A Phosphorylation Cluster in the Chromatin-binding Region Regulates Chromosome Association of LAP2α*

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LAP2α is a LEM family protein associated with nucleoplasmic A-type lamins and chromatin in interphase. Like lamins and other lamina proteins LAP2α is cytoplasmic in metaphase, but it associates with chromosomes prior to nuclear envelope formation in late anaphase to telophase. In vitro phosphorylation analysis and mass spectrometry identified a cluster of at least three mitotic cyclin-dependent kinase 1 phosphorylation sites in the C-terminal chromatin-binding region of LAP2α as well as four additional potential sites in the cluster, some of which were targeted alternatively in alanine mutants at the major sites. LAP2α mutants containing serine→alanine mutations at all seven sites revealed a clear phenotype. Mutated LAP2α remained associated with chromosomes throughout mitosis, but the dissociation of lamins into the cytoplasm and nuclear envelope disassembly were not affected. These data demonstrate the in vivo significance of mitotic phosphorylation for the dynamic behavior of LAP2α in the cell cycle and show that, unlike the interaction with lamins, the chromatin association of LAP2α is regulated by multiple mitosis-specific phosphorylation at sites clustered within a defined region in the C terminus of the protein.

In higher eukaryotes, lamins (1) and lamin-binding proteins form the lamina at the nuclear envelope, providing mechanical stability for the nuclear membrane (2, 3). Lamins are also found in stable complexes in the nuclear interior (4–6), where they are involved in numerous functions (7, 8), including DNA replication (9), RNA polymerase II-dependent transcription (10), chromatin organization, and spacing of nuclear pores (11) and possibly RNA splicing (12). A growing number of lamin-associated proteins at the nuclear periphery and in the nucleoplasm regulate lamin complex assembly and function (7, 13, 14). Lamin-binding proteins in the inner nuclear membrane include the lamin B receptor (LBR, Ref. 15, emerin, Ref. 16, and various isoforms of lamina-associated polypeptide (LAP1, Refs. 17 and 18 and LAP2, Refs. 19–21). In the nuclear interior, lamins have been found to interact with histones (22, 23), retinoblastoma protein pRb (24, 25), and a nucleoplasmic LAP2 isoform, LAP2α (26).

The mouse and human LAP2α gene encodes six alternatively spliced isoforms (21), most of which are inner nuclear membrane proteins and closely related in structure to LAP2β that binds B-type lamins (18). LAP2α shares only the N-terminal constant region with the other isoforms and contains a unique C terminus. LAP2α forms nucleoskeletal complexes with A-type lamins throughout the nuclear interior (26, 27). All LAP2 isoforms belong to the LEM (LAP-emerin-MAN1) protein family (28), characterized by the presence of a 40-residue long structural motif (LEM domain) (28–30). The LEM domain interacts with the DNA cross-bridging protein barrier-to-autointegration factor (BAF; Ref. 31) and an N-terminal LEM-like motif binds DNA (29). Thus, LAP2 isoforms interact with chromatin at the nuclear envelope and in the nucleoplasm and may regulate higher order chromatin structure.

Multicellular eukaryotes reversibly disassemble the nuclear lamina, nuclear pore complexes, and nucleoskeletal structures during mitosis (8, 32). Mitosis-specific phosphorylation of lamins (33, 34) was found to be essential for lamina disassembly by expressing lamin mutants with mutations in their cyclin-dependent kinase 1 (cdk1) target sites (35). Lamin-binding proteins, such as LAP2β and LAP2α, are also phosphorylated in mitosis (18, 27). However, unlike for lamins, the role of mitosis-specific phosphorylation of lamin-binding proteins for their cell cycle-dependent dynamics has never been demonstrated in vivo.

LAP2α showed a particularly intriguing dynamic behavior in the cell cycle. Like lamins and LAP2β, LAP2α was dispersed throughout the cytoplasm in metaphase cells, but associated with chromosomes early during nuclear reassembly, prior to accumulation of LAP2β and assembly of membranes (18, 26, 27, 36). While the LEM and LEM-like motifs in the LAP2α N terminus were dispensable for the LAP2α-chromosome interaction, a chromatin-binding region in the unique C-terminal domain was found to be essential and sufficient for interaction (36, 37). Chromatin-binding LAP2α fragments dominantly inhibited nuclear assembly (37), suggesting that LAP2α relocalization to chromosomes is essential for proper nuclear assembly. In this study we focused on identifying molecular regulatory mechanisms, controlling the association of LAP2α with chromosomes. We identify three major mitosis-specific

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∥ The abbreviations used are: LAP, lamina-associated polypeptide; LEM, LAP-emerin-MAN1 protein family; PBS, phosphate-buffered saline; DTT, dithiothreitol; CHO, Chinese hamster ovary cells; cdk, cyclin-dependent kinase; MS, mass spectrometry.
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phosphorylation sites clustered in the core of the chromatin-binding region as well as four potential sites in the cluster. We further show that eliminating LAP2α phosphorylation by mutating serines to alanines at major and potential target sites renders the protein incapable of dissociating from chromosomes in mitosis. These studies demonstrate for the first time the in vivo significance of phosphorylation of a lamin-binding protein for its redistribution during mitosis and show that chromosome association, unlike its interaction with lamin, is regulated by phosphorylation of LAP2α.

EXPERIMENTAL PROCEDURES

Construction of LAP2α Expression Plasmids—Mutations replacing serine to alanine residues in the LAP2α cDNA were subcloned from pTBD15 derivatives encoding full-length LAP2α with a C-terminal Myc tag (36) as template. The following pairs of oligonucleotide primers were used: 5'-GGTCTCTGAGCTGTCTCTCAGTTAGT-AAGC-3' and 5'-CTCTAATGACGGAGGCTGAGCAGGAC-C-3' to replace serine 309; 5'-GGATCAATAGGATTTTTATGAT-CAATGCC-3' and 5'-GGGCGGTCTGATATATCTTGGGTCGTTTCC-3' to replace serine 317; 5'-CATATTTTTAGATTTTTAGTCCCTTC-3' and 5'-GGGCGGCTTGGGAGGATCCCCTC-3' to replace serine 350; 5'-GGGCGCTTGGGAGGATCCCCT-3' and 5'-ATCTGGAAATG-3' to replace serine 362; 5'-GGGATACCCATATTGACGGAG-3' and 5'-CTGAGGAGCTTGATTTCT-3' to replace serine 363; 5'-GGGCGGTCTGATATATCTTGGGTCGGTTTCC-3' and 5'-ATCTGGAAATG-3' to replace serine 392; 5'-GGGAAAGGATCAGTGAAGGCGCTTGAGT-3' and 5'-CTGAGGAGCTTGATTTCT-3' to replace serine 433.

All mutations were confirmed by sequencing. For bacterial expression of mutated proteins, the respective cDNAs were subcloned into pTBD15 derivatives into the bacterial expression vector pET-23a(+)(Novagen, Madison, WI) via Nhel-XhoI.

Cell Culture and Transfections—HeLa, HeLa Tet-on (Clontech Laboratories, Palo Alto, CA), and CHO cells were routinely maintained in Dulbecco's modified Eagle's medium containing high glucose, 10% fetal calf serum, 50 units/ml penicillin, 50 µg/ml streptomycin (all from Invitrogen) at 37 °C in a humidified atmosphere with 5% CO₂. Culture media for HeLa Tet-on cells were supplemented with 100 µg/ml G418 (Invitrogen), for transfected stable HeLa Tet-on clones with additional 200 µg/ml G418. Transfections were performed according to the manufacturer's instructions using Lipofectamine 2000 reagent, Opti-MEM (Invitrogen), and DNA prepared with a Jet Star plasmid kit (Genomed, Bad Oeynhausen, Germany). Transient transfections were done in Falcon 4 chamber tissue culture glass slides (BD Biosciences, Franklin Lakes, NJ) for 4 h in transfection reagent containing 0.6 µg of DNA per chamber. For stable transfections, 5 × 10⁶ HeLa Tet-on cells on 6-cm culture dishes were co-transfected with 0.4 µg of pT-Hyk selection vector (Clontech Laboratories) and 6 µg of response plasmid. After 48 h of culture in complete medium, cells were split into ten 10-cm culture dishes and cultured in medium containing 200 µg/ml hygromycin B for 14 days. Single clones were transferred into 24-well plates and grown in the presence or absence of doxycycline.

Chromosome Spreads—Mitotic cells harvested from an unsynchronized cell culture were incubated in 0.075 M KCl for 20 min at room temperature and lysed by addition of 0.1% Tween 20. Samples were spun on coverslips at 2,000 rpm for 3 min in a Cytospin 2 (Thermo Shandon, Pittsburgh, PA), fixed in 2% formaldehyde for 10 min, and processed for immunofluorescence microscopy.

Immunofluorescence Microscopy—Cells were fixed in 2 or 3% formaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature, incubated with 50 µl of mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA) in PBS, and permeabilized with 0.5% Triton X-100 in PBS for 5 min each. After incubation in PBS-0.1% gelatin and 3% normal goat serum, the antibodies were applied in PBS for 1 h each. Primary antibodies used were monoclonal antibodies to LAP2α, and LAP2β (27), antisera to LAP2α (37), monoclonal Myc 1–9E10.2 antibody (American Type Culture Collection CRL-1729), monoclonal lamin A antibody (I33A2, abcam, Cambridge, UK). Secondary antibodies used were affinity-purified goat anti-rabbit or anti-mouse IgG conjugated to Alexa 488 or Texas Red (Jackson Immunoresearch Laboratories, West Grove, PA). DNA was stained with 1 µg/ml Hoechst dye 33258 applied together with the secondary antibody. Samples were mounted in Mowiol and viewed in a Zeiss Axiosvert 100M equipped with either a LSM 510 confocal microscope or an Axioscam.

Protein Expression in Bacteria and in Vitro Phosphorylation—For bacterial protein expression Escherichia coli strain BL21 was transformed with pET-23a(+) (Novagen)-derived plasmids. Bacteria were grown in 100 ml of LB medium plus 100 µg/ml ampicillin until an OD₆₀₀ of 0.6–0.8, and protein expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside for 4 h. Bacteria were lysed in 5 ml of lysis buffer (20 mM HEPES pH 7.4, 100 mM NaCl, 1 mM DTT, 0.1 mg/ml lysozyme, and 0.1% Triton X-100, and 1 mM MgCl₂) for 30 min at 37 °C and centrifuged at 3,000 × g for 30 min. Pellets were resuspended in urea buffer (7 M urea, 100 mM NaCl, 20 mM HEPES pH 7.4, and 1 mM DTT) and stored frozen at −20 °C. For in vitro phosphorylation, samples were dialyzed into buffer A (50 mM HEPES pH 7.4, 5 mM MgCl₂, 1 mM EGTA, 0.1% Triton X-100, 100 mM NaCl, and 0.1 mM DTT). 80–µl samples were mixed with a 10-µl interphase or mitotic CHO cell extract or immunoprecipitated cdk1 (38), and 10 µl of an ATP[γ-32P]ATP mixture (1 µCi for 50 µl of a 1 mM ATP solution), incubated for 30 min at 37 °C, and separated on 3%–SDS sample gels and analyzed by autoradiography. The following inhibitors were used: chloramphenicol (84 µg/ml, Calbiochem, La Jolla, CA); staurosporine (0.2 µM, Sigma); H7 (0.3 µM, Calbiochem); EDTA (10 mM, Sigma). Two-dimensional phosphopeptide mapping was done according to Boyle et al. (39) with slight modifications (38).

Immunoprecipitation of LAP2α and Mass Spectrometry—Mitotic or interphase cells (38) were lysed in 500 µl of buffer A containing 1% Triton X-100 and 0.4% SDS and pressed through a 27-gauge needle to shear DNA. Buffers A was reduced to SDS concentration to 0.1%, and lysates were precleared by incubation with 20 µl of 50% protein G-Sepharose beads (Sigma) and centrifuged. 20 µl of protein G-Sepharose beads, preincubated in 100 µl of hybridoma supernatant coated with anti-Myc antibodies or to the Myc tag, were harvested by centrifugation following a 2-h incubation. The beads were washed five times in buffer A and proteins separated by SDS-PAGE. Coomassie-stained LAP2α bands were cut out from gels and digested as described elsewhere (40). High quality water for the in-gel digestion and the mass spectrometric experiments was prepared using an ELGA Maxima water purification system (Vivendi Water Systems, High Wycombe, Bucks, UK). Amonium hydroxide hydrogen carbonate was obtained from FLUKA (Sigma-Aldrich), DTT for the reduction of proteins before in-gel digestion was purchased from Roche Applied Science, iodoacetamide was supplied by Sigma, and trypsin was obtained from Roche Applied Science. Tryptic peptides were extracted by 5% formic acid, purified, and concentrated. The chromatographic media used was Poros 20 R2 (PerSeptive Biosystems, Foster City, CA) filled into the non-coated porous pipette tip of Poros Biosystems (Proxeon Biosystems, Denmark). Hydrophilic peptides were captured by Poros Oligo material (PerSeptive Biosystems, Foster City, CA). Concentrated peptides were eluted with a mixture of 50% methanol, 5% formic acid directly into a metal-plated silica capillary (Proxeon Systems A/S; Odense, Denmark). The measurements were carried out on a QSTAR (Applied Biosystems; Foster City, CA) hybrid mass spectrometer. The instrument was externally calibrated with the MS/MS fragments of a synthetic peptide (ALILTLVS). First a peptide spectrum was obtained, and the charge state of the peptides was determined. Doubly or triply charged peptides were chosen for MS/MS experiments. Fragment ions were labeled according to the nomenclature proposed by Roepstorff and Fohlman (41). MS/MS spectra were interpreted with the aid of the Mascot (Matrix Science Ltd, London, UK) search engine.

Polyacrylamide Gel Electrophoresis, Transfer, and Detection—SDS-PAGE was performed according to Laemmli (42). For autoradiography gels were dried and exposed to an x-ray film. For immunoblotting, proteins were electrophoretically transferred onto nitrocellulose (0.2 µm; Schleicher and Schuell, Dassel, Germany) in 48 mM Tris-HCl, pH 9.4, 39 mM glycine using the Mini Transblot system (Bio-Rad). Primary antibodies used were: hybridoma supernatants of anti-LAP2α antibodies (37), monoclonal Myc 9E10 antibody (ATCC), and LAP2α antibody 245-2 (diluted 1:10,000; Ref. 37); secondary antibodies, alkaline phosphatase- or peroxidase-conjugated goat antibodies against the primary antibodies (diluted 1:7,500). For detection of proteins, the Proteoblot immunoscreening system (Promega) or the Super Signal ECL (Pierce) were used.
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LAP2α Is Differentially Phosphorylated by Interphase and Mitotic Kinases in Vitro—We have previously shown that LAP2α isolated from metaphase cells had a slightly reduced mobility on SDS-PAGE as compared with LAP2α from interphase cells. Furthermore, isoelectric focusing revealed a more acidic pI of mitotic versus interphase LAP2α (27). As these properties were consistent with a mitosis-specific phosphorylation of LAP2α, we set out to test this possibility by various means. First, we performed in vitro phosphorylation assays using bacterially expressed, recombinant LAP2α as substrate, or histone H1 as a control, and mitotic or interphase cell extracts as a source for kinases. Samples were analyzed by SDS-PAGE and autoradiography (Fig. 1A) and specific protein phosphorylation (cpm per microgram of protein, Fig. 1B) was determined. Extracts of nocodazole-arrested metaphase cells phosphorylated both LAP2α and histone H1 to a 3–4-fold higher extent than interphase extracts. Endogenous LAP2α present in cell extracts was highly diluted and not detectable in these assays (data not shown). Three potent kinase inhibitors, olomoucine, staurosporine, and H7, which affect predominantly cdk-type kinases, protein kinase C, and a broad range of different kinases, respectively, reduced in vitro phosphorylation significantly, while the calcium chelator EDTA blocked kinase activity completely. Interestingly, unlike EDTA, none of the inhibitors significantly affected interphase kinase activity toward LAP2α. Thus, we concluded that LAP2α is primarily phosphorylated by mitotic kinases.

cdk1 Phosphorylates LAP2α at Mitosis-specific Site(s) in Vitro—The sensitivity of mitotic LAP2α kinase activity to olomoucine suggested an involvement of mitosis-specific kinase cdk1. To test this hypothesis, we immunoprecipitated cdk1 from nocodazole-arrested metaphase cells using an antiserum generated against a synthetic peptide representing the C terminus of human cdk1 (43) and performed in vitro phosphorylation assays. Recombinant LAP2α (Fig. 2, A and B) and histone H1 (not shown) were significantly phosphorylated by cdk1 in vitro.

To demonstrate that cdk1 also represented a major kinase activity for LAP2α in whole mitotic cell lysates, we performed two-dimensional tryptic phosphopeptide mapping of in vitro phosphorylated LAP2α. The phosphopeptide analysis of LAP2α phosphorylated by whole mitotic cell lysate yielded several strongly labeled spots (Fig. 2C), indicating targeting of various phosphorylation sites during mitosis. Most of the phosphopeptides generated from LAP2α phosphorylated by interphase extracts were only weakly labeled, and interphase and mitotic peptide patterns were clearly different. While only one spot was detected in both mitotic and interphase samples (Fig. 2C, arrowhead), two of three major mitotic peptides were exclusively detected in mitotic samples, indicating mitosis-specific phosphorylation. Interestingly, one of these mitosis-specific peptides was also detected in phosphopeptide maps of LAP2α phosphorylated by immunoprecipitated cdk1 (arrow). Thus, the major phosphorylation sites in LAP2α were predominantly or exclusively targeted in mitosis and involve cdk1 as well as other kinases.

To narrow down the cdk1-dependent, mitosis-specific target site in LAP2α, we expressed LAP2α fragments covering different domains of the protein in bacteria and tested their in vitro phosphorylation by immunoprecipitated cdk1 (Fig. 2B) or mitotic extracts (Fig. 2D) using SDS-PAGE/autoradiography and two-dimensional phosphopeptide mapping, respectively. While the LAP2α-specific C-terminal region (amino acids 188–693) was phosphorylated as efficiently as full-length protein (amino acids 1–693) and produced the mitotic, cdk1-dependent spot was not detected in peptide maps of LAP2α phosphorylated by immunoprecipitated cdk1 (arrow). Thus, the major phosphorylation sites in LAP2α were predominantly or exclusively targeted in mitosis and involve cdk1 as well as other kinases.

Serine 423 Is the Main cdk1-dependent Phosphorylation Site in LAP2α—Searching for minimal cdk1-consensus phosphorylation sites in the LAP2α primary sequence, serine/threonine followed by proline (44), revealed two potential targets between LAP2α amino acids 410 and 615, serine 423 and threonine 547, the former being an optimal consensus site with a basic residue at position +2 ((S/T)XP[ KR]) (Fig. 3). Thus, we hypothesized that serine 423 may be the predominant cdk1-dependent in
vivo phosphorylation site in LAP2α. To confirm this hypothesis, we exchanged serine 423 with an alanine by site-directed mutagenesis and tested the in vitro phosphorylation of LAP2α S423A mutant by mitotic extract. The specific phosphorylation of LAP2α S423A was reduced 2-fold compared with wild-type protein (Fig. 4A), and the mutated protein phosphorylated by mitotic cell extracts (Fig. 4B) or cdk1 (not shown) did not generate the cdk1-dependent spot in two-dimensional peptide maps (arrow). Endogenous LAP2α from mitotic cell lysates was not detectable at significant levels because of its low concentration in the assay, and it may incorporate radioactive phosphate less efficiently than recombinant protein, as it was already phosphorylated at mitotic sites. In contrast to serine 423, mutation of serine 309 or serine 369 did not significantly re-
duce the mitotic in vitro phosphorylation of LAP2α, although the extent of phosphorylation of double and triple mutants containing the S423A mutation plus S309A and/or S369A mutations was further reduced as compared with that of the single S423A LAP2α mutant. Thus, serines 309 and 369 may be targeted by mitotic kinases with different efficiencies in wild type and S423A mutants.

Mass Spectrometry Reveals a Cluster of at Least Three Mitotic Phosphorylation Sites in Vivo—Our in vitro assays have shown that cdk1 phosphorylated LAP2α predominantly at serine 423. To test if this site is also phosphorylated in vivo and to identify additional potential targets for mitosis-specific kinases, we immunoprecipitated LAP2α from nocodazole-arrested metaphase and from interphase cells in the presence of kinase and phosphatase inhibitors and analyzed the proteins by mass spectrometric sequencing. To minimize contamination of interphase cell fractions with mitotic cells we removed round mitotic cells from adherent interphase cells by vigorous shaking prior to cell harvest. Mass spectrometry confirmed serine 423 as a mitosis-specific in vivo phosphorylation site in LAP2α, as fragments containing phosphorylated serine 423 were consistently detected only in the mitotic, but not in several independent interphase protein samples (Fig. 5A). The relative molecular mass of the tryptic peptide containing the phosphorylated serine 423 differed by only 0.05 Da from a non-phosphorylated form with a completely different sequence. The resolution of the QSTAR mass spectrometer was not high enough to separate the triply charged ions, but Fig. 5A clearly shows that the fragment spectra of the ions at m/z 715 Da are different in the samples prepared from the mitotic and interphase cells. The peptide VIEEEWQQVDR was present in both interphase and mitotic samples. Additional fragment ions were only detected in the sample prepared from the mitotic cells. These ions can be assigned to the peptide FQETEFLS*PPR containing the phosphorylated serine 423. The ion generated by the loss of a phosphoric acid residue from the parent molecule was also detected (labeled with an asterisk in Fig. 5A). In addition we observed phosphorylation at the minimal cdk1 consensus site at serines 369 (Fig. 5B) and 350 (Fig. 5C), as well as at serine 443 (data not shown), which does not represent a known kinase consensus motif. With the exception of serine 443, for which we never got sequence coverage in interphase samples, we showed that these phosphorylation sites were targeted exclusively in mitosis. Thus, LAP2α was phosphorylated during metaphase at least at four sites, including three mitosis-specific potential cdk1-dependent sites. Interestingly, all sites clustered within a 100-amino acid long region in

![Fig. 3. In vivo phosphorylation of LAP2α analyzed by mass spectrometry.](image)

![Fig. 4. Mutation of serine 423 to alanine reduced in vitro phosphorylation of LAP2α significantly.](image)
the center of the previously identified chromatin-binding domain of LAP2α (36). In addition, the mass spectrometric analyses provided evidence that serines 344 and 362 (Fig. 3), located within the identified phosphorylation cluster, may also be targeted in mitosis. However, the quality of the fragment spectra was not good enough to unambiguously localize the target sites within these peptides. Aside from the phosphorylation sites in the chromatin-binding region, mass spectrometric analyses uncovered permanent cell cycle-independent phosphorylation of serines 65 and 66 within a protein kinase CKII consensus motif (Fig. 3).

Alternative Phosphorylation Sites Can Be Used in Mutated LAP2α—The clustering of mitosis-specific phosphorylation sites in the chromatin-binding region of LAP2α suggested that the dissociation of the protein from chromosomes in metaphase, as described previously (27), may be regulated by phos-

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**Fig. 5. Positive ionization product ion spectra of tryptic peptides.** Peptides shown are at m/z 715 Da (A), acquired from the samples prepared from mitotic (containing phosphorylated serine 423) and interphase cells; at m/z 783 Da (B), containing the phosphorylated serine 369 from mitotic cells; at m/z 761 Da (C), containing the phosphorylated serine 350 in mitotic cells; and at m/z 503 Da (D), containing the phosphorylated serine 309 in mutated LAP2α in mitotic cells. Ions generated by the loss of a phosphoric acid residue from the phosphorylated parent ion were labeled with an asterisk (*).
phorylation. To test the physiological relevance of LAP2α phosphorylation, we generated various LAP2α mutants containing different combinations of mutated phosphorylation sites (serine to alanine exchanges) and expressed C-terminally Myc-tagged mutants in HeLa cells using the doxycyclin-inducible expression system. None of the cell clones expressing LAP2α mutant protein with different single or multiple serine to alanine mutations showed a phenotype like mislocalization or unusual reorganization of mutant versus wild-type endogenous proteins in the cell cycle (data not shown).

To analyze this phenomenon in more detail, we focused on generating stable clones expressing LAP2α mutants to analyze the phosphorylation of mutated LAP2α in vivo. We obtained a stable cell clone expressing the LAP2α phosphorylation mutant S344A/S362A/S369A/S423A/S443A, in which two of the three mitotic in vivo targets as well as the potential phosphorylation sites at serine 344, 362, and 443 (see above) were mutated (Fig. 3); yet we did not observe any clear cellular phenotype. We precipitated mutated LAP2α from lysates of mitotic cells using antibodies to the Myc tag. As expected mass spectrometry revealed phosphorylation of serine 350, the cdk1 target site identified also in wild-type LAP2α, but surprisingly, the mutated protein contained an additional phosphate group at the cdk1 target serine 309 (Figs. 3 and 5D), which was never observed to be phosphorylated in the wild-type protein. Thus, we concluded that upon mutation of cdk1 target sites within the phosphorylation cluster, the cells used a different alternative phosphorylation site in mutated LAP2α, thus probably retaining a partial phosphorylation-dependent regulation.

Expression of a 7-fold LAP2α Mutant Caused a Stable Cellular Phenotype.—In view of the observed alternative phosphorylation in LAP2α phosphorylation mutants, we decided to mutate all potential cdk1 target sites within the phosphorylation cluster (serines 309, 350, 369, and 423) as well as potentially phosphorylated sites revealed by mass spectrometry of wild-type proteins (serines 344, 362, and 443) (Fig. 3) in order to avoid any alternative phosphorylation of LAP2α phosphorylation mutants in the cluster region in vivo. The 7-fold LAP2α phosphorylation mutant with a C-terminal Myc tag was stably expressed in HeLa cells using the doxycyclin-dependent expression system. Immunoblot analyses of cell lysates of stable clones, using antibodies to Myc or to LAP2α, revealed that the expression of the mutated protein was tightly controlled by doxycyclin (Fig. 6A). The level of ectopic protein was 2–3-fold higher than that of endogenous protein as checked by densitometric scanning of bands on the blots. As a control, expression of LAP2β did not change upon addition of doxycyclin.

In order to specifically detect the mutated LAP2α in mitotic cells we performed immunofluorescence microscopy using an antisum to the Myc tag. Mutated LAP2α was predominantly localized on chromosomes in metaphase and anaphase, while endogenous LAP2α in untransfected cells was cytoplasmic at these stages of the cell cycle (Fig. 6B). In order to detect both ectopic mutated and endogenous wild-type LAP2α in stable cell clones we stained the cells with monoclonal antibody 9E10 to the Myc tag and antisum to LAP2α, which detects both ectopic and mutated protein. These studies revealed that endogenous LAP2α was still released from chromosomes in cell clones expressing mutated protein (Fig. 6C). Interestingly, the LAP2α antibody seemed to react only weakly with mutated protein, which can be explained by tight association of mutated LAP2α with chromosomes causing epitope masking. Together these data indicated that mutated LAP2α bound tightly to chromosomes, but did not interfere with the dissociation of wild-type endogenous LAP2α. To confirm the tight association of mutated LAP2α with metaphase chromosomes, we generated chromosome spreads of stable clones and performed immunofluorescence microscopic analysis. The Myc-tagged protein was clearly detected all over the chromosomes (Fig. 6D, +Doxycyclin), whereas endogenous protein in cells not expressing the mutated LAP2α (Fig. 6D, −Doxycyclin) localized exclusively to the tips of chromosomes.

The LAP2α-binding partner in interphase cells, lamin A (Fig. 6E), and other lamina proteins (data not shown) were unaffected in mutant cells and were exclusively cytoplasmic during metaphase/anaphase. Thus, lamin A also efficiently dissociated from chromosomes and mutated LAP2α, indicating that the LAP2α-lamin interaction was not regulated by phosphorylation of LAP2α, but may involve lamin phosphorylation. Mutated, unphosphorylated LAP2α remained firmly bound to chromosomes throughout completion of mitosis, while wild-type LAP2α accumulated efficiently at chromosomes in telophase (Fig. 6C), similar to untransfected control cells (compare Refs. 27 and 36). In interphase, both mutated and wild-type protein localized throughout the nucleus, although the merged image indicated a more peripheral localization of endogenous versus mutated protein. Since mutated LAP2α in the center of the nucleus was weakly stained by the antisum to LAP2α, we concluded that also in interphase the epitopes in the mutated protein may be masked, possibly reflecting the strong association of mutated protein with chromosomes.

Altogether, we observed a mislocalization of mutated LAP2α at chromosomes in mitosis, demonstrating the essential role of LAP2α phosphorylation in its dynamic behavior during the cell cycle. Nuclear envelope reassembly following sister chromatid separation was, however, not affected, as indicated by the translocation of nuclear membrane protein LAP2β to chromosomes in telophase and formation of a continuous nuclear envelope around chromatin in interphase (Fig. 6F).

DISCUSSION

In this article we have shown that LAP2α is predominantly phosphorylated by mitotic kinases in its chromatin-binding domain, and demonstrated the in vivo significance of LAP2α phosphorylation in its dynamic behavior during mitosis.

**In Vitro versus in Vivo Phosphorylation**—Several observations indicated that serine 423 is the major cdk1 phosphorylation site in LAP2α. (i) Mutation of this site reduced in vitro phosphorylation by 50%. (ii) Serine 423 represents a classical cdk1 consensus motif followed by a basic residue in position +2 (44) and is conserved between mouse and humans. (iii) Mass spectrometry of LAP2α immunoprecipitated from mitotic and interphase cells confirmed serine 423 as a mitotic phosphorylation target in vivo.

Besides the major site at serine 423, we unambiguously identified two additional mitosis-specific minimal cdk1 consensus sites and one site of unknown kinase specificity and interphase modification. In addition we also obtained evidence for two additional phosphorylation sites of unknown kinase specificity in the vicinity of these sites. Although mass spectrometry did not allow distinguishing major from minor phosphorylation sites, serine 423 may also represent the major target site in vivo, assuming that all kinases phosphorylating LAP2α in vivo are also present in the mitotic extract used for the in vitro phosphorylation assays. Nevertheless, if serine 423 were the major target site in vivo, its phosphorylation was not sufficient for regulating LAP2α association with chromosomes, because expression of LAP2α mutants containing a single serine to alanine mutation at residue 423, behaved like the wild-type protein.

Previous studies by Dreger et al. (45) have identified five phosphorylated sites in the membrane-anchored LAP2 isoform, LAP2β, during interphase in neuroblastoma Neuro2a cells.
Although all sites were located in the N-terminal constant region of LAP2β, which is identical to the N terminus of LAP2α, we did not find any of these sites phosphorylated in LAP2α in HeLa cells. Instead, we identified a permanent phosphorylation in the N terminus within a casein kinase II phosphorylation consensus motif. Thus, the isoforms LAP2α and LAP2β may be differentially phosphorylated in the common N terminus, consistent with their different cellular localization, or alternatively, LAP2 proteins are differentially phosphorylated in a cell type-specific manner in interphase.

Significance of LAP2α Phosphorylation—The importance of lamina protein phosphorylation for nuclear disassembly has been postulated many times based on the observation that lamina proteins were phosphorylated in a mitosis-specific manner, that phosphorylation correlated with lamin-complex disassembly, and that phosphorylated proteins failed to interact with their binding partners (for review see Ref. 46). However, except for lamins (35), phosphorylation sites have not been identified in most lamina proteins, and the significance of phosphorylation for nuclear disassembly has not been demonstrated in vivo. Here, we show for the first time for a lamin-binding protein that phosphorylation is essential for its dissociation from chromosomes during mitosis in cells. LAP2α has previously been shown to localize to the cytoplasm in meta-

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**Fig. 6.** Expressed 7-fold mutated LAP2α permanently associates with chromosomes. Stable HeLa Tet-on cells expressing LAP2α S309A/S344A/S350A/S362A/S369A/S423A/S443A mutant were cultured in the absence (−) or presence (+) of doxycyclin and cell lysates analyzed by immunoblotting (A) using antibodies to Myc, to the LAP2 N terminus (LAP2), or to LAP2α, or processed for immunofluorescence microscopy (B, C, E, F) or for preparation of chromosome spreads (D) using antibodies as indicated. Epifluorescence (C) or confocal (B, D–F) images are shown. Bars in B (for B, C, E, F) and in D represent 10 μm.
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