrane, such as LAP2β. In contrast, LAP2α shares only the N-terminal 187 amino acids with the other isoforms, including the LEM and LEM-like domains, but otherwise possesses a unique 56-amino-acid C-terminal region without a transmembrane domain (see Fig. 1A), encoded by one large exon found only in mammals (10).

LAP2α is exclusively located in the nucleoplasm in interphase and interacts with lamin A/C (11) and hypophosphorylated retinoblastoma protein (pRb) via distinct C-terminal domains (12, 13). The LAP2α-lamin A/C-pRb complex is thought to regulate cell proliferation and differentiation in adult stem cells (3, 12). During mitosis, LAP2α dissociates from chromosomes in a phosphorylation-dependent manner and is redistributed throughout the mitotic cytoplasm, like most nuclear lamina components (14, 15). However, during anaphase, LAP2α associates with the telomeres of separated sister chromatids and subsequently forms stable structures associated with decondensing chromatin before the nuclear envelope is formed (14). Although LAP2α can interact with BAF and DNA via its common LEM and LEM-like motifs, its C

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4 The abbreviations used are: LAP, Lamina-associated polypeptide; BAF, barrier-to-autointegration factor; DCM, dilated cardiomyopathy; LEM, Lamina-associated, polypeptide2-emerin-MAN1; pRb, retinoblastoma; SEC-MALS, size exclusion chromatography combined with multilangle light scattering; GFP, green fluorescent protein; DTT, dithiothreitol; DSP, dithio-bis-(succinimidylpropionate); RFP, red fluorescent protein; mRFP, monomeric RFP.
terminus was shown to be essential and sufficient for chromatin association during mitosis (16, 17). Intriguingly, a mutation causing an amino acid substitution (Arg-690 to Cys) near the C terminus of LAP2α has been associated with dilated cardiomyopathy (DCM) (18), a condition also known to be caused by mutations in the LMNA gene (5). The mutation altered the observed LAP2α interaction with A-type lamins in vitro and may represent a rare cause of DCM.

In this study, we show that LAP2α is engaged in homo-oligomerization via its unique C-terminal domain and forms stable trimers in vivo and in vitro. These homo-trimers may be the building blocks of higher order structures containing LAP2α and other proteins.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Mouse monoclonal anti-LAP2 (6E10) and rabbit polyclonal anti-Myc antibodies were obtained from Abcam. Rabbit anti-LAP2α antiserum and monoclonal anti-LAP2α antibody 15/2 and 12 were described (16, 17). Polyclonal anti-GFP antibodies were from Clontech; polyclonal anti-DsRed antibody was from Roche Diagnostics; protein G-agarose conjugate was from Sigma.

**Vector Construction and Expression of Recombinant Proteins**—cDNA coding for monomeric red fluorescent protein 1 (mRFP1) was amplified from pRSETB-mRFP1 (a kind gift of Dr. R. Tsien, Howard Hughes Medical Institute, University of California, San Diego, CA) and inserted into the vector pCI (Promega). The resulting plasmid was used to create N-terminally tagged mRFP1-LAP2α and mRFP1-LAP2α-(188–693) by ligating amplified fragments of LAP2α in-frame with mRFP1. mRFP1-LAP2α and mRFP1-LAP2α-(188–693) were then excised and ligated blunt into the EcoRV site of eukaryotic expression vector pEF-puro.PL3 (19). Plasmids pEF.mRFP1-LAP2α and pEF.mRFP1-LAP2α-(188–693) were transfected into HeLa cells using Lipofectamine (Invitrogen), and clones were selected in 2 μg/ml puromycin.

For the construction of PC-Myc-mRFP1-LAP2α-(188–693) and PC-Myc-LAP2α-(188–693), a sequence coding for protein C epitope EDQVDPRLIDGK fused to the Myc-tag EKLISEEDL was inserted into the EcoRV site of pEF-puro.PL3. The resulting plasmid was linearized with EcoRV and ligated in-frame with mRFP1-LAP2α-(188–693) or LAP2α-(188–693).

For GFP-LAP2α carrying the DCM mutation, vector gAG43 was constructed by shuttling wild-type LAP2α cDNAs from gAG41 (18) into GFP destination vector pcDNA-DEST53 by the LR reaction (Invitrogen). The bacterial expression vectors encoding His-tagged LAP2α fragments are described elsewhere (16).

For bacterial expression, proteins were expressed in *Escherichia coli* strain BL21(DE3) using the inducible T7 RNA polymerase-dependent pET vector system as described previously (11, 16). Protein expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside for 3 h. Bacteria were harvested by centrifugation at 4,000 rpm for 5 min (Heraeus Megafuge, 1.0R) and lysed in one-tenth of the original culture volume of Tris buffer (20 mM Tris-HCl, pH 8, 500 mM NaCl, 5 mM imidazole, 1 mM dithiothreitol, protease inhibitors) by freezing and thawing and the addition of 0.1 mg/ml lysozyme, 0.1% Triton X-100, 10 mM MgCl₂, 50 μg/ml DNase, and 20 μg/ml RNase. Following a 30-min incubation at 30 °C, the samples were centrifuged for 10 min at 14,000 rpm, and pellets were resuspended in one-tenth of the original culture volume of Tris buffer plus 7 M urea and incubated for 1 h at room temperature. Cell lysates were centrifuged at 45,000 rpm for 30 min, and supernatants were stored as aliquots at −20 °C. If fragments were soluble, urea was added directly to the cell extract prior to centrifugation at 14,000 rpm.

Renaturation of recombinant proteins was achieved by dialyzing twice against KHM buffer (78 mM KCl, 50 mM HEPES, pH 7.4, 8.4 mM CaCl₂, 10 mM EGTA, 4 mM MgCl₂, 1 mM dithiothreitol) and cleared by centrifugation at 4,000 rpm for 5 min. For light scattering, full-length LAP2α was expressed in *E. coli* strain Rosetta 2 (DE3) (Novagen) grown in Luria-Bertani medium containing 100 mg/liter ampicillin at 30 °C. Expression was induced at an A₆₀₀ of 0.6 by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside. Cultures were further grown at 30 °C for 20 h. Cells were harvested by centrifugation at 4,500 g and 4 °C. The bacterial pellet was resuspended in 100 mM potassium phosphate, pH 8.0, 100 mM NaCl, 1 mM dithiothreitol (DTT), containing 50 μg/ml DNase and a protease inhibitor mixture (Roche Applied Science) and sonicated. The homogenate was clarified by centrifugation and filtering, and the resulting supernatant was applied to a Ni²⁺-chelating HiTrap column (GE Healthcare) equilibrated in lysis buffer. Upon serial column washes of buffer supplemented with 50 mM imidazole, the protein was eluted in 300 mM imidazole. The eluant was subjected to gel filtration in buffer supplemented with 10 mM DTT (Superdex 200, HiLoad 16/60 PG; GE Healthcare). The protein so obtained was pure according to SDS-PAGE.

**Immunoblotting**—Cell extracts in 20 mM Tris-HCl, pH 8.0, 130 mM NaCl, 1% Triton X-100, and protease inhibitor mixture were centrifuged at 13,000 × g for 10 min, and pellet and supernatant fractions were analyzed on 10% polyacrylamide gels and electrotransferred to nitrocellulose (Schleicher & Schuell). Blots were blocked for 60 min in Tris-buffered saline, pH 8.0, 0.1% bovine serum albumin, and 5% nonfat dry milk, incubated with primary antibody in Tris-buffered saline, 0.1% bovine serum albumin for 60 min and with anti-mouse (Sigma) or anti-rabbit (DAKO) alkaline phosphatase-conjugate, and visualized using BM purple AP substrate, (Roche Diagnostics). Semi-native gels contained SDS only in the running buffer (0.1% SDS); no SDS was present in the sample buffer or the polyacrylamide gels themselves.

**Immunoprecipitations**—Confluent cell monolayers in 10-cm dishes were lysed in TNCT (20 μl Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Ca²⁺, 1% Triton X-100, and protease inhibitors) and centrifuged for 10 min at 13,000 × g. Supernatants were incubated with 25 μl of anti-protein C matrix for 1 h. Bound complexes were washed four times with TNCT and eluted by boiling in SDS-PAGE sample buffer. Samples were processed for immunoblotting. Alternatively, supernatants were divided in two parts; one half was incubated on ice with monoclonal anti-Myc (2 μg) or monoclonal anti-LAP2 (15 μg) antibody, and the other half was incubated with a control monoclonal antibody (mouse monoclonal anti-HRV2, a kind gift of Dr. Blaas, Max
Perutz Laboratories, Vienna, Austria) for 30 min. Immunocomplexes were bound to 12.5 μl of protein G-agarose for 1 h, washed with TNCT, and eluted in sample buffer.

**Immunofluorescence Microscopy**—Cells on coverslips were fixed with 3% paraformaldehyde in phosphate-buffered saline and quenched in 50 mM NH₄Cl. They were permeabilized in 0.1% Triton X-100, blocked in 5% fetal calf serum, and incubated with monoclonal anti-Myc antibody at 1 μg/ml in phosphate-buffered saline containing 1% fetal calf serum. The secondary antibody was anti-mouse Alexa Fluor 488 (Molecular Probes). Preparations were mounted using Citifluor AP1 (PLANO) and examined with a Nikon Eclipse 800 fluorescent microscope.

**Chemical Cross-linking**—HeLa cells were resuspended in KHM buffer and homogenized on ice by pressing the suspension 10–15 times through a metal ball cracker (European Molecular Biology Laboratory (EMBL), Heidelberg, Germany). Cell lysates and dialyzed recombinant proteins were mixed with various concentrations of cross-linking agent dithiobis(succinimidylpropionate) (DSP, Pierce) for 2 h on ice, and the reaction was stopped by quenching free active groups with 50 mM Tris-HCl, pH 6.8, for 1 h on ice. Samples were analyzed by SDS-PAGE in the presence or absence of 100 mM DTT.

**Blot Overlay Assays**—Recombinant polypeptides were resolved by SDS-PAGE and blotted to nitrocellulose membranes as described (11). Membranes were stained with Ponceau S, washed with phosphate-buffered saline containing 0.05% Tween 20, and incubated in overlay buffer (10 mM Hepes, pH 7.4, 100 mM NaCl, 5 mM MgCl₂, 2 mM EGTA, 0.1% Triton X-100, 1 mM DTT) for 1 h. After blocking with 2% bovine serum albumin in overlay buffer, membranes were probed 3 h with [³⁵S]methionine-labeled polypeptides diluted 1:50 in overlay buffer plus 1% bovine serum albumin. Membranes were washed extensively with overlay buffer, and bound proteins were detected by autoradiography.

**In Vitro Transcription/Translation and GFP Immunoprecipitation**—150 μl of protein A-Sepharose were coupled overnight with 6 μl of polyclonal anti-GFP antibody and 150 μl of protein G-Sepharose with 1 ml of monoclonal anti-LAP2 antibody 12. ³⁵S-labeled wild-type or mutated LAP2α was expressed by in vitro transcription/translation using the TNT quick-coupled transcription/translation reaction mix (Promega) either alone or together with GFP-tagged wild-type LAP2α (gAG49, gAG49 and gAG43, gAG50, gAG50 and gAG43). After incubation at 30 °C for 3 h, binding buffer (50 mM HEPES, pH 7.4, 50 mM NaCl, 5 mM MgCl₂, 1 mM EGTA, 0.1% Triton, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride) was added to 225 μl. For preclearing, samples were incubated with 20 μl of protein A-Sepharose or protein G-Sepharose for 15 min and centrifuged at 1,000 rpm for 3 min. Supernatants were incubated with antibody-coupled beads (see above) for 2 h at 4 °C, and beads were centrifuged through a 30% sucrose cushion. Supernatants and beads were mixed with SDS sample buffer and analyzed by gel electrophoresis and autoradiography.

**Size Exclusion Chromatography Combined with Multichannel Light Scattering (SEC-MALS)**—The oligomeric state of LAP2α in solution was determined by SEC-MALS measurements performed on an ÄKTA explorer 10 system (GE Healthcare) connected to a tri-angle light scattering detector and a differential refractometer (miniDAWN Tristar and Optilab, respectively; Wyatt Technology). A Superdex 200 10/300 GL column (GE Healthcare) was equilibrated in 100 mM potassium phosphate, pH 8.0, 100 mM NaCl, 10 mM DTT at a flow rate of 0.5 ml/min. A sample volume of 100 μl was injected at a concentration of 1 mg/ml. Data were processed using Astra software (Wyatt Technology) assuming a specific refractive index increment (dn/dc) of 0.185 ml/g. To determine the detector delay volumes and the normalization coefficients for the MALS detector, a bovine serum albumin sample (Pierce) was used as reference. Neither despiking nor band broadening correction was applied.

**RESULTS**

**The C Terminus of LAP2α Is Involved in the Formation of Oligomeric Complexes in Vivo**—Our previous studies revealed the formation of stable, chromatin-associated LAP2α structures during early nuclear assembly stages (14, 17), indicating oligomerization of the protein. To analyze oligomeric LAP2α structures in vivo and to determine the domain of LAP2α responsible for oligomerization, either full-length LAP2α or the LAP2α-specific C-terminal domain, LAP2α-(188–693), fused to the C terminus of monomeric red fluorescent protein 1 (mRFP1) (Fig. 1A), were stably expressed in HeLa cells. Unlike GFP and DsRed, mRFP1 remains entirely monomeric and therefore precludes misinterpretations of LAP2α oligomerization due to dimerization or tetramerization of the tag (20). Immunoblot analyses of cell extracts prepared from stable cell clones revealed the presence of fusion proteins of expected sizes, in amounts comparable with that of the endogenous LAP2α (Fig. 1B, left panel). Both constructs reacted with polyclonal antibodies against LAP2α C terminus (anti-LAP2αc) and antibodies against red fluorescent protein (anti-RFP), whereas a monoclonal antibody directed against the N-terminal region of LAP2α that is common to all LAP2 isoforms recognized only mRFP1-LAP2αC plus the endogenous protein (anti-LAP2). Both mRFP1-LAP2αC and mRFP1-LAP2α-(188–693) were readily extracted in buffer containing 1% Triton X-100 and 130 mM NaCl (Fig. 1B, left panel). However, although a small fraction of endogenous LAP2α and of mRFP1-LAP2α remained in the pellet fraction, mRFP1-LAP2α-(188–693) was completely soluble, indicating that full-length LAP2α is more tightly bound to nuclear complexes than the LAP2α C terminus.

Next, we examined the cellular distribution of the tagged proteins at various stages of the cell cycle by using fluorescence microscopy. As shown in Fig. 1C, both mRFP1-LAP2α-(188–693) and mRFP1-LAP2α localized to the nucleoplasm in interphase, to the cytoplasm in metaphase, and to chromatin in anaphase/telophase as reported previously for the endogenous protein and GFP-LAP2α (14, 16, 17, 21). Unlike the full-length protein, a fraction of mRFP1-LAP2α-(188–693) remained in the cytoplasm during anaphase (Fig. 1C, arrow), again indicating a less stable integration of the C terminus to chromatin-associated structures as compared with full-length protein. Nevertheless, these data show that the C terminus is capable of interacting with chromatin or nuclear lamina components during nuclear assembly in vivo.
and a Myc tag at the N terminus (PC-Myc-mRFP1-LAP2α-(188–693), Fig. 1A), facilitating efficient isolation of the fusion protein from cell extracts. In addition, we generated a fusion protein containing protein C-epitope and a Myc tag fused directly to the C terminus of LAP2α (PC-Myc-LAP2α-(188–693)). Upon stable transfection into HeLa cells, the fusion constructs were expressed at similar levels as endogenous LAP2α, as revealed by immunoblot analyses of cell extracts with anti-LAP2α and anti-Myc antibodies (Fig. 1B, right panel). Both proteins behaved like mRFP1-LAP2α-(188–693) upon Triton extraction (Fig. 1B) and in immunofluorescence microscopy (Fig. 1C). PC-Myc-mRFP1-LAP2α-(188–693) and PC-Myc-LAP2α-(188–693) were precipitated from Triton X-100-soluble cell fractions using the anti-protein C matrix (Fig. 2A). A protein of about 80 kDa reacting with the anti-LAP2 common domain antibody was consistently found in the immunoprecipitates from these clones but not in non-transfected HeLa cells or clones expressing unrelated constructs (Fig. 2A and data not shown). As the epitope recognized by the monoclonal antibody is not present in the recombinant proteins, the 80-kDa protein could be unambiguously identified as the endogenous LAP2α protein. The other LAP2 isoforms were not co-immunoprecipitated (data not shown). Similarly, when the tagged LAP2α C terminus was precipitated with anti-Myc antibody, full-length LAP2α was detected in the immunoprecipitates, whereas a control antibody did not bring down any of those proteins (Fig. 2B). Likewise, unlike control antibodies, the anti-LAP2 common domain antibody co-precipitated PC-Myc-mRFP1-LAP2α-(188–693) and PC-Myc-LAP2α-(188–693) with the endogenous protein (Fig. 2C). Overall, our data demonstrate that the LAP2α-C terminus associated with full-length LAP2α in vivo, and indicate a role of the C-terminal region in higher order structure organization of LAP2α complexes.

To investigate whether the C terminus can also be incorporated into higher order LAP2α structures, we developed a co-immunoprecipitation approach. The mRFP1-LAP2α-(188–693) fusion protein was modified by adding a protein C-epitope and data demonstrate that the LAP2α-C terminus associated with full-length LAP2α in vivo, and indicate a role of the C-terminal region in higher order structure organization of LAP2α complexes.

**FIGURE 1. Stable expression of LAP2α and the α-specific C terminus in HeLa cells.** A, schematic presentation of fusion proteins of full-length LAP2α and LAP2α-(188–693) with mRFP1, protein C epitope, and Myc tag, and for comparison, the domain organizations of LAP2α and LAP2β are shown. The light gray region represents the LAP2α-specific C terminus; gray and black boxes show positions of LEM, LEM-like, and transmembrane domains, respectively. The arrow indicates the epitope recognized by the monoclonal anti-LAP2 common domain antibody. B, untransfected HeLa cells (HeLa) or HeLa cells stably expressing the indicated constructs (Fig. 1A) were lysed in 1% Triton X-100 and total cell lysates (T) or pellet (P) and supernatant (S) fractions following centrifugation were analyzed by immunoblotting using polyclonal anti-LAP2α (anti-LAP2α), monoclonal anti-LAP2α common domain (anti-LAP2), and polyclonal anti-DsRed (anti-RFP) or polyclonal anti-Myc antibodies, as indicated. The numbers show molecular masses in kDa. C, cells expressing mRFP1-LAP2α or mRFP1-LAP2α-(188–693) or PC-Myc-LAP2α-(188–693) were analyzed at various cell cycle stages by fluorescence microscopy. Left images show mRFP1 or anti-Myc staining, and right images show 4',6-diamidino-2-phenylindole-stained DNA. The arrow indicates anaphase cells with remaining cytoplasmic staining. Bar, 10 μm.
LAP2α Forms Stable Complexes of Defined Molecular Weight

To obtain further evidence for the existence of oligomeric LAP2α complexes, we chemically cross-linked protein complexes in a total HeLa cell lysate using DSP. DSP-mediated cross-links are stable in non-reducing conditions but can be removed by the addition of reducing agents. HeLa cell extracts treated with different concentrations of DSP were analyzed by reducing and non-reducing SDS-PAGE and immunoblotting using a monoclonal antibody against the LAP2α-specific C terminus (Fig. 3A). Depending on the concentration of the cross-linking agent, LAP2α was detected in a complex with an apparent molecular mass larger than 200 kDa. When the cross-linking agent was cleaved by the addition of dithiothreitol, complexes were dissociated, yielding monomeric LAP2α. Intriguingly, semi-native, non-denaturing electrophoresis of HeLa cell lysates also revealed a LAP2α complex slightly larger than 200 kDa (Fig. 3A, left lane). Thus, LAP2α may exist in a stable complex of ~200–250 kDa in vivo.

To test whether LAP2α is able to form oligomeric complexes of similar sizes in vitro, we cross-linked highly enriched recombinant, bacterially expressed LAP2α. Immunoblot analyses

FIGURE 3. Analysis of LAP2α complexes by chemical cross-linking and semi-native gel electrophoresis. A, HeLa cell lysates were analyzed on semi-native polyacrylamide gels (Endogenous complex, left panel) or incubated with the indicated amounts of DSP for 2 h, dissolved in sample buffer containing (+) or lacking (−) DTT, and analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting using monoclonal anti-LAP2α antibody 15/2. B, bacterially expressed full-length LAP2α and LAP2α fragments, as indicated, were incubated with 1 mM DSP, mixed with sample buffer with (+) or without (−) DTT, and analyzed by immunoblotting using monoclonal anti-LAP2α antibody 15/2 (left and central panels) or anti-LAP2 antibody 12 (right panel). C, in vitro translated, [35S]methionine-labeled LAP2α was analyzed by semi-native PAGE and autoradiography. The numbers denote molecular masses in kDa, the arrows indicate the position of monomeric proteins, and the arrowheads indicate the position of complexes.

FIGURE 2. Co-immunoprecipitation of endogenous LAP2α with expressed LAP2α C terminus from HeLa cell extracts. A, soluble cell extracts of non-transfected HeLa cells, two HeLa clones expressing PC-Myc-mRFP1-LAP2α-(188–693), and one clone expressing PC-Myc-LAP2α-(188–693) were incubated with anti-protein C matrix, and immunoprecipitates were analyzed by immunoblotting using anti-LAP2 common domain antibody. Note that in SDS-PAGE, LAP2α runs seemingly faster in the first two lanes as compared with lane 3 due to comigration of PC-Myc-mRFP1-LAP2α-(188–693) with endogenous LAP2α under these conditions. Furthermore, the amount of PC-Myc-mRFP1-LAP2α-(188–693) is higher in the sample shown in lane 2 as compared with that in lane 1. B, cell extracts were incubated with monoclonal anti-Myc (+) or with control (−) antibody, and immunoprecipitates were probed with anti-LAP2 antibody.
revealed a LAP2α complex of more than 200 kDa in non-reducing conditions (Fig. 3A, −DTT), whereas mostly the monomeric protein of ∼80 kDa was detected in reducing conditions (Fig. 3B). Intriguingly, the C terminus of LAP2α-(188–693) also formed larger complexes in non-reducing conditions, whereas the N-terminal region (1–187) remained mostly monomeric under these conditions. Since both the N terminus and the C terminus contain lysine residues, which can be targeted by the cross-linker, we concluded that LAP2α forms higher order, oligomeric complexes through self-association of its C-terminal region.

To further show the homo-oligomerization of LAP2α, we generated LAP2α by in vitro translation in a reticulocyte lysate and analyzed the complex by semi-native PAGE (Fig. 3C). Interestingly, a major band between 200 and 250 kDa was detected by the cross-linker, we concluded that LAP2α forms homo-oligomeric complexes. Autoradiography revealed a LAP2α-(188–693) band, whereas mostly the mono-meric protein of ∼80 kDa was detected in reducing conditions, which may be specific interaction partners for LAP2α, it is very likely that the >200-kDa complex represents homo-oligomeric LAP2α complexes.

The LAP2α Self-interaction Is Mediated by the C-terminal Region—Our data suggest direct interaction of LAP2α C-terminal regions mediating homo-oligomerization of the protein. To further demonstrate that self-interaction of LAP2α polypeptides occurs via the C terminus, we performed additional in vitro binding assays. Bacterially expressed full-length LAP2α (1–693), LAP2α C terminus (188–693), and LAP2 N terminus (1–187) were separated by SDS-PAGE, blotted onto a membrane, and overlaid with radioactively labeled in vitro translated full-length LAP2α or LAP2α C terminus. Autoradiography revealed binding of both labeled proteins to full-length LAP2α and to the C terminus, whereas the N terminus did not interact (Fig. 4A). Thus, only the C terminus of LAP2α can mediate self-interaction of the protein. To narrow down the self-interaction domain within the LAP2α C terminus, we expressed different fragments of LAP2α in bacteria and performed solid phase overlay assays with radioactively labeled, in vitro translated, full-length LAP2α or LAP2α C terminus. Results presented in Fig. 4B and summarized in Fig. 4C show that removal of 78 residues from the C terminus of LAP2α only moderately affected binding of the truncated protein to LAP2α and LAP2α C terminus, removal of the C-terminal 279 residues significantly reduced binding (for LAP2α C terminus to an undetectable level), and deletion of the last 439 residues abolished binding completely. On the other hand, the C-terminal 284 residues of LAP2α-(410–693) showed a binding that was slightly weaker than that of the entire C terminus (188–693). These results show that the extreme C-terminal domain of LAP2α-(410–693) has some binding affinity to the LAP2α C terminus, but strong interaction requires upstream regions between residues 254 and 410. Vimentin as a negative control did not interact with LAP2α.

Purified Soluble LAP2α Is a Homo-trimeric Complex—Our results demonstrate a self-association of LAP2α, and crosslinking experiments revealed complexes of larger than 200 kDa, which suggests trimeric structures. To show the level of assembly of LAP2α in solution accurately, we performed SEC-MALS and refractive index measurements on soluble, purified samples of LAP2α. This technique yields an accurate determination of the molecular mass independent of molecular shape or hydrodynamic parameters. SEC-MALS data revealed that
LAP2α Forms Homo-trimers

LAP2α migrates as a monodisperse molecule with an estimated molecular mass of 207.2 kDa (Fig. 5). Since the calculated theoretical mass of a LAP2α trimer is 226.0 kDa, this result shows that LAP2α exists as a stable homo-trimer in solution. It is also in accordance with the molecular mass observed in the cross-linking and the semi-native gel electrophoresis experiments (Fig. 3).

The Self-association of LAP2α Is Not Affected by a Disease-linked Mutation—A mutation in the extreme C terminus of LAP2α has been linked to dilated cardiomyopathy in humans (18). The molecular mechanism of this disease is currently unknown, but we have shown that the disease-linked mutation in LAP2α decreased its binding affinity for lamin A and C. Therefore, we wondered whether the mutation could also interfere with the self-association of LAP2α molecules and whether this could contribute to the cellular defect in patient cells. To test this hypothesis, GFP-LAP2α and untagged wild-type or disease-linked LAP2α variants were \textit{in vitro} translated simultaneously using a reticulocyte extract, and LAP2α complexes were immunoprecipitated with anti-GFP antibody. Immunoblot analyses using LAP2α antibodies detected untagged LAP2α in the immunoprecipitates only when GFP-LAP2α was present in the samples, whereas untagged protein alone was not precipitated by anti-GFP antibodies (Fig. 6). These data clearly support the direct self-interaction of LAP2α molecules. Both wild-type and mutated LAP2α co-precipitated with GFP-LAP2α with similar efficiencies, indicating that the disease-linked mutation in LAP2α does not interfere with its self-association.

DISCUSSION

The studies presented here revealed a so far unappreciated biochemical property of LAP2α, the formation of homo-oligomeric structures, and revealed the presence of LAP2α homo-trimers in cell lysates and in solution \textit{in vitro}. Co-immunoprecipitation of endogenous LAP2α with exogenously expressed full-length LAP2α or different fusion proteins containing the α-specific C-terminal domain demonstrated that higher order LAP2α structures occur in living cells, are stable, and require the unique C terminus of the protein. Cross-linking of LAP2α complexes in cell lysates and size exclusion chromatography of purified LAP2α combined with multilight scattering measurements showed that LAP2α forms stable homo-trimers.

LAP2α C Terminus Is Involved in Multiple Interactions—\textit{In vitro} overlay experiments suggested that the homo-trimerization of LAP2α is mediated through the direct interaction of its C-terminal α-specific domain. Although the last 284 amino acids were sufficient for self-interaction \textit{in vitro}, additional upstream regions within the unique LAP2α domain contribute to the formation of stable oligomers. The C terminus has been shown to contain several additional binding domains. The last 78 amino acids of LAP2α are involved in the interaction with lamin C (11), whereas further upstream regions mediate the interaction with pRb (12). One may argue that the binding regions of LAP2α for pRb, for lamin A/C, and for self-association may overlap and influence or even compete with each other. However, our observation that stable LAP2α trimers exist in the cell argues that these trimers form the building block of higher order LAP2α complexes. Thus, it is much more likely for a LAP2α trimer \textit{versus} a monomer to interact simultaneously with pRb and lamin A/C and act as a platform for the assembly of transcriptional regulator complexes as proposed in previous studies (12).

Intriguingly, mutated LAP2α (R690C) expressed in the human disease DCM showed reduced binding to lamin A tail \textit{in vitro} (18), whereas LAP2α self-association was not impaired by the R690C substitution. This observation is consistent with the results of overlay blots indicating a relatively broad interface of self-interaction between LAP2α monomers potentially involving several regions within its unique C terminus.

Size of Stable Homo-oligomeric LAP2α Structures—Analysis of HeLa cell extracts after chemical cross-linking or in semi-native non-denaturing gels showed the presence of stable LAP2α-containing complexes of molecular masses between 200 and 250 kDa. Complexes of the same size were detected...
after cross-linking of bacterially expressed LAP2α and by seminative PAGE of LAP2α expressed in reticulocyte lysate, indicating that LAP2α is the only protein in the 200–250-kDa complex. According to their size, these complexes might accommodate three LAP2α molecules of 75–80 kDa, suggesting that the protein exists as a homo-trimeric complex. Size exclusion chromatography combined with light scattering analyses of highly purified bacterially expressed LAP2α revealed a high monodispersity of the sample with a molecular mass consistent with a trimeric organization.

Functional Implication of the LAP2α Self-interaction—By fluorescent microscopy, LAP2α C-terminal domain stably expressed in HeLa cells revealed a cellular distribution identical to that of endogenous LAP2α, except for anaphase-telophase, where it showed a slightly less efficient association with chromosomes. This observation supports previous findings, showing that the C terminus is required and sufficient for chromosome association of LAP2α during nuclear assembly (17). The less efficient binding of LAP2α C terminus to chromatin also indicates that, although the C terminus is sufficient for targeting LAP2α to chromosomes, its stable association also requires the N-terminal common domain. However, based on the data presented here, we cannot rule out completely that the C-terminal fragment associated with chromosomes mainly through its interaction with full-length endogenous LAP2α during nuclear assembly in vivo.

In any case, the engagement of LAP2α in a self-interaction has important implications for its previously reported functions. The formation of stable LAP2α trimers as basic building blocks for higher order structures clearly affects the chromatin binding properties of LAP2α. A trimer brings three LEM and LEM-like domains in close vicinity in a complex and generates multiple binding sites for BAF and DNA, thus significantly increasing the affinity of the complex for chromatin. Secondly, the N termini of the oligomeric complex could interact simultaneously with several DNA fibers and thus perform efficient cross-linking of chromatin regions. In line with this model, it was shown that full-length LAP2α and BAF are essential components of the preintegration complex of retroviruses (22). The proposed role of LAP2α in stabilizing the interaction of BAF with preintegration complexes required both the N-terminal common domain and the α-specific C-terminal domain of LAP2α. Our results, showing that the C terminus is the determinant for oligomerization of LAP2α, provide a possible explanation for this observation. Finally, the proposed role of LAP2α as a transcriptional regulator of E2F target genes (12) by forming complexes with pRB and lamin A/C can be better accomplished by a higher order structure, such as a trimer, than by a monomer.

In conclusion, our data suggest a model where a homo-trimeric core complex of LAP2α, formed via self-association of the C-terminal domain, serves as the building block for higher order homo- and hetero-oligomeric structures of LAP2α that are involved in chromatin organization and in transcriptional regulation.

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