Identification of Phosphorylation Sites in Native Lamina-Associated Polypeptide 2β†

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ABSTRACT: Lamina-associated polypeptide 2β (LAP 2β), an integral protein of the inner nuclear membrane, appears to be involved in the spatial organization of the interface between nucleoplasma, lamina, and nuclear envelope. Its ability to interact with other proteins and the structural integrity of the nuclear envelope is probably regulated by phosphorylation. Here, we report nonmitotic LAP 2β phosphorylation sites that are phosphorylated in the native protein when purified from nuclear envelopes of mouse neuroblastoma Neuro2a cells. Five phosphorylation sites were detected by nano-electrospray mass spectrometric analysis of tryptic LAP 2β peptides using parent ion scans specific for phosphopeptides. By mass spectrometric sequencing of these peptides, we identified as phosphorylated residues Thr 74, Thr 159, Ser 176, and Ser 179. Two of the phosphorylation sites, Thr 74 (within a region known to bind chromatin) and Thr 159, are part of consensus sequences of proline-directed kinases. Ser 179 is part of a consensus site for protein kinase C which is able to highly phosphorylate LAP 2β in vitro. Three phosphorylation sites, Thr 159, Ser 176, and Ser 179, are located within a stretch of 20 amino acids, thereby forming a highly phosphorylated protein domain which may integrate signaling by multiple protein kinases. Additionally, we identified for the first time at the protein level the LAP 2 splice variant LAP 2ϵ in nuclear envelopes.

The nuclear envelope (NE[1]) comprises an outer nuclear membrane that is continuous with the membrane of the endoplasmic reticulum and an inner nuclear membrane that is attached to the nuclear lamina at its nucleoplasmic face. These membranes enclose the intermembrane space. At the nuclear pore complexes, the outer membrane folds into the inner membrane. The NE is disassembled during mitosis. After mitosis, the NE reassembles: nuclear envelope proteins as well as lamins attach to the chromatin in an ordered manner suggesting specialized roles for the inner membrane proteins despite having in part the same binding partners (*1*). Only a handful of proteins residing in the inner nuclear membrane have been identified so far, including the lamin B receptor (*2*, *3*), emerin (*4*, *5*), the lamina-associated polypeptides (LAPs) (*6*, *7*), and otefin (*8*, *9*).

Protein phosphorylation is likely to be one of the key mechanisms to control interactions between proteins of the inner nuclear membrane and components of the nuclear lamina as well as chromatin (*10*, *11*). In the present study, we focus our work on the phosphorylation of LAP 2β (LAP 2β), one of the inner membrane proteins.

The LAP 2 proteins (earlier referred to as thymopoietin proteins) constitute a family of five proteins derived from a single gene as splice variants sharing a common N-terminal domain (aa 1−187) (*12*). As deduced from its amino acid sequence, LAP 2β contains a large nucleoplasmic domain (amino acids 1−409), a putative transmembrane region (410−439), and a short perinuclear tail (440−453), whereas LAP 2α apparently lacks any transmembrane domain. LAP 2β was demonstrated to bind both chromatin and B-type lamins (*10*). A critical role of LAP 2β in the organization of the nuclear envelope architecture was suggested by the finding that overexpression of the lamin-binding fragment of the protein in HeLa cells as well as the addition of exogenous recombinant LAP 2β in *Xenopus laevis* nuclear assembly reactions prevents nuclear volume increase after formation of the NE (*13*, *14*). In the latter case, the addition of the recombinant protein also enhanced replication in the assembled nuclei (*14*) suggesting the existence not only of structural but also of functional interactions between nuclear envelope and chromatin with LAP 2β emerging as one of the key players.

Cell cycle-dependent phosphorylation of LAP 2β was shown to abolish the binding of lamin B1 and chromatin (*10*). The amino acid sequence of LAP 2β comprises multiple predicted phosphorylation consensus sequences, but up to now no phosphorylation sites have been confirmed, neither in vitro nor in vivo.

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[1] Abbreviations: NE, nuclear envelope; LAP, lamina-associated polypeptide; BAC, benzyldimethylhexadecylammonium chloride; ESI, electrospray ionization; MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight; PSD, post source decay; PKC, protein kinase C; res, residue.

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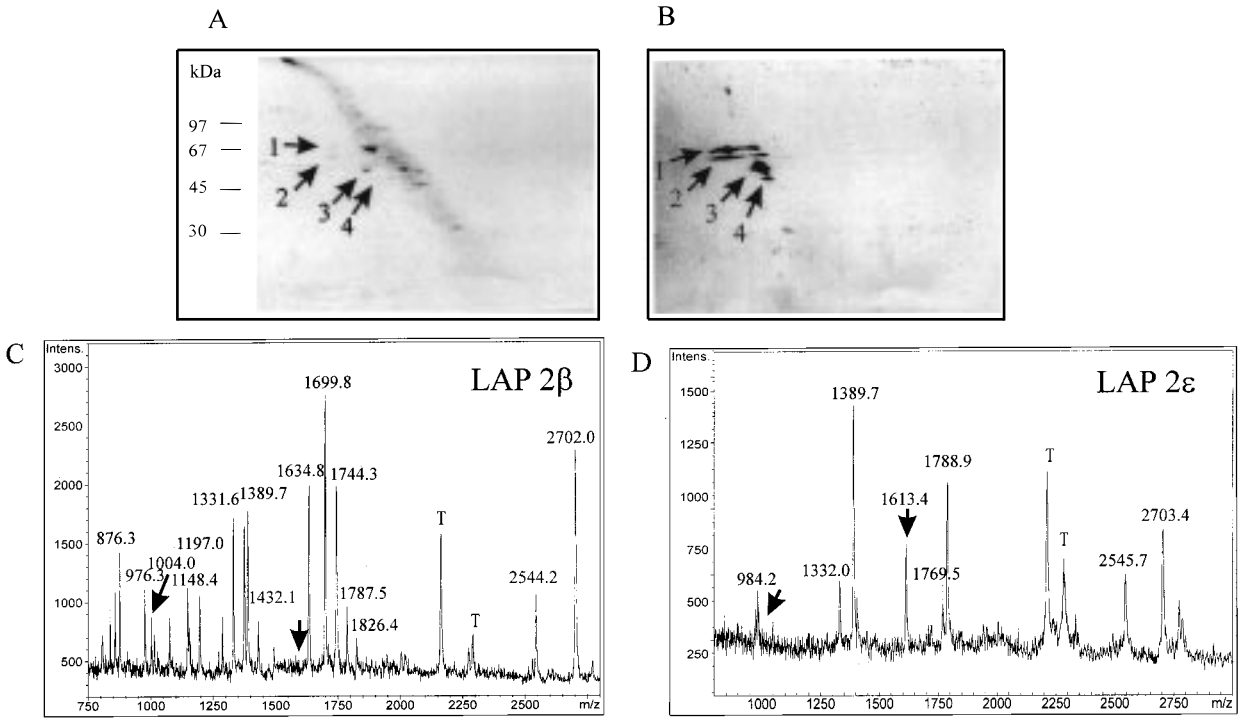

FIGURE 1: Identification of LAP 2β in nuclear envelopes separated by BAC gel electrophoresis: (A) Coomassie-stained BAC gel. Arrows indicate proteins as identified by MALDI-MS: (1) lamin A; (2) lamin C; (3) LAP 2β; (4) LAP 2ϵ. (B) Detection of the four proteins as indicated in (A) by western blotting. Immunodecoration was carried out by sequentially using anti-lamin A/C antibody (a kind gift of Dr. F. Mc Keon, Harvard Medical School, Boston, MA) and anti-LAP 2β antibody R1. (C) Peptide pattern detected by MALDI-MS as obtained from tryptic in-gel digestion of LAP 2β (spot 3). (D) Peptide pattern obtained from in gel-digestion of LAP 2ϵ (spot 4). Both proteins were identified by database searching using PeptideSearch and MS-Fit (see methods). Arrows in the spectra indicate mass peaks that are diagnostic in the discrimination between the two LAPs. T indicates autodigestion products of trypsin.

As a starting point for studying LAP 2β function and regulation, we aimed to identify native phosphorylation sites of the protein. Endogenous LAP 2β was prepared from nuclear envelopes of mouse neuroblastoma Neuro2a cells by two-dimensional electrophoretic separation of the proteins. Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) and nano-electrospray mass spectrometry were used for detection of LAP 2β and localization of the native phosphorylation sites. As the preparation of the protein started from the isolation of intact nuclei, these phosphorylation sites reflect the phosphorylation state of LAP 2β in interphase nuclei.

EXPERIMENTAL PROCEDURES

Cell Culture. Mouse neuroblastoma neuro 2a cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 μg/mL streptomycin, and 100 units/mL penicillin at 37 °C in a humidified atmosphere with 5% CO_2.

Antibodies. The polyclonal antiserum (termed R1) was raised against a C-terminal peptide of LAP 2β (residues 438−451, GNPFTNFLQDTKIS) in the laboratory of Prof. Viktor Tsetlin, Shemyakin Ovchinnikov Institute for Bioorganic Chemistry, Moscow. The antiserum recognizes the LAP 2β, γ, δ, and ϵ proteins as deduced from the apparent molecular weight in gel electrophoresis and, in the case of LAP 2β and ϵ, as confirmed by MALDI-MS analysis (Figure 1). For immunoprecipitation the antibodies were cross-linked to protein G-sepharose beads (Pharmacia) with dimethylsuberimidate by standard procedures (*15*). For use in western blots of immunoprecipitated samples, the protein G-sepharose purified antibodies were biotinylated by sulfo-NHS-biotin (Pierce) according to the supplier's instructions. Streptavidin−horseradish peroxidase conjugate (Sigma) visualized by enhanced chemoluminescence (SuperSignal detection reagent, Pierce) was used as the detection system when western blots were probed with biotinylated antibody, otherwise alkaline phosphatase conjugates of antirabbit IgGs were used.

Recombinant Proteins. For expression of full length LAP 2β bearing C-terminally a combination of a His_6- and myc-tag, LAP 2β was subcloned into the vector pHO4c.

This vector was produced by first annealing the partially overlapping oligonucleotides HIS6MYCS (5′-GCGAATTCGGGCCACCATCACCACCATCACGGCGAACAGAAACTGATCAGC-3′) and MYCABH1 (5′-GCCGGATCCTAGTTCAGATCTTCTTCGCTGATCAGTTTCTGTTC-3′) and filling in the missing 3′ ends by Pfu polymerase. The product was cut using EcoR1 and BamH1, gel-purified, and inserted between the same sites of the previously described vector pHO2c (*16*).

The LAP 2β sequence was amplified via PCR from full length LAP 2β-cDNA in pBluescript II SK (+) (kindly provided by A. J. Simon and G. Rechavi (*12*)) using Taq polymerase and the primers 98101TPB (5′-GTCTCAGCATATGCCGGAGTTCCTAGAGG-3′) and 98103TBP (5′-CTGCAATTGGCGTTGGATATTTTAGTATCTTGAAG-3′). The PCR fragment was subcloned into the pHO4c vector using standard techniques (*17*). It was inserted into the Nde 1 and EcoR1 site of the vector via its Nde 1 and Mfe 1-sites, thereby adding C-terminally the sequence ANSGHHHHHHGEQKLISEEDLN.

His_6-myc-tagged LAP 2β was purified using immobilized metal chelate chromatography (IMAC) essentially as described previously (*18*). Proteins were eluted from a 1 mL column of Ni^{2+}−NTA−agarose (Qiagen) by stepwise adding buffer containing 25, 50, 100, and 400 mM imidazole. Two 1 mL fractions were eluted at each imidazole concentration. The purity of the eluted LAP 2β was checked by SDS−PAGE and Coomassie stain or by western blotting using the anti-myc antibody 9E10 (*19*) for immunodecoration. Fractions were pooled accordingly, dialyzed into 20 mM HEPES × NaOH, pH 7.6, 150 mM NaCl, 0.5% sodium cholate, 1 mM DTT, and concentrated by ultrafiltration to a concentration of approximately 0.2 mg/mL LAP 2β.

Preparation of Nuclear Envelopes. Nuclei from neuroblastoma Neuro2a cells were prepared according to Emig et al. (*20*). Nuclear envelopes were prepared by a modification of the method described by Otto et al. (*21*). Briefly, the nuclei (5−8 mg of protein) were suspended in 40 mL of ice cold TP buffer (10 mM Tris/HCl pH 7.4, 10 mM $Na_xH_xPO_4$ pH 7.4, 1 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin) containing 250 μg/mL of Heparin, 1 mM Na_3VO_4, 10 mM NaF, and 400 units of Benzon Nuclease (Merck). The suspension was stirred for 90 min at 4 °C. Nuclear envelopes were then sedimented by centrifugation for 30 min at 10 000*g* at 4 °C and resuspended in STM 0.25 buffer (20 mM Tris/HCl pH 7.4, 0.25 M sucrose, 5 mM $MgSO_4$, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/mL aprotinin, 10 μg leupeptin) at roughly 1 mg/mL. Protein determination was performed afterward according to Bradford (*22*). Nuclear envelope preparations typically contained 6−9% of total nuclear protein.

Two-Dimensional Gel Electrophoresis and In-Gel Digestion. Nuclear envelope proteins were separated by two-dimensional benzyldimethyl-*n*-hexadecylammonium chloride (16-BAC)/SDS−polyacrylamide gel electrophoresis (BAC-/SDS PAGE) which is, due to the presence of ionic detergents in both dimensions, especially useful for the separation of integral membrane proteins (*23*). Differences in the migration of proteins in the presence of the cationic detergent 16-BAC under acidic conditions in the first dimension as compared to their mobility in SDS−PAGE according to Laemmli (*24*) used as the second dimension lead to a sickle-shaped separation pattern of proteins. In a typical experiment, 40 μg of protein per lane was loaded onto the first dimension. After two-dimensional separation of the NE proteins, the gel spots of interest were excised. The in-gel digestion was carried out as previously described (*25*). The resulting peptides were lyophilized in a vacuum centrifuge and stored at −20 °C until usage.

Immunoprecipitation. In a typical experiment, 150 μg of nuclear envelopes were initially solubilized for 15 min by incubation in 0.5% (w/v) SDS, 2% (w/v) Triton X-100, 0.3 M NaCl, and 20 mMTris/HCl pH 7.4 in the presence of 1 mM PMSF, 10 μg/mL aprotinin, and 10 μg/mL leupeptin. The samples were then diluted to obtain the final immunoprecipitation conditions of 20 mM Tris/HCl, pH 7.4, 100 mM NaCl, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, 1 mM Na_3VO_4, 10 mM NaF, 1 mM PMSF, 10 μg/mL leupeptin, and 10 μg/mL aprotinin (immunoprecipitation buffer) and solubilization was continued for another 30 min at 4 °C. The samples were then centrifuged for 10 min at 13 000 rpm in a Biofuge (Heraeus). A 50 μL volume of R1-antibody beads was added to the supernatant and constantly agitated overnight. R1-beads were allowed to settle by gravity, and the beads were washed three times with 1 mL of immunoprecipitation buffer. The beads were then incubated in Laemmli sample buffer for 5 min at room temperature. This allowed the recovery of LAP 2β from the beads whereas residual antibody dissociating from the beads remained in a high molecular weight form when subjected to SDS−PAGE. The success of immunprecipitation was confirmed by western blotting using biotinylated R1-antibody and by MALDI-MS analysis of tryptic digests from samples that were run in parallel.

In Vitro Phosphorylation and Two-Dimensional Phosphopeptide Mapping. A 0.5 mg amount of nuclear envelopes was phosphorylated by endogenous protein kinases in vitro immediately after NE preparation from either freshly prepared nuclei or nuclei that were frozen in liquid nitrogen and stored at −70 °C. The reaction was performed at a protein concentration of 0.5 mg/mL in 20 mM Tris/HCl pH7.4, 1 mM Ouabain, 1 mM Na_3VO_4, 10 mM NaF, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 0.1 mM EGTA, 1 mM PMSF, 10 μg/mL aprotinin, and 10 μg/mL leupeptin in the presence of 10 μM γ-^{32}P-ATP with an specific activity of approximately 1 μCi/nmol for 15 min at 30 °C. BAC sample buffer was added to stop the reaction. For in vitro phosphorylation using recombinant PKC α, the reaction buffer was supplemented with 100 nM phorbol-12-myristate-13-acetate-4-*O*-methyl ester (PMA). Alternatively, 10 μg of recombinant LAP 2β instead of nuclear envelopes was used as PKC α substrate. The samples were subjected immediately to BAC/SDS−PAGE or SDS−PAGE. The separated proteins were then blotted on polyvinylidene difluoride (PVDF) membranes, stained with india ink (*26*) and analyzed by autoradiography. The high reproducibility of the position of LAP 2β on the blotting membrane allowed us to pool LAP 2β spots from several blot membranes. These protein spots were excised and digested on the membrane by trypsin, and two-dimensional phosphopeptide mapping was performed (*27*) using 10 × 10 cm Cellulose-HPTLC plates (Merck). The identity of LAP 2β was checked by MALDI-MS analysis of an aliquot of the digest supernatant. The spots containing labeled phosphopeptides as visualized by autoradiography were recovered from the thin layer plates. Peptides were then eluted from the cellulose (*27*), lyophilized, and subjected to nano-electrospray-MS or MALDI-MS.

Desalting for Nano-Electrospray Mass Spectrometry. The lyophilized peptide mixture was reconstituted in 1 μL of 80% formic acid, which was quickly diluted to 10 μL by the addition of water. The peptide mixture was desalted using a pulled glass capillary filled with Poros oligoR3 sorbent (Perseptive Biosystems, Framingham, MA) to a bed volumn of approximately 100 nL. The column was equilibrated in 0.5% formic acid. After the peptide mixture was applied to the column, it was washed twice with 0.5% formic acid. The peptides were generally eluted in three steps of roughly 1 μL of 20% methanol, 50% methanol, and 50% methanol/5% ammonia. Each fraction was directly eluted into the spraying needle of the nano-electrospray ion source and analyzed individually. In one experiment, a "double column alignment" (*28*) was used for the separation of very complex phosphopeptide mixtures. In this case, two equilibrated columns filled with Poros R2 and oligoR3 sorbent (both from

Perseptive Biosystems, Framingham, MA) were aligned for the peptide loading and washing steps. The elution from each column was done as described above for the oligoR3 column.

Nano-Electrospray Mass Spectrometry. All experiments were carried out as described (*29*, *30*) on a API III triple quadrupole mass spectrometer equipped with an updated collision cell (Perkin-Ellmer Sciex Instruments, Thornhill, Canada) and the nano-electrospray ion source developed at the EMBL, Heidelberg, Germany (*29*). For Q1 scans a step width of 0.1 atomic mass units (amu) and a dwell time of 1 ms were used. Precursor and product ion scans were acquired with a step width of 0.2 amu and a dwell time of 3 ms. Argon was used as a collision gas at a collision gas thickness of 2.8×10^{14} to 3.0×10^{14} molecules/cm^2. For parent ion scans the collision energy setting was 50 V (difference between R0 and R2 potentials on the Sciex API III). The collision energy for product ion scans was adjusted individually for each experiment.

MALDI Mass Spectrometry. MALDI-TOF-MS was performed on a Bruker REFLEX mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) equipped with an ion gate and operating with continuous extraction. Aliquots of tryptic digest supernatants obtained from either in-gel digestion, on-blot digestion, or two-dimensional phosphopeptide mapping were analyzed using the fast evaparation/nitrocellulose matrix preparation as decribed (*25*). All measurements were performed in the positive ion mode using α-cyano-4-hydroxycinnamic acid as matrix compound. For analysis of fragment ions generated by post source decay (*31*), the FAST method (Bruker Daltonik) was used. For protein identification, the programs PeptideSearch (at http://www.mann.embl-heidelberg.de/Services/PeptideSearch/PeptideSearchIntro.html), MS-Fit (http://prospector.ucsf.edu/ucsfhtml/msfit.htm) and MS-Tag (http://prospector.ucsf.edu/ucsfhtml/mstag.html) were applied.

RESULTS

Detection of LAP 2β in Nuclear Envelope Preparations from Neuroblastoma Neuro2a Cells. The separation of nuclear envelope proteins by BAC/SDS−PAGE yielded a complex two-dimensional protein pattern as shown in Figure 1a. Some of the major protein spots were identified by MALDI-MS after tryptic in-gel digestion matching the obtained peptide masses to protein databases with support of the program PeptideSearch and by sequencing of the major peptides from the unseparated mixture by post source decay fragment ion analysis with support of the program MS-Tag (see methods). One of the major spots which is clearly separated from the other NE proteins was identified as LAP 2β (Figure 1c). This was also confirmed by western blotting using the antibody R1 raised against the C-terminus of the transmembrane domain of lamina-associated proteins $\beta-\epsilon$ (Figure 1b). The sequence coverage obtained from MALDI-MS spectra of the unseparated LAP 2β digest was in the range of 50−60% in a typical experiment when 0.5 μg (≈10 pmol representing the amount recovered from a single BAC-gel) of LAP 2β (as estimated from the Coomassie stain) was digested. About 5 μg of LAP 2β was typically obtained from gel spots after separation of 500 μg total nuclear envelope protein.

The antibody R1 also recognized a 45 kDa protein spot. From its apparent molecular weight, its mobility in BAC-gel electrophoresis, and the peptide pattern after tryptic digestion we assigned this spot to LAP 2ϵ (Figure 1d). This is to our knowledge the first time that LAP 2ϵ is detected at the protein level.

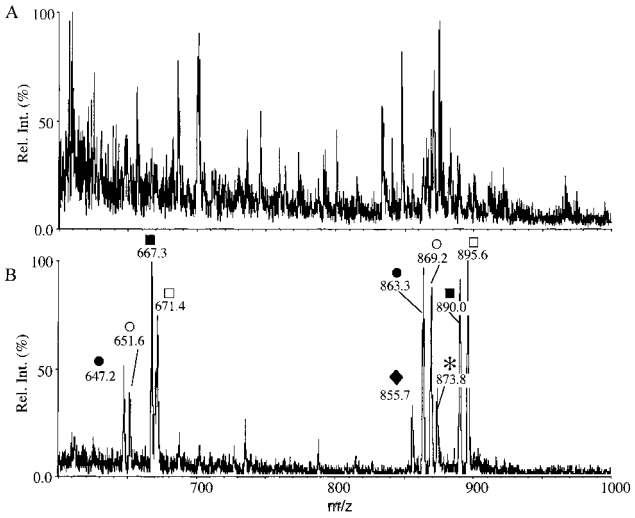

FIGURE 2: Detection of phosphopeptides of LAP 2β by parent ion scan: (A) Q1 scan of the tryptic digest in negative ion mode. All peptide ions are detected. (B) Parent ion scan of *m*/*z* 79 on the same analyte solution. Only the phosphopeptides are detected. The molecular masses are 2624 Da (∗; res 61−85, monophosphorylated), 1713 Da (♦; res 157−172, monophosphorylated), 2610 Da (■; res 173−197; monophosphorylated), 2690 Da (□; res 173−197; diphosphorylated), 2593 Da (●; res 172−197 Da; monophosphorylated with an N-terminal pyroglutamic acid), 2673 Da (○; res 172−197 Da; diphosphorylated with an N-terminal pyroglutamic acid).

All major mass peaks that were observed in the spectra could be matched to the LAP 2β sequence (GenBank accession no. U39074 (*12*)), except for the peptide of 2543.5 Da (see below). However, analysis of phosphopeptides revealed the presence of a minor amount of apparently highly phosphorylated ovary testis transcribed protein (Ott (*32*)) comigrating with LAP 2β in samples of total nuclear envelope (see below).

Detection and Identification of Phosphorylated Peptides of Native LAP 2β. The peptide mixture obtained from in-gel digestion of LAP 2β was desalted over a Poros oligoR3 microcolumn, and the peptides were eluted from this column in a stepwise fashion. Each fraction was then analyzed individually by nano-ESI-MS. For the specific detection of phosphopeptides in complex mixtures, parent ion scanning was applied. In this scanning mode, only those peptides are detected which produce a specific fragment upon collision-induced dissociation. In the case of phopsphopeptides, the "reporter" fragment is PO^{3-} (*m*/*z* 79) (*28*, *30*, *33*). By application of this technique to the unseparated digest mixture of LAP 2β after desalting, six different phosphopeptides were detected in one or two different charge states in the fraction eluted with 50% methanol (Figure 2). Elutions with 20% methanol and 50% methanol/5% ammonia did not yield any additional phosphopeptides in this experiment. From the mass spectrum, the molecular masses were calculated to be 1713, 2593, 2610, 2624, 2673, and 2690 Da. Only three of these peptides matched the masses of the predicted tryptic peptides in their mono- or diphosphorylated form (1713 Da, SST-PLTVSSSAENTR (res 157−172, monophosphorylated); 2610 and 2690 Da, QNGSNDSDRYSDNDEDSKIELK (res

173−194, mono- and diphosphorylated)). The other peptides could only be assigned to the sequence upon mass spectrometric sequencing. In the case of the 2624 Da peptide, the unphosphorylated and the phosphorylated form (2544 Da) were sequenced by both post source decay on a MALDI-TOF instrument and by CID with the nano-electrospray/triple quadrupole mass spectrometer. A sequence conflict to the database entry was discovered: instead of the predicted tryptic peptide (GPPDFSSDEERDATPVLGSGASVGR, res 61−85; calculated mass, 2503 Da (*12*)), the sequence was found to be GPPDFSSDEEREPTPVLGSGASVGR. This amino acid sequence is identical to the corresponding sequence stretch in the rat homologue of LAP 2β (*6*). Thus, the difference between the mouse and the rat sequence at this position is most likely due to a database error.

The peptides with the molecular masses 2593 and 2673 Da were also sequenced by nano-electrospray mass spectrometry, and the sequence was found to be identical to the 2610 and 2690 Da peptides (res 173−194), the difference in mass being due to a N-terminal pyroglutamate residue. This modification is often observed in peptides containing N-terminal glutamine residues and may be introduced during sample preparation.

Localization of the Phosphorylation Sites. Once the phosphopeptides were identified with a parent ion scan of *m*/*z* 79 in negative ion mode, the mode of ionization was switched to positive and product ion scans were performed on the same sample. The mass spectrum of the product ion of *m*/*z* 857.5 ($(M + 2H)^{2+}$ of the 1713 Da peptide) allowed the localization of the phosphorylation site to residue Thr 159 by a N-terminal B ion series (Figure 3a). Using the same approach for the detection of phosphopeptides by parent ion scans and localization of the phosphorylated residue byproduct ion scans, the phosphorylation sites of the other phosphopeptides were also determined (see Table 1) in an additional experiment, using more starting material than in the first experiment. In the case of the 2624 Da peptide, the phosphorylation site was determined to be Thr 74 by two partial Y″ ion series (C-terminal ion series, Figure 3b). The doubly phosphorylated form of this peptide (2705 Da) was also observed (data not shown), but it was not possible to localize the phosphate moiety. Both Thr 74 and Thr 159 are part of the consensus sequences for phosphorylation by proline-directed kinases, e.g. cyclin-dependent kinase 2 (*34*). Thr 74 is located within the proposed chromatin binding region (*35*); Thr 159, in a region of unknown function.

The phosphorylation site of peptide 2610 Da (or 2593 Da, for the pyroglutamate form, see above) (QNGSNDSDRYSDNDEDSKIELK, res 173−194, monophosphorylated) was localized to Ser 179, and the diphosphorylated peptide (2690 or 2673 Da for the pyroglutamate form) was found to be additionally phosphorylated at Ser 176, since the partial sequence obtained excludes a phosphorylation site C-terminal of Ser 179, leaving S 176 as the only residue which could be phosphorylated in the sequence strech QNGSND (res 173−178, data not shown). Ser 179 is part of a consensus sequence for phosphorylation by conventional protein kinase C isoforms (*36*); Ser 176 is not part of a known consensus phosphorylation sequence.

In the second experiment with more starting material than in the first one, two additional minor phosphopeptides were observed (4226 and 4306 Da), which were not detected in the first experiment and are derived by incomplete tryptic digestion. These masses fit for the mono- and diphosphorylated forms of a tryptic peptide spanning residues 157−194 and, therefore, are combining two of the already identified phosphopeptides (1713 and 2610 Da). Interestingly, five additional phosphopeptides were detected by parent ion scan, which could not be assigned to the sequence of LAP

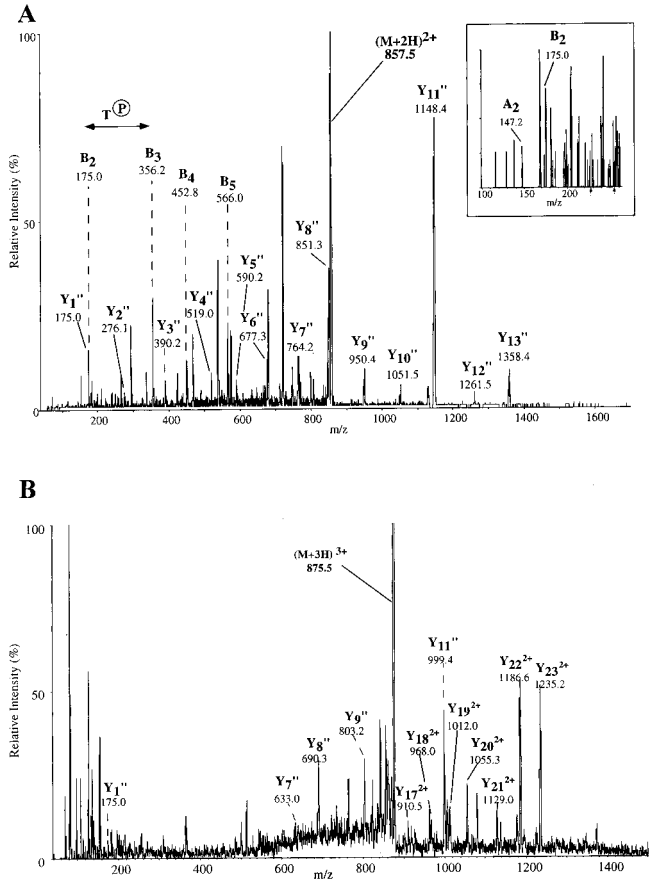

FIGURE 3: Identification of phosphorylation sites. Product ion scan spectra obtained by collision-induced dissociation of the phosphopeptides: (A) $(M + 2H)^{2+} = 857.5$ Da (corresponding to 1713 Da, res 157−172, monophophorylated); (B) $(M + 3H)^{3+} = 875.5$ Da (corresponding to 2624 Da, res 61−85, monophosphorylated). The most prominent fragment ion series in product ion scan spectra is the Y″ ion series derived from C-terminal fragments. (A) The peptide sequence is SSTPLPTVSSSAENTR. As the Y''_{13} ion has the expected mass of the nonphosphorylated fragment, the phosphate moiety must be located within the three N-terminal amino acids. Due to the presence of a strong N-terminal or B-ion series in the spectrum, the phosphate moiety can be unambiguously located to Thr 159 (the B_2 ion has the expected mass of the nonphosphorylated fragment, the B_3 ion of the phosphorylated fragment). Insert: Fragment ion spectrum of the phosphopeptide acquired in the low *m*/*z* range with high collision energy. The A_2 and the B_2 ions are both observed for the peptide. If the phosphate group was attached to either of the first two Ser in the peptide, the A_2 and B_2 ions would be expected at 227 and 255 Da, respectively (marked with small arrows). Additional proof is a weaker B ion series which has lost phosphoric acid (B-98). This sequence starts only with the B_3 ion (which includes Thr 159), as expected for the threonine being the phosphorylated residue. (B) The peptide sequence is GPPDFSSEEREPTPVLGSGASVGR. Thus, if Thr 74 was phosphorylated, the phosphate moiety should first be observable in the Y''_{12} ion. In the spectrum, the Y''_{11} ion is in the nonphosphorylated state and the (double charged) Y''_{17} ion in the phosphorylated state. This indicates that the phosphate moiety must be located in the stretch from Glu 69 to Thr 74. Thr 74 is the only phosphorylatable amino acid within this stretch.

Table 1: Phosphopeptides Detected by Nano-Electrospray Parent Ion Scan and Sequenced by Tandem Mass Spectrometry[a]

peptide (m/z)/ charge state	peptide mass unphos-phorylated (Da)	phosphopeptide mass (Da)	sequence (residues)	phosphorylated residue
855.8/(M − 2H)$^{2-}$	1632.8	1713.4 (1P)	SS***T***PLPTVSSSAENTR (157−172)	Thr 159
651.6/(M − 4H)$^{4-}$ 869.3/(M − 3H)$^{3-}$	2529.5	2610.5 (1P)	QNGSND***S***DRYSDNDEDSKIELK (173−194)	Ser 179
671.4/(M − 4H)$^{4-}$ 895.6/(M − 3H)$^{3-}$	2529.5	2689.7 (2P)	QNG***S***ND***S***DRYSDNDEDSKIELK (173−194)	Ser 179, Ser 176
647.2/(M − 4H)$^{4-}$ 863.2/(M − 3H)$^{3-}$	2512.6	2592.8 (1P)	pyENGSND***S***DRYSDNDEDSKIELK (173−194)	Ser 179
667.3/(M − 4H)$^{4-}$ 890.0/(M − 3H)$^{3-}$	2512.6	2673.1 (2P)	pyENG***S***ND***S***DRYSDNDEDSKIELK (173−194)	Ser 179, Ser 176
873.4/(M − 3H)$^{3-}$	2543.7	2624.4 (1P)	GPPDFSSDEEREP***T***PVLGSGASVGR (61−85)	Thr 74
540.0/(M − 5H)$^{5-}$ 675/(M − 4H)$^{4-}$	2543.7	2704.6 (2P)	GPPDFSSDEEREP***T***PVLGSGASVGR (61−85)	Thr 74, second phosphate moiety unknown
603.0/(M − 7H)$^{7-}$ 703.0/(M − 6H)$^{6-}$	4146.3	4226.3 (1P)	SSTPLPTVSSSAENTRQNGSNDSDRYSDNDEDSKIELK (157−194)	combination of 1713.4 + 2610.5 with an uncleaved site
614.0/(M − 7H)$^{7-}$	4146.3	4306.3 (2P)	SSTPLPTVSSSAENTRQNGSNDSDRYSDNDEDSKIELK (157−194)	combination of 1713.4 + 2610.5 with an uncleaved site

[a] This table comprises data from several parent ion scan experiments as shown in Figure 2. Phosphoamino acids are written in bold italicized letters. These data refer to CID experiments such as those shown in Figure 3. pyE: pyroglutamic acid (considered as an artificial modification probably arising during sample preparation).

2β. Mass spectrometric sequencing of one of these peptides and a subsequent database search identified ovary testis transcribed protein (Ott (*32*)) as a second protein migrating at the same position as LAP 2β. However, the protein constituted likely a minor contamination, as no mass peaks derived from Ott were observed in MALDI peptide mapping.

Pattern of LAP 2β Phosphopeptides after in Vitro Phosphorylation by Endogenous Kinases. In vitro phosphorylation of nuclear envelope proteins by endogenous kinases in the presence of γ-^{32}P-ATP followed by two-dimensional phosphopeptide mapping was applied as an alternative approach for the micropreparation of LAP 2β phosphopeptides. The phosphorylation reaction was performed under conditions optimal for both protein serine/threonine as well as protein tyrosine kinases. LAP 2β was isolated as described in the methods section. In this experiment, approximately 100 cpm as determined by Cerenkov counting and corresponding to 100 fmol of total phosphopeptide was recovered from the blotting membrane and subjected to the thin layer plate.

The two-dimensional separation yielded a complex pattern of ^{32}P-labeled peptides (Figure 4a). The areas on the thin layer plate containing labeled peptides were recovered. The results of the measurements are shown in Figure 4b (see also Table 1 for the assignment of the mass peaks). Mass spectrometric analysis of samples from the spots not shown in Figure 4b revealed no interpretable phosphopeptide signals despite even more intense signals in the autoradiography. This is most likely due to ^{32}P incorporation into previously already partly phosphorylated sites. The ^{32}P incorporation during in vitro phosphorylation alone did not yield detectable amounts of phosphopeptides. Thus, rather than allowing the detection of phosphorylation sites formed de novo during in vitro phosphorylation, in our experiment the ^{32}P-label apparently served to visualize the position of phosphopeptides containing phosphorylation sites already partly occupied prior to in vitro phosphorylation. All of the phosphopeptide masses detected (2624, 2690, 2592, and 1713 Da) corresponded to phosphopeptides also detected by the nano-electrospray parent ion scan of the entire digest of wild-type LAP 2β. This suggests that the same phosphorylation sites that are used in vivo during interphase can be phosphorylated under in vitro phosphorylation conditions by kinases present in the nuclear envelope preparation.

LAP 2β Is a Substrate for Protein Kinase C α. Our finding that, in endogenous LAP 2β prepared from nuclear envelopes of Neuro2a cells, Ser 179, which is part of a predicted protein kinase C phosphorylation consensus sequence, is partially phosphorylated suggests that LAP 2β is a nuclear substrate of protein kinase C. To investigate whether LAP 2β in its native environment is a substrate for exogenous PKC, NE were incubated with recombinant PKC α (kindly provided by Stefan Wagner) in the presence of γ-^{32}P-ATP and the PKC activator phorbol ester. Aliquots of the reaction mixture were separated by BAC/SDS−PAGE. LAP 2β was immunoprecipitated from the remaining material, separated by SDS−PAGE, electroblotted, and analyzed by autoradiography. Considering the overall phosphorylation pattern of total NE proteins, LAP 2β turned out to be a major substrate for activated exogenous PKC α (Figure 5a,b). The capability of PKC α to phosphorylate LAP 2β was further confirmed by performing the phosphorylation reaction using recombinant LAP 2β (Figure 5c), demonstrating that LAP 2β can be directly phosphorylated by PKC α.

DISCUSSION

The proof that a predicted phosphorylation site is indeed a target for an endogenous kinase is a key step toward the detailed understanding of the regulation of protein function. LAP 2β as well as other proteins residing in or associated with the inner nuclear membrane are known to be regulated by phosphorylation. This is best understood in the context of disassembly of nuclear structures at the beginning of mitosis (*1*). LAP 2β was demonstrated to be phosphorylated at the beginning of mitosis probably accompanied by disruption of its interaction with lamins and chromatin (*10*).

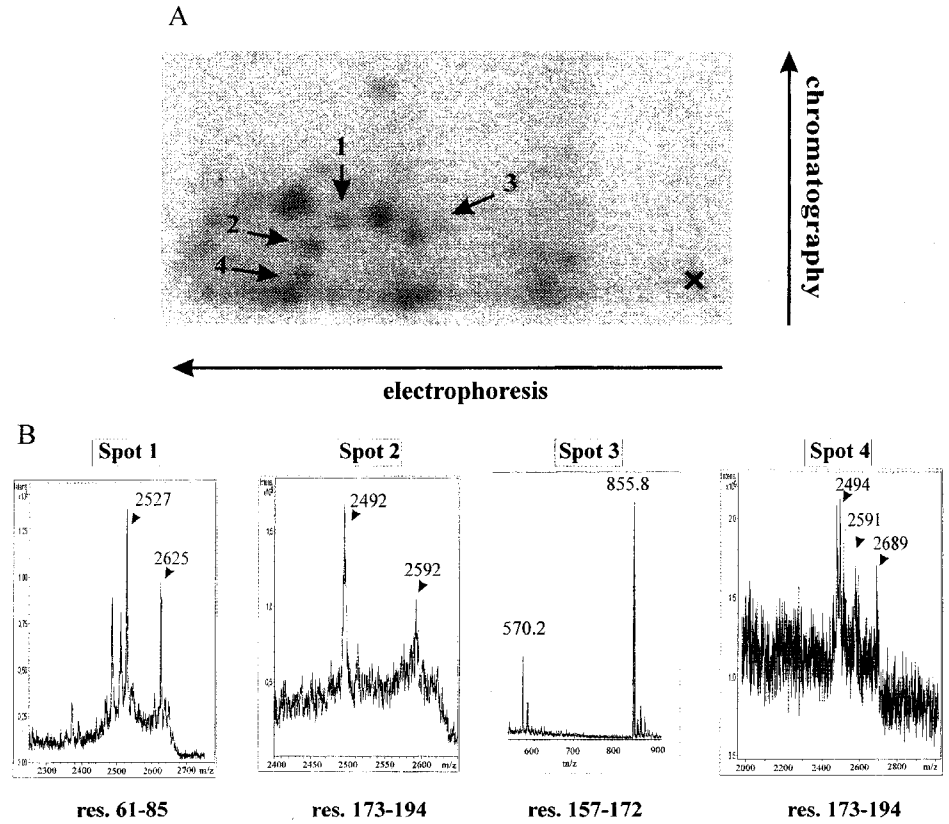

FIGURE 4: Analysis of LAP 2β phosphopeptides by two-dimensional phosphopeptide mapping: (A) Autoradiography of the thin layer plate after separation of the LAP 2β digest; arrows indicate spots corresponding to the peptides that were detected by mass spectrometry as shown in (B). (B) Spots 1, 2, and 4 (MALDI-MS spectra in the reflectron mode): spot 1, 2625 Da (res 61−85, monophosphorylated); spot 2, 2592 Da (res 173−194 with N-terminal pyroglutamic acid, monophosphorylated); spot 4, 2689 Da (res 173−194, diphosphorylated). The loss of 98 Da or 98 + 98 Da in MALDI-MS reflectron mode measurements corresponding to one or two phosphate moieties from the parent ion mass represents a hallmark for Ser/Thr phosphorylation. Spot 3: 1713 Da (res 157−172) in double and triple negatively charged state detected by nano-electrospray parent ion scan.

Furthermore, the existence of at least five differentially phosphorylated isoforms of the nontransmembrane LAP 2α, a splice variant sharing the first 187 amino acids with LAP 2β (*7*, *12*), was demonstrated at the onset of mitosis (*37*). However, no phosphorylation site of any LAP has been identified yet.

In the present study, we found that LAP 2β is phosphorylated at various sites in interphase nuclei. As the isolation of the protein started with the preparation of intact nuclei, mitotic phosphorylation states can be ruled out. The protein was also observed as one of the major proteins phosphorylated in vitro by kinases endogenous to nuclear envelope preparations from mouse neuroblastoma Neuro2a cells. Our approach for the identification of the phosphorylation sites was based on the two-dimensional separation of NE proteins by BAC/SDS−PAGE. This technique enabled us to isolate in one step 5−10 μg LAP 2β in sufficient purity for a detailed analysis of phosphorylation sites by mass spectrometric methods.

Three major phosphopeptides of LAP 2β prepared from nuclear envelopes of Neuro2a cells without prior stimulation of the cells or in vitro phosphorylation were detected. Two of these peptides occurred in the mono- as well as in the diphosphorylated state. Thr 74, Thr 159, Ser 179, and Ser 176 were identified as phosphorylated residues by mass spectrometric sequencing of the tryptic phosphopeptides corresponding to res 61−85 (observed as mono- and diphosphorylated; the second phosphorylation site was not identified), res 157−172 (observed monophosphorylated), and res 173−194 (observed mono- and diphosphorylated). Due to incomplete tryptic cleavage, we also detected peptides combining the residues 157−194 in different phosphorylated states (see Table 1).

All of these sites reside in the N-terminal portion of LAP 2β shared by the entire LAP subfamily (Figure 6). They provide consensus phosphorylation sites for proline directed kinases such as cdc2 kinase and cdk2 or MAP kinase (Thr 74 and Thr 159) and for protein kinase C (Ser 179). This portion of the protein was recently demonstrated to prevent membrane binding to chromatin when added as recombinant fragment to a cell-free *Xenopus laevis* nuclear assembly reconstitution system (*14*).

In particular the results obtained from parent ion scans by nano-electrospray-MS (see below) specific for phosphopeptides demonstrate the unique power of this technique in the de novo characterization of protein phosphorylation. However, it should be noted that not all phosphopeptides may be detectable. Also, neither MALDI-MS nor nano-electrospray-MS measurements allow determination of the stoichiometry of the phosphorylation. As can be seen in the MALDI-spectra in Figure 1, at least some of the major phosphopeptides were also detected in the nonphosphorylated state. This may be due to losses during the sample preparation or due to different phosphorylation states of LAP 2β within interphase nuclei. As phosphopeptides are less efficiently ionizable in positive mode of MALDI-MS and nano-

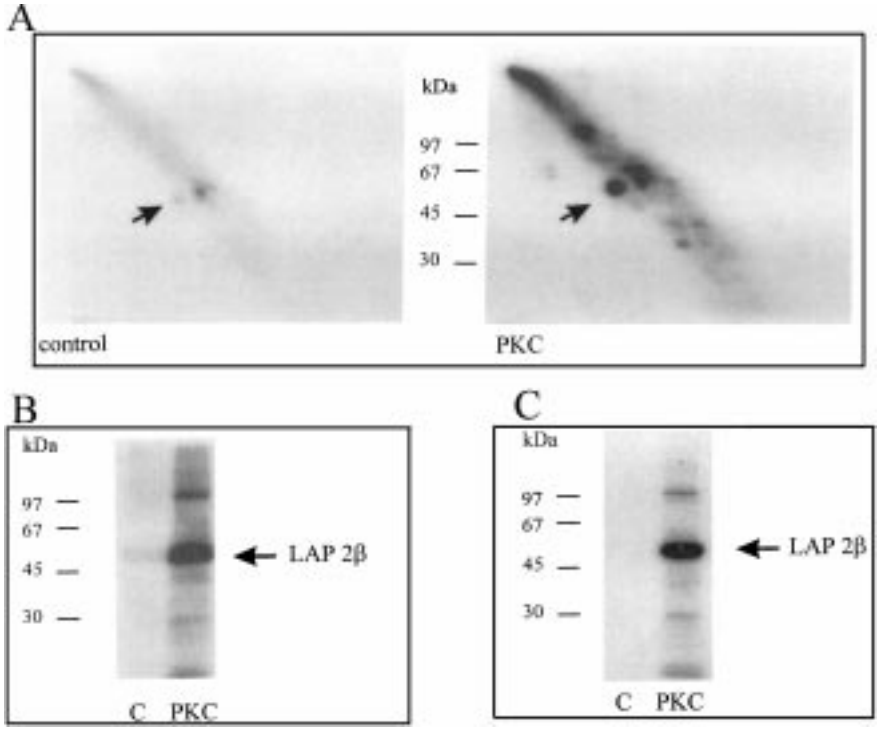

FIGURE 5: LAP 2β is a substrate for activated PKC α *in vitro*: (A) Autoradiographies of BAC gels showing separated nuclear envelope proteins after in vitro phosphorylation in the presence of γ-^{32}P-ATP by endogenous kinases (control) or in the presence of additional activated PKC α (PKC). The arrows indicate the position of LAP 2β. (B) Autoradiography of immunoprecipitated proteins from samples corresponding to (A). Lane C: LAP 2β immunoprecipitated by R1-protein G sepharose after phosphorylation of NE proteins by endogenous kinases. Lane PKC: LAP 2β immunoprecipitated after the phosphorylation of NE proteins by added PKC α. Equal amounts of immunoprecipitated LAP 2β were loaded as detected by western blotting using the biotinylated R1 antibody and Streptavidin−peroxidase for visualization by enhanced chemoluminescence. No LAP 2β was immunoprecipitated when the immunization peptide was present during the immunoprecipitation. (data not shown). (C) Autoradiography of recombinant LAP 2β separated by SDS−PAGE after in vitro phosphorylation in the absence of exogenous kinases (lane C) or in the presence of activated PKC α (lane PKC). Equal amounts of recombinant LAP 2β were loaded as assessed by western blotting.

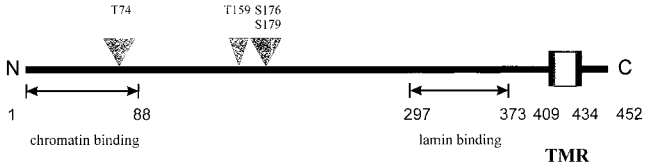

FIGURE 6: Schematic representation of the localization of the LAP 2β phosphorylation sites at interphase. All phosphorylation sites detected are localized within the residues 1−187, a region of the protein that is shared by the entire LAP 2 protein subfamily. Thr 74 is located within the chromatin binding region (*34*); the other phosphoamino acids are located within a region of unknown function. No phosphorylation was found in the lamin-binding region (*34*).

electrospray product ion scan, the signal intensity of the nonphosphorylated species is likely to be exaggerated as compared to that of the phosphorylated species.

What do our findings mean in the context of the current knowledge about structure and function of LAP 2β? It is known that the chromatin binding region of the rat homologue of LAP 2β was mapped to res 1−88 and the minimal lamin binding fragment was identified as res 297−373 (*35*). Thus, the phosphorylated residue Thr 74 is located within the putative chromatin binding region, and actually its sequence context creates the only consensus sequence for phosphorylation by proline-directed kinases such as cdc2 kinase within this chromatin binding region.

Thus, our finding that this residue is phosphorylated in LAP 2β prepared from intact interphase nuclei is not consistent with the simple assumption that a possible mitotic phosphorylation by cdc2 kinase in this region might be responsible for the phosphorylation-dependent abolishment of the LAP 2β-chromatin interaction described by Foisner and Gerace (*10*). As the knowledge about phosphorylation consensus sequences for different proline directed kinases at the level of the substrate primary structure does not allow an exact prediction which kinase may phosphorylate LAP 2β at the identified sites in vivo (in other substrates, there exist, e.g., both "optimal" and "suboptimal" sites for cdc2 kinase which both may be used (*38*)), other proline directed kinases such as MAP kinase may be capable of phosphorylating LAP 2β. Cdc 2 kinase is regarded as a candidate kinase for mitotic LAP 2β phosphorylation in analogy to the phosphorylation of other nuclear envelope proteins, e.g. the B-type lamins and the lamin B-receptor by this kinase (*39*, *40*). However, other proline-directed kinases are active in the nucleus throughout the cell cycle like Cdk 2 (*41*).

No phosphorylation site was detected so far in the minimal lamin binding fragment (res 297−373). According to the notion that the LAP 2β/B-type lamin interaction is abolished by mitotic phosphorylation (*10*), one would expect phosphorylation to affect this region of the protein. Although we cannot rule out that we fail to detect some phosphopeptides, our results are consistent with the assumption that phosphorylation of this region may be primarily mitotic.

Ser 176 was found to be phosphorylated but is not located within a known consensus phosphorylation sequence. How-

ever, the partly simultaneous detection of phosphothreonine 159, phosphoserine 179, and Ser 176 as third phosphorylated residue within a sequence stretch of only 17 amino acids (Table 1) favors the existence of a highly phosphorylated region in LAP 2β in the interphase NE (Figure 6). This region might serve as target for different protein kinases and thus may be capable of integrating signals from different signal transduction pathways.

Our in vitro phosphorylation experiments suggest that LAP 2β is one of the major substrates for activated PKC α within the nuclear envelope. Nuclear PKC isoenzymes appear to play multiple roles during the cell cycle, e.g. at the G1/S-phase and at the G2/M-phase transition (*42*). Phosphorylation of lamin B1, one of the binding partners of LAP 2β in interphase nuclei (*10*), by PKC βII isoenzyme leads to disassembly of the nuclear lamina and is regarded as one of the first steps of nuclear envelope disassembly at the onset of mitosis (*43*). Recently Collas demonstrated that nuclear envelope disassembly in the zebrafish requires sequential phosphorylation of both the zebrafish homologues of B-type lamin and LAP 2β by PKC and Cdc 2 kinase (*44*). Translocation of PKC α to the nuclear envelope and intranuclear structures of neuroblastoma Neuro2a cells upon stimulation by phorbol ester has been reported (*45*). Assuming that LAP 2β is a PKC α substrate in vivo, this notion may link LAP 2β phosphorylation to nuclear lipid signaling, e.g. in response to mitogenic signals.

To clarify the role of phosphorylation of LAP 2β by multiple protein kinases, a detailed analysis of its phosphorylation state resolved with respect to physiological signals is required.

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