

Phosphorylation on Protein Kinase C Sites Inhibits Nuclear Import of Lamin B₂

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Abstract. The nuclear lamina is a karyoskeletal structure at the nucleoplasmic surface of the inner nuclear membrane. Its assembly state is regulated by phosphorylation of the intermediate filament type lamin proteins. Strong evidence has been obtained for a causal link between phosphorylation of lamins by the p34^{cdc2} protein kinase and disassembly of the nuclear lamina during mitosis. In contrast, no information is currently available on the role of lamin phosphorylation during interphase of the cell cycle. Here, we have identified four protein kinase C phosphorylation sites in purified chicken lamin B₂ as serines 400, 404, 410, and 411. In vivo, the tryptic peptide containing serines 400 and 404 is phosphorylated throughout interphase, whereas serines 410 and 411 become phosphorylated

specifically in response to activation of protein kinase C by phorbol ester. Prompted by the close proximity of serines 410/411 to the nuclear localization signal of lamin B₂, we have studied the influence of phosphorylation of these residues on nuclear transport. Using an in vitro assay, we show that phosphorylation of lamin B₂ by protein kinase C strongly inhibits transport to the nucleus. Moreover, phorbol ester treatment of intact cells leads to a substantial reduction of the rate of nuclear import of newly synthesized lamin B₂ in vivo. These findings have implications for the dynamic structure of the nuclear lamina, and they suggest that the modulation of nuclear transport rates by cytoplasmic phosphorylation may represent a general mechanism for regulating nuclear activities.

THE nuclear lamina is a protein meshwork composed of intermediate filament type proteins called lamins (Aebi et al., 1986; Fisher et al., 1986; McKeon et al., 1986; for reviews see Gerace and Burke, 1988; McKeon, 1991; Nigg, 1992a and b). As is characteristic of all intermediate filament proteins, lamins display a central α -helical rod domain, flanked by NH₂- and COOH-terminal non- α -helical end domains (for reviews see Gerace and Burke, 1988; Nigg, 1989; Stewart, 1990; McKeon, 1991). In addition, lamins contain a nuclear localization signal (NLS)¹ (Loewinger and McKeon, 1988; Chelsky et al., 1989), and a COOH-terminal tetrapeptide motif known as the CaaX box (C = Cys; a = aliphatic amino acid, X = any amino acid). The CaaX box is subject to three successive posttranslational modifications (farnesylation, proteolytic trimming, and carboxyl-methylation), which are required for association of newly synthesized lamins with the nuclear membrane (Vorbürger et al., 1989a; Farnsworth et al., 1989; Krohne et al.,

1989; Holtz et al., 1989; Beck et al., 1990; Pollard et al., 1990; Kitten and Nigg, 1991).

In vertebrates, lamins can be classified as A- or B-type, depending on structural characteristics and biochemical properties (for reviews see McKeon, 1991; Nigg, 1992a and b). Two distinct B-type lamins (B₁ and B₂) are expressed in virtually all somatic cells (Lehner et al., 1986a; Peter et al., 1989; Vorbürger et al., 1989b; Weber et al., 1990; Höger et al., 1988, 1990), but the expression of A-type lamins is developmentally controlled (Lehner et al., 1987; Stewart and Burke, 1987; Wolin et al., 1987; Röber et al., 1989, 1990), suggesting that differential expression of A-type lamins might functionally relate to developmental processes (for discussion see Nigg, 1989; Röber et al., 1989; Peter and Nigg, 1991). A- and B-type lamins display distinct fates also during cell division. When the lamina depolymerizes at the onset of mitosis, A-type lamins are solubilized, but B-type lamins remain associated with membranous structures (Gerace and Blobel, 1980; Stick et al., 1988; for discussion see Nigg et al., 1992).

The mitotic disassembly of the nuclear lamina has long been proposed to result from hyperphosphorylation of lamin proteins (Gerace and Blobel, 1980; Ottaviano and Gerace, 1985; Miake-Lye and Kirschner, 1985). In support of this notion, several recent studies indicate that nuclear lamins are

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1. *Abbreviations used in this paper:* NCS, N-chlorosuccinimide; NLS, nuclear localization signal; PKA, cAMP-dependent protein kinase; PKC, protein kinase C.

direct substrates of the p34^{cdc2}/cyclin B protein kinase, a major regulator of the cell cycle (Peter et al., 1990a, 1991; Dessev et al., 1991; Lüscher et al., 1991; Enoch et al., 1991). The major sites phosphorylated by the p34^{cdc2}/cyclin B complex were mapped to SP/TP motifs flanking the central α -helical rod domain of lamin proteins (Peter et al., 1990a; Ward and Kirschner, 1990), and mutational analyses demonstrate that phosphorylation of cdc2 phosphoacceptor sites is required for lamina disassembly both in vivo (Heald and McKeon, 1990) and in vitro (Peter et al., 1991).

Lamins are phosphorylated not only during mitosis but also during interphase (Gerace and Blobel, 1980; Ottaviano and Gerace, 1985; Peter et al., 1990a; Ward and Kirschner, 1990). However, while the role of lamin phosphorylation during mitosis is comparatively well understood, the functional significance of lamin phosphorylation during interphase remains to be clarified. In vitro, purified lamins can be phosphorylated by several kinases, including cAMP-dependent protein kinase (PKA; Peter et al., 1990a; Foisner et al., 1991), protein kinase C (PKC; Fields et al., 1988, 1990; Hornbeck et al., 1988; Peter et al., 1990a; Ward and Kirschner, 1990; Tsuda and Alexander, 1990; Kasahara et al., 1991) and MAP kinases (Peter et al., 1992). The PKA and PKC sites also become phosphorylated in vivo, when cells are treated with agonists inducing the activation of PKA or PKC, respectively (Hornbeck et al., 1988; Fields et al., 1988, 1990; Peter et al., 1990a; Tsuda and Alexander, 1990).

The purpose of the present study was to identify major in vivo phosphorylation sites in chicken lamin B₂ during interphase of the cell cycle, to assess the role of PKC in the phosphorylation of these sites, and to explore the possible functional consequences of these phosphorylations. Our results indicate that major interphase phosphorylation sites are located in the COOH-terminal end domain of lamin B₂, and that PKC is likely to phosphorylate these sites not only in vitro but also in vivo. Furthermore, we show that phosphorylation on PKC sites substantially reduces the rate of nuclear transport of lamin B₂. Inhibition of nuclear transport is observed in vitro, as well as in living cells that have been treated with PMA. These results demonstrate that nuclear transport rates depend on the state of activity of cytoplasmic protein kinases in vivo, and that the nuclear import of newly synthesized proteins is subject to regulation by extracellular signals.

Materials and Methods

Cell Culture and Metabolic Labeling

Chicken hepatoma (DU249) and HeLa cells were cultured as described previously (Nakagawa et al., 1989; Kitten and Nigg, 1991). For in vivo phosphorylation studies, exponentially growing DU249 cells were incubated for 4 h in 90% phosphate-free MEM, 10% DME, 10% dialyzed FCS, 1% dialyzed chicken serum and 500 μ Ci/ml of ³²P-orthophosphate (Amersham Corp., Arlington Heights, IL). Where indicated, cells were treated with PMA (Sigma Chemical Co., St. Louis, MO) during the last 30 min of the labeling period. PMA was added to a final concentration of 0.32 μ M (from a 1,000 \times stock solution prepared in ethanol); control cultures received the corresponding volumes of ethanol.

Pulse-chase labeling experiments were carried out essentially as described by Lehner et al. (1986b). Briefly, exponentially growing DU249 cells were washed with prewarmed methionine-free MEM and incubated for 30 min in methionine-free MEM, supplemented with 4% dialyzed FCS

(GIBCO BRL, Gaithersburg, MD) and 1% penicillin/streptomycin (GIBCO BRL). Then, they were labeled for 15 min by incubation in prewarmed labeling medium (methionine-free MEM containing 4% dialyzed FCS, 1% penicillin/streptomycin, 0.2 mCi/ml Tran[³⁵S] label [New England Nuclear, Boston, MA]). Where indicated, PMA (to a final concentration of 0.32 μ M) was included into the labeling medium. After removal of the labeling medium, cells were washed twice with normal growth medium and incubated further at 37°C in growth medium containing 5 mM unlabeled methionine.

Immunochemical Techniques

Lamin B₂ was studied using either the lamin B₂-specific mAbs E-3 and L-20, or a lamin A/B₂-specific rabbit serum (Lehner et al., 1986a; Kitten and Nigg, 1991). For immunoprecipitations, metabolically labeled cells were washed twice with ice-cold PBS and lysed in P-RIPA (1% Triton X-100, 1% deoxycholate, 0.1% SDS, 20 mM sodium phosphate (pH 7.2), 100 mM NaCl, 20 mM NaF, 0.02% Na₃N₃). Immunoprecipitations using the mAb E-3 were performed as described previously (Nakagawa et al., 1989). In the case of immunoprecipitations with the rabbit serum, immune-complexes were collected using 50 μ l protein A-Sepharose (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ).

Immunoblotting experiments were carried out as described previously (Borer et al., 1989), and the processing of samples for indirect immunofluorescence microscopy has also been described (Nigg et al., 1985; Kitten and Nigg, 1991). Immunofluorescence experiments were carried out using the chicken-specific anti-lamin B₂ mAb L20 (Lehner et al., 1986a).

Enucleation Experiments

The protocol for enucleation experiments has been described in Krek et al. (1992). In brief, cell monolayers were grown on round (20-mm diam) plastic supports that had been cut from tissue culture dishes. After carrying out the appropriate pulse-chase labeling experiments (see above), the plastic supports were placed, cell side down, into Corex^R tubes containing 10 ml of prewarmed DME, supplemented with cytochalasin B (5 μ g/ml). Samples were immediately centrifuged for 8 min at 30–37°C (9,500 rpm; Sorvall HB4 rotor). Cytoplasts and nuclear pellets were solubilized separately in P-RIPA buffer and incubated for 15 min on ice. Then, DNase and RNase were added to a final concentration of 100 μ g/ml each. After a further 15 min incubation on ice, and a 10 min centrifugation (13,000 g; 4°C), supernatants were used for immunoprecipitation experiments. For control, total extracts were prepared from unfractionated cells and analyzed in parallel. Data were quantified by laser densitometric scanning and analyzed assuming first-order kinetics of nuclear uptake.

In Vitro Transport Assays

In vitro transport assays were carried out essentially as described by Adam et al. (1990), with the following slight modifications: coverslips with digitonin-permeabilized HeLa cells were placed on a 50 μ l drop of prewarmed (37°C) transport mix containing 200 ng bacterially expressed purified lamin B₂ as a transport substrate. The transport mix consisted of transport buffer supplemented with 25 μ l of reticulocyte lysate (Promega Corp., Madison, WI; precleared by centrifugation at 100,000 g for 30 min), 1 mM ATP, 5 mM creatine phosphate, 300 μ g/ml creatine kinase (Boehringer Mannheim Corp., Indianapolis, IN), 0.27 TIU/ml aprotinin, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin, 1.5 μ M quercetin, 80 mM β -glycerophosphate, 2 μ M okadaic acid. For controls, samples were incubated in parallel at 4°C; alternatively, ATP as well as the ATP regenerating system were omitted from the transport mix. All samples were then placed in a humidified cell incubator (5% CO₂) at 37°C. After the times indicated, cells were washed three times for 5 min with ice cold transport buffer and either directly lysed in gel sample buffer for immunoblotting, or processed for indirect immunofluorescence microscopy.

In Vitro Kinase Assays

Wild-type lamin B₂ as well as NH₂- and COOH-terminal deletion mutants were expressed in *Escherichia coli* as described previously (Heitlinger et al., 1992). For in vitro phosphorylation reactions, proteins were either purified (Heitlinger et al., 1991) or immunoprecipitated from bacterial lysates using the rabbit anti-lamin A/B₂ serum. Bacterial lysates were prepared exactly as described previously (Peter et al., 1991). Immunoprecipitates were washed four times with P-RIPA buffer, twice with appropriate

kinase buffer, and then subdivided into aliquots. One aliquot was routinely used for controlling the efficiency of immunoprecipitation by immunoblotting, the others were used for *in vitro* phosphorylation reactions.

The conditions for phosphorylating lamin proteins with purified PKC and p34^{cdc2}/cyclin B have been reported previously (Peter et al., 1990a, 1991). As sources of PKC we used either a mixture of the α , β , and γ isoforms purified from bovine brain (Marais and Parker, 1989), or the γ -subunit obtained from insect cells infected with recombinant baculovirus (Stabel et al., 1991), with virtually identical results.

For phosphorylation of purified lamin B₂ proteins, these were dialyzed into 50 mM Tris-HCl pH 7.5. Then conditions were adjusted to 10 mM MgCl₂, 0.5 mM CaCl₂, 5 μ g/ml dicaproyl (Sigma Chemical Co.) and 50 μ g/ml phosphatidylserine (Sigma Chemical Co.) and 1 mM ATP or, where indicated, 10 μ Ci [γ -³²P]-ATP. Approximately 2 μ g of lamin B₂ were phosphorylated with 5 μ l PKC (1.5 pmol/min/ μ l) or 0.5 μ l p34^{cdc2}/cyclin B (100 pmol/min/ μ l). After incubation for 30 min at 30°C, reactions were stopped, either by freezing the samples at -20°C, or by adding gel sample buffer and boiling for 3 min.

Peptides used for *in vitro* phosphorylation reactions were prepared by solid phase synthesis. They were phosphorylated as described (Peter et al., 1990a and b).

Phosphatase Treatments

To determine the activity of phosphatase 2A on phosphorylated lamin B₂, purified lamin B₂ was incubated with PKC in the presence of 10 μ Ci [γ -³²P]ATP. Lamin B₂ was then dephosphorylated as described in Peter et al. (1991), except that purified phosphatase 2A was used instead of phosphatase 1.

To study the effect of phosphatase treatment on nuclear transport of lamin B₂, purified lamin B₂ was phosphorylated with PKC as described above. For control, a sample which had not been phosphorylated with PKC was analyzed in parallel. The various samples were dialyzed for 1 h against phosphatase buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1% β -mercaptoethanol). MgCl₂ was then added to a final concentration of 1 mM, and aliquots were incubated for 1 h at 30°C in the presence or absence of purified phosphatase 2A. Then, samples were frozen at -20°C until being used in transport assays.

Tryptic Phosphopeptide Mapping and Phosphoamino Acid Analysis

Two-dimensional mapping of tryptic phosphopeptides and phosphoamino acid analyses were carried out as described previously (Peter et al., 1990b; Boyle et al., 1991). Individual phosphopeptides were eluted from TLC-plates as described by Krek and Nigg (1991).

CNBr and NCS Cleavages

Purified lamin B₂ was phosphorylated with PKC as described above. For CNBr cleavage, phosphorylated lamin B₂ was excised and eluted from SDS-polyacrylamide gels, lyophilized, and resuspended in 90 μ l of 70% formic acid. Cleavage was initiated by the addition of 10 μ l of a stock solution containing CNBr (100 mg/ml) in 70% formic acid, and allowed to proceed for 16 h at room temperature in the dark. The sample was then lyophilized three times with 0.5 ml H₂O, taken up in 50 μ l gel sample buffer and analyzed on a 10–18% SDS-polyacrylamide gradient gel.

N-chlorosuccinimide (NCS) cleavages were carried out essentially as described by Vorburget et al. (1989a). In brief, lyophilized phosphorylated lamin B₂ was resuspended in 40 μ l of 0.1% urea in 50% (vol/vol) acetic acid. Then, 40 μ l of 80 mM NCS (Sigma Chemical Co.) dissolved in the same solution was added, and the sample was incubated for 1 h at room temperature. After lyophilization for three times in 0.5 ml H₂O each, the sample was taken up in 50 μ l gel sample buffer and analyzed by SDS-PAGE on a 10–18% gradient gel.

Lamin Assembly-disassembly Assays

Purified lamin B₂ proteins were assembled into head-to-tail polymers as described previously (Heitlinger et al., 1991). Then, conditions were adjusted to 10 mM MgCl₂, 0.5 mM CaCl₂, 5 μ g/ml dicaproyl (Sigma Chemical Co.), 50 μ g/ml phosphatidylserine (Sigma Chemical Co.), and ATP was added to a final concentration of 1 mM. Disassembly assays were started by the addition of 1 μ l of starfish p34^{cdc2}/cyclin B kinase (Labbé et al., 1989, 100 pmol/min/ μ l), 10 μ l of PKC (Marais and Parker, 1989; 1.5

pmol/min/ μ l) or 20 μ l of PKA (Sigma Chemical Co., 35 pmol/min/ μ l). Incubations were carried out for 1 h at 30°C. To control for the efficiencies of phosphorylation, aliquots were incubated in parallel, in the presence of 10 μ Ci [γ -³²P]-ATP. At the times indicated, aliquots were removed from the disassembly assays and placed on ice. At the end, all reactions were stopped by the addition of EDTA to a final concentration of 10 mM, and samples were centrifuged for 1 h (4°C, 100,000 g, Beckman TL-100.2 rotor). The partitioning of lamin B₂ between supernatant and pellet fractions was determined by immunoblotting as described previously (Peter et al., 1991).

Results

Phorbol Ester Treatment of DU249 Cells Induces Phosphorylation of Lamin B₂ on a Peptide Which Is also Phosphorylated by PKC *In Vitro*

Fig. 1 shows that treatment of DU249 cells with phorbol ester (PMA) caused increased phosphorylation of lamin B₂ (Fig. 1A, compare lane 1 with lane 2), confirming and extending earlier studies with other cell types (Hornbeck et al., 1988; Fields et al., 1988, 1990; Tsuda and Alexander, 1990; see also Peter et al., 1990a). Whereas the quantitative increase in PMA-induced phosphorylation of lamin B₂ was comparatively modest (\sim 1.7-fold), tryptic phosphopeptide mapping (Fig. 1B, a and b) revealed phosphorylation of a peptide (phosphopeptide 2) which was not phosphorylated in non-PMA-treated cells (Fig. 1B, b, see also Peter et al., 1990a). Phosphorylation of this peptide occurred exclusively on serine (not shown).

Because treatment of cells with PMA is known to rapidly activate PKC (for review, see Nishizuka, 1988), we tested purified PKC for its ability to phosphorylate lamin B₂ *in vitro*. Consistent with earlier studies (Hornbeck et al., 1988; Peter et al., 1990a), purified PKC readily phosphorylated lamin B₂ (Fig. 1A, lane 3). Phosphorylation occurred on three tryptic peptides (Fig. 1B, c) and exclusively on phosphoserine (not shown). The two major phosphopeptides (peptides 1 and 2) comigrated exactly with two of the peptides that were phosphorylated also in DU249 cells *in vivo* (Figure 1B, d). Whereas phosphopeptide 1 was phosphorylated throughout interphase of the cell cycle, peptide 2 was phosphorylated specifically in response to PMA. Importantly, neither phosphopeptides 1 and 2 were phosphorylated in mitotic cells (Peter et al., 1990a; Nigg et al., 1991; see also Lamb et al., 1991).

In vitro, PKC phosphorylated a third peptide in lamin B₂ (phosphopeptide 3 in Fig. 1B, c, and Fig. 6C, a). This peptide most likely maps to a region immediately COOH-terminal to the central α -helical rod domain of lamin B₂ (data not shown) and probably corresponds to a site shown to be phosphorylated by PKC in human lamin C (Ward and Kirschner, 1990). However, phosphopeptide 3 was not observed in interphase cells *in vivo*, and its physiological relevance is therefore doubtful.

Both Major Peptides Containing PKC Phosphorylation Sites Map to the COOH-Terminal End Domain of Lamin B₂

To obtain information on the approximate location of the two major peptides phosphorylated by PKC (phosphopeptides 1 and 2), three deletion mutants of lamin B₂ were tested as substrates. These mutants lack either the NH₂-terminal end domain, the COOH-terminal end domain, or both end do-

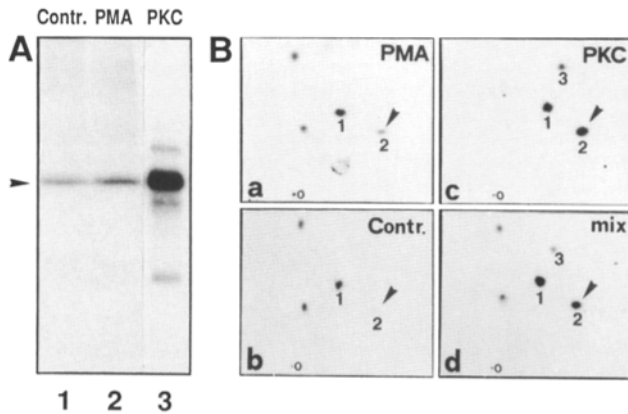


Figure 1. Phosphopeptide analysis of lamin B₂ after phosphorylation with PKC in vitro and after in vivo stimulation of cells with PMA. Lamin B₂ was either phosphorylated in vitro by PKC (A [lane 3] and B [c]) or immunoprecipitated from exponentially growing, ³²P-orthophosphate labeled DU249 cells; these had been treated either with the phorbol-ester PMA (A [lane 2] and B [a]) or with solvent for control (A [lane 1] and B [b]). Phosphorylated lamin B₂ was excised from polyacrylamide gels and digested with trypsin. The resulting phosphopeptides were separated by electrophoresis at pH 3.5 (horizontal direction, anode to the right) and ascending chromatography. Origins of sample application are marked by 0. The arrowhead marks a phosphopeptide that is specifically phosphorylated in vivo after stimulation of DU249 cells with PMA, as well as after in vitro phosphorylation of lamin B₂ by PKC. The result of mixing equal Cerenkov counts of in vitro and in vivo phosphorylated lamin B₂ is shown in B, (d).

mains (Heitlinger et al., 1992). The results of these studies demonstrated that both phosphopeptides 1 and 2 originated from the COOH-terminal end domain of lamin B₂ (not shown). To map the PKC phosphorylation sites more precisely, bacterially expressed wild-type lamin B₂ was phosphorylated with PKC, purified by SDS-PAGE, and then cleaved with either cyanogen bromide (CNBr; Fig. 2 B) or NCS (Fig. 2 C). Treatment of lamin B₂ with CNBr (i.e., a reagent cleaving specifically after methionine residues) generated several phosphorylated fragments including two peptides of ~9 and 10 kD, respectively (Fig. 2 B, lane 2, arrows). When CNBr fragments were eluted individually from SDS gels and analyzed by two-dimensional tryptic peptide mapping, they all contained phosphopeptides 1 and 2 (shown for the 9-kD fragment in Fig. 2 D, a). This indicates that all CNBr peptides larger than the 9-kD fragment represented partial cleavage products. As illustrated in Fig. 2 A, only two CNBr fragments are predicted to lie within the COOH-terminal end domain of chicken lamin B₂. Of these, the fragment lying close to the COOH-terminal end of the central rod domain (Fig. 2 A, fragment a; residues 366–447) has a predicted molecular weight of 8,560 and thus fits the size of the 9-kD phosphopeptide.

To confirm that phosphopeptides 1 and 2 map to fragment a, phosphorylated lamin B₂ was treated with NCS, a reagent known to cleave preferentially after tryptophan residues (Lischwe and Sung, 1977). Chicken lamin B₂ contains four closely spaced tryptophans, all of which lie COOH-terminal to the CNBr fragment a (Fig. 2 A, arrows). Thus, if major PKC phosphorylation sites were indeed located within the 9-kD peptide designated as fragment a in Fig. 2

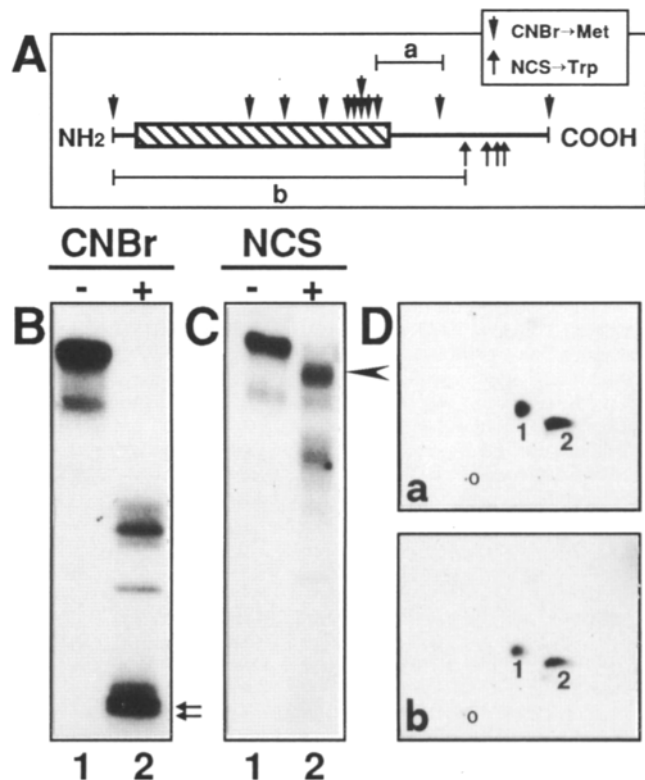


Figure 2. Major PKC-phosphorylation sites map to a region COOH-terminally adjacent to the central rod domain of lamin B₂. A illustrates the fragments generated after cleavage of lamin B₂ with CNBr (arrowheads) or NCS (arrows), as predicted by the primary sequence of the chicken lamin B₂ cDNA (Vorburger et al., 1989b). (B, C, and D) Purified, bacterially expressed lamin B₂ was phosphorylated in vitro with PKC and eluted from SDS-gels. Aliquots were either cleaved with CNBr (B, lane 2), NCS (C, lane 2) or, for control, incubated with the respective buffers alone (B and C, lane 1). Phosphorylated polypeptides were separated on a 10–18% gradient SDS polyacrylamide gel and visualized by autoradiography. D shows (a) the tryptic phosphopeptide map of the phosphorylated 9 kD CNBr-fragment (marked by the lower arrow in B, lane 2), and in b the 56-kD fragment obtained after NCS-cleavage (marked by arrowhead in C, lane 2). Note that the small 9-kD CNBr-fragment contains both major PKC phosphorylation sites.

A, NCS cleavage would be expected to generate a major phosphorylated fragment of about 56 kD (Fig. 2 A, fragment b), and tryptic cleavage of the purified 56-kD NCS fragment should yield both phosphopeptides 1 and 2. As shown in Fig. 2 B, lane 2, Fig. 2 D, b, these predictions were met.

Identification of the PMA Inducible Phosphorylation Sites as Serines 410 and 411

The sequence of the 9-kD CNBr fragment (Vorburger et al., 1989b) predicts several potential serine phosphoacceptor sites. Three considerations suggested that phosphopeptide 2 represents the tryptic peptide SSR, corresponding to residues 410 to 412. First, phosphopeptide 2 was not retained on a C18 HPLC column, indicating that it is likely to be small and very hydrophilic; second, during manual Edman degradation, radioactive phosphate was released in cycles 1 and 2. Third, both serines 410 and 411 are predicted to be poten-

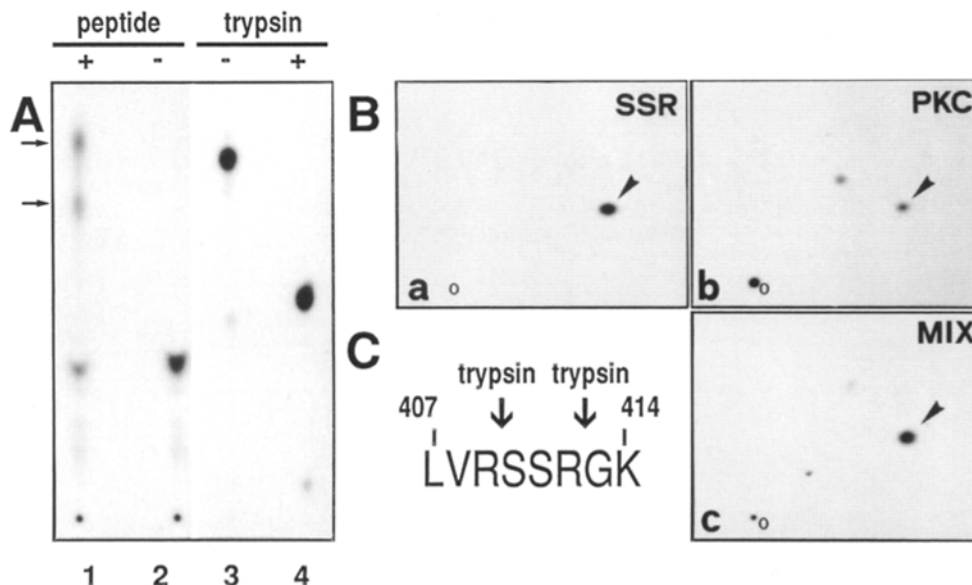


Figure 3. Identification of serines 410/411 as PKC phosphorylation sites. (A) The synthetic peptide LVRSSRGK was phosphorylated in vitro with purified PKC and analyzed by ascending chromatography on a TLC-plate (lane 1). For control, the phosphorylation reaction was carried out in the absence of peptide substrate (lane 2). Both phosphorylated forms of the peptide (arrows) were eluted from the TLC-plate, pooled and subjected to electrophoresis at pH 1.9 either before (lane 3) or after (lane 4) digestion with trypsin. (B) The tryptic map of phosphorylated SSR-peptide is shown in panel a. For comparison, b illustrates the tryptic map of lamin B₂

after in vitro phosphorylation by PKC. The result of mixing equal amounts (Cerenkov counts) of these tryptic digests is shown in c (mix). Note the exact comigration of the SSR-peptide with one of the peptides obtained after phosphorylation of lamin B₂ with PKC. The position of sample applications is marked by 0. (C) Sequence of the synthetic peptide corresponding to amino-acids 407–414 of chicken lamin B₂. The arrows indicate the positions of trypsin cleavage.

tial phosphoacceptor sites for PKC (Pearson and Kemp, 1991).

To provide a more direct identification of phosphopeptide 2, an octapeptide with the sequence LVRSSRGK was synthesized; this sequence corresponds to amino acids 407–414 of chicken lamin B₂. As shown in Fig. 3 A, this synthetic peptide was readily phosphorylated by purified PKC (lane 1). No phosphorylation was observed when the peptide was omitted from the phosphorylation reaction (lane 2). For unknown reasons, the phosphorylated peptide migrated as two spots when chromatography was used to separate it from free [γ -³²P]ATP (lane 1), but the two spots comigrated during electrophoresis (lane 3). When the in vitro phosphorylated peptide was eluted from TLC plates and digested with trypsin, it displayed a substantially reduced electrophoretic mobility (Fig. 3 A, lane 4), consistent with the expected loss of two positive charges (see Fig. 3 C). Fig. 3 B shows the migration of the tryptic phosphopeptide of TLC plates (a), in comparison with a phosphopeptide map obtained after in vitro phosphorylation of lamin B₂ with PKC (b). As demonstrated by mixing of the two samples (c), the tryptic fragment derived from the synthetic peptide comigrated precisely with phosphopeptide 2. These results identify serines 410 and 411 as PKC phosphorylation sites on lamin B₂. Based on the results of Edman degradation, we further conclude that phosphopeptide 2 contains a mixture of peptides carrying phosphate on either serine 410 or 411. We have not observed the doubly phosphorylated version of this peptide, neither in vitro nor in vivo, indicating that phosphorylation of serines 410 and 411 occurs in a mutually exclusive way. Phosphopeptide 2 was observed also after stimulation of DU249 cells with PMA (see Fig. 1), indicating that the same residues were phosphorylated also in vivo.

As demonstrated above (Fig. 2) the second major tryptic peptide containing PKC phosphorylation sites (phosphopeptide 1) must also be located within the 9-kD CNBr fragment

(i.e., residues 366–447). Phosphorylation of serine residues 386, 388, 390, and 391 can be excluded, because phosphopeptide 1 was still observed when a mutant lamin B₂ protein was analyzed in which serines 386, 388, 390, and 391 had been replaced by nonphosphorylatable alanine residues (the SPSP mutant; Peter et al., 1991). Also, we note that the migration of phosphopeptide 1 on TLC plates was very different from that of a tryptic peptide in which residues 386 and 388 had been phosphorylated by p34^{cdc2} (see Nigg et al., 1991, and Fig. 5C). Edman degradation of phosphopeptide 1 revealed that the bulk of the radioactive phosphate was released in the fifth cycle, with a second, weaker peak of radioactivity in the ninth cycle (not shown). The only tryptic peptide within the 9-kD CNBr fragment that contains serine residues at both position 5 and 9 is a 14-mer spanning residues 396–409, and we conclude, therefore, that PKC most probably phosphorylates purified lamin B₂ on serine 400, and to a lesser extent on serine 404. This conclusion is supported by the fact that the experimentally observed migration of phosphopeptide 1 on TLC plates is in good agreement with computer predictions for a phosphorylated peptide of the corresponding sequence (Boyle et al., 1991). As shown above, a phosphopeptide with identical mobility was constitutively phosphorylated during interphase of the cell cycle in DU249 cells (Fig. 1), but the identity of the in vivo phosphorylated residues has not been definitively established.

To facilitate the interpretation and discussion of the data shown below, Fig. 4 provides a schematic summary of the location of p34^{cdc2} and PKC phosphorylation sites within chicken lamin B₂.

Phosphorylation by PKC Does Not Induce Disassembly of Lamin B₂ Head-to-Tail Polymers

Phosphorylation by PKC is well known to trigger disassem-

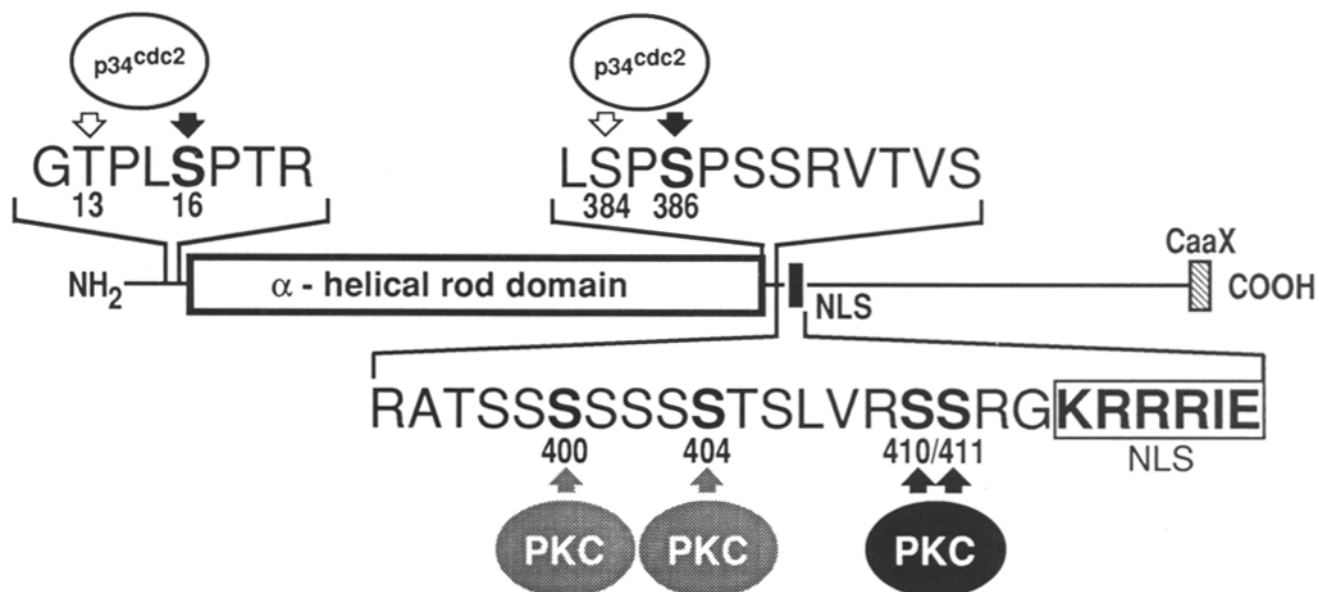


Figure 4. Schematic illustration of the location of p34^{cdc2} and PKC phosphorylation sites within chicken lamin B₂. Note that p34^{cdc2} phosphorylation sites flank the central α -helical rod domain (Peter et al., 1990a, 1991): major p34^{cdc2} sites are serines 16 and 386 (filled arrows), while minor sites have been tentatively identified as threonine 13 and serine 384 (open arrows). In contrast, PKC phosphorylation sites are all located within the COOH-terminal end domain, i.e., between the central α -helical rod and the nuclear localization signal (NLS; boxed; Loewinger and McKeon, 1988): serines 410/411 (filled symbols) have been shown here to be phosphorylated by PKC in vitro, as well as in response to PMA in vivo; serines 400 and 404 (shaded symbols) have been shown to be phosphorylated by PKC in vitro, but the precise location of phosphorylation sites within the corresponding tryptic peptide have not been determined in vivo.

bly of in vitro formed polymers of vimentin and other cytoplasmic IF proteins (e.g., Inagaki et al., 1987; Hisanaga et al., 1990). To determine whether PKC could also induce in vitro depolymerization of lamin proteins, preformed lamin head-to-tail polymers (Heitlinger et al., 1991) were incubated in the presence of ATP with purified PKC, and their assembly state was monitored using a sedimentation assay (Peter et al., 1991). As shown in Fig. 5 A, lanes 1–4, no solubilization of lamin B₂ could be detected, even after a 60-min incubation with PKC (Fig. 5 A, compare lanes 3 and 4 with lanes 1 and 2; see also Peter et al., 1990a). For control, parallel experiments were carried out using the p34^{cdc2}/cyclin B protein kinase to disassemble lamin polymers (Fig. 5 A, lanes 5 and 6). PKC phosphorylated lamin polymers at least as efficiently as p34^{cdc2}/cyclin B (Fig. 5 B, compare lanes 1 and 2), indicating that the inability of PKC to disassemble lamin head-to-tail polymers cannot be attributed to an insufficient stoichiometry of phosphorylation. Instead, the difference in the results obtained with the two kinases is likely to be due to the fact that they phosphorylated different sites on lamin B₂ (see tryptic phosphopeptide analyses in Fig. 5 C, a–c).

A Permeabilized Cell System Supports Nuclear Import and Lamina Incorporation of Exogenous Lamin B₂

Two of the major PKC phosphorylation sites (serine 410 and 411) are located immediately upstream of the NLS of lamin B₂ (spanning residues 414–420; see Fig. 4). This prompted us to ask whether phosphorylation by PKC might interfere with nuclear transport of lamin B₂. In a first series of experiments, we adapted an in vitro transport system based on permeabilized HeLa cells (Adam et al., 1990) to mon-

itor nuclear transport of bacterially expressed avian lamin B₂. Time-dependent nuclear uptake of purified lamin B₂ could be demonstrated by indirect immunofluorescence microscopy (Fig. 6 A, compare panels b and a), as well as by immunoblotting (Fig. 6 B; lanes 2–6). In both assays, the avian lamin B₂ was distinguished from the human homolog by the use of a chicken-specific monoclonal antibody (L-20; Lehner et al., 1986a); no immunoreactivity was observed in the absence of transport substrate, demonstrating the specificity of the antibodies for avian lamin B₂ (Fig. 6 B, lane 9). Nuclear import of lamin B₂ was routinely observed in 70–85% of all nuclei, although the extent of import was variable (see Fig. 6 A, b). As expected for an active process, nuclear transport of lamin B₂ was temperature dependent (Fig. 6 A, compare b with c; Fig. 6 B, compare lane 8 with lane 6), and it required ATP (Fig. 6 B, compare lane 7 with lane 6). Furthermore, nuclear accumulation of lamin B₂ was inhibited by WGA (not shown), a lectin known to interfere with active nuclear transport (for review, see Garcia-Bustos et al., 1991).

Interestingly, the distribution of lamin B₂ within the nucleus was not homogenous. Compared to the distribution of DNA in the permeabilized HeLa cells (Fig. 6 A, d–f), the antilamin B₂ antibodies clearly produced a ringlike peripheral staining (Fig. 6 A, b). This indicates that chicken lamin B₂ was not only transported to the nucleus, but was also incorporated into the endogenous lamina. However, nuclear transport could be uncoupled from lamina assembly: when an assembly-incompetent preparation of lamin B₂ was used as a transport substrate, this resulted in nucleoplasmic localization rather than membrane association (not shown). Taken together, these results establish that the permeabilized cell

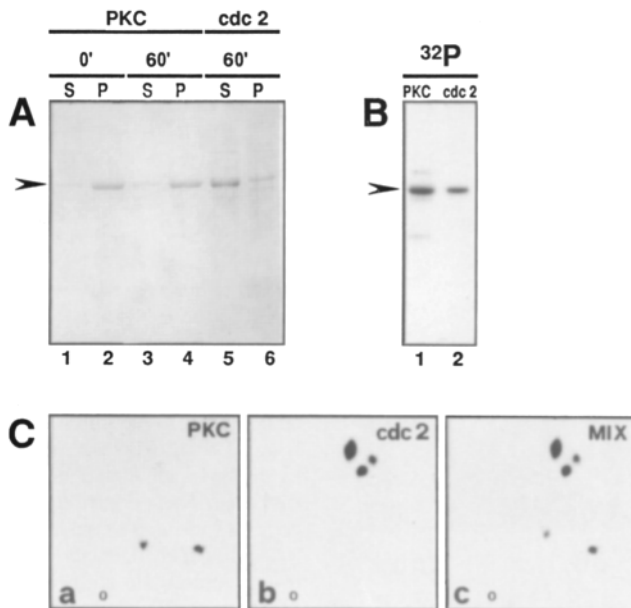


Figure 5. Phosphorylation of lamin B₂ by PKC does not induce disassembly of head-to-tail polymers. (A) Lamin B₂ head-to-tail polymers were incubated with PKC (lanes 1–4) or p34^{cdc2}/cyclin B (lanes 5 and 6). At the times indicated, aliquots were removed from the incubations and subjected to centrifugation; the partitioning of lamin B₂ (arrowhead) between supernatant (S) and pellet (P) fractions was analyzed by SDS-PAGE followed by Coomassie brilliant blue staining. (B) To monitor the extents of phosphorylation of lamin B₂ by PKC (lane 1) and p34^{cdc2}/cyclin B (lane 2), aliquots of the disassembly reactions were incubated in the presence of [³²P]-ATP, and phosphorylated lamin B₂ was visualized by SDS-PAGE and autoradiography. (C) Lamin B₂, phosphorylated either by PKC (a) or p34^{cdc2}/cyclin B (b), was eluted from SDS-gels, and digested with trypsin. The resulting phosphopeptides were separated on TLC-plates as described in the legend to Fig. 1. A mix of the tryptic digests is shown in c.

system used here supports nuclear transport of lamin B₂, as well as its incorporation into the preexisting lamina.

Phosphorylation by PKC Interferes with Nuclear Transport of Lamin B₂ In Vitro

To determine whether phosphorylation by PKC would interfere with nuclear transport of lamin B₂, similar experiments were carried out using *in vitro* phosphorylated lamin B₂ as a transport substrate (Fig. 7). Compared to unphosphorylated lamin B₂ (Fig. 7A, lower; Fig. 7B, b), the nuclear transport of *in vitro* phosphorylated lamin B₂ was strongly inhibited (Fig. 7A, upper; Fig. 7B, a). To demonstrate that this inhibition was due to phosphorylation, phosphorylated lamin B₂ was treated with phosphatase 2A. This treatment efficiently dephosphorylated the protein (Fig. 7C, compare lanes 2 and 3 with lanes 1 and 4), and it restored nuclear transport (Fig. 7D; left).

To determine whether or not phosphorylation of other sites within the COOH-terminal end domain of lamin B₂ would also affect nuclear transport, we tested a lamin B₂ mutant (the LSPTR mutant) in which phosphorylation by p34^{cdc2} occurs almost exclusively on serines 384 and 386 (Peter et al., 1991). These residues are located approxi-

mately 30 amino acids upstream of the NLS (see Fig. 4). As shown in Fig. 7D, right, phosphorylation of serines 384 and 386 by p34^{cdc2} did not interfere with nuclear transport, indicating that inhibition of nuclear transport is specific to the phosphorylation of PKC sites on lamin B₂.

PMA Stimulation Reduces the Rate of Nuclear Import of Newly Synthesized Lamin B₂ In Vivo

During interphase of the cell cycle, the bulk of the nuclear lamin proteins exists as a karyoskeletal structure inside the nucleus. Thus, phosphorylation of lamin B₂ by PKC could only play a role in controlling nuclear transport *in vivo*, if PKC were to act on newly synthesized, cytoplasmic protein. To determine whether this might be the case, we studied the rate of nuclear import of newly synthesized lamin B₂ in cells that had been incubated with or without the PKC-activator PMA. Exponentially growing DU249 cells were pulse-labeled for 15 min with ³⁵S-methionine, in the presence or absence of PMA. Then, they were fractionated after different periods of time, using a rapid enucleation protocol (Krek et al., 1992), and the partitioning of lamin B₂ was monitored by immunoprecipitation. Enucleation yields nuclei with minimal contamination by cytoplasm, and cytoplasts with a minimal contamination by intact cells (Gordon et al., 1981; Baeuerle and Baltimore, 1988). In the absence of PMA stimulation, the nuclear import of newly synthesized lamin B₂ was very rapid (Fig. 8A), consistent with earlier studies (Lehner et al., 1986b). As a consequence, most of lamin B₂ in untreated cells was already nuclear after the 15-min labeling period (i.e., chase time 0 min; Fig. 8A), and nuclear uptake was virtually complete after chase times of 30 and 60 min (Fig. 8A). In contrast, in cells in which PKC had been activated by PMA treatment, the bulk of the newly synthesized lamin B₂ was still cytoplasmic after the labeling period, and ~30–40% remained cytoplasmic after a 30 min chase (Fig. 8B). After 60 min, most of the lamin was nuclear even in the PMA treated cells, indicating that the inhibition of nuclear accumulation was transient. A quantitative analysis of data from three independent experiments indicates that PMA treatment reduced the rate of nuclear import of newly synthesized lamin B₂ to ~30%.

Discussion

In the present study, we have explored the relationship between *in vivo* phosphorylation of lamin B₂ during interphase of the cell cycle, and *in vitro* phosphorylation of this protein by PKC. We have mapped the major *in vitro* PKC phosphorylation sites within lamin B₂ and show that these are located within tryptic peptides that are phosphorylated also *in vivo*. Most interestingly, we found that phosphorylation of lamin B₂ on PKC sites leads to almost complete inhibition of nuclear transport in an *in vitro* assay, and that PMA stimulation of cells reduces the rate of nuclear uptake of newly synthesized lamin B₂ *in vivo*.

Identification of Major Interphase Phosphorylation Sites in Avian Lamin B₂

As shown by a comparison of tryptic phosphopeptide maps, PKC phosphorylates lamin B₂ on two peptides that are also

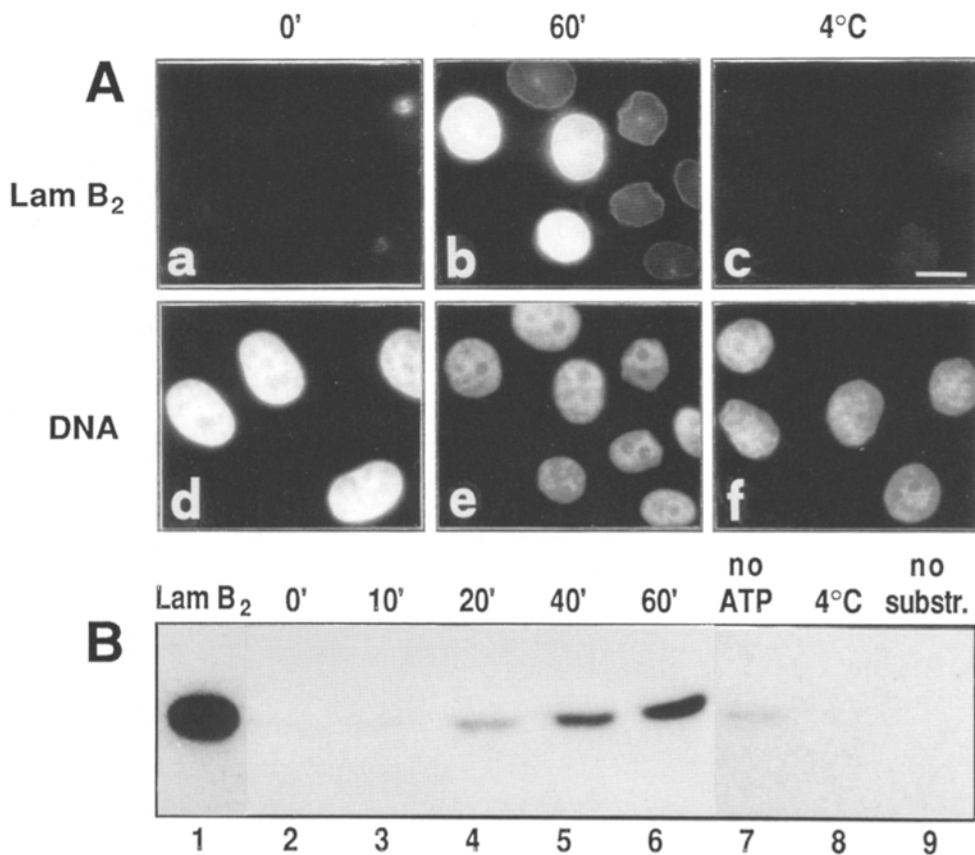


Figure 6. In vitro import of bacterially expressed lamin B₂ into nuclei of permeabilized HeLa cells. HeLa cells were grown on coverslips, permeabilized with digitonin, and incubated with transport mix containing purified lamin B₂. After the times indicated, cells were washed and nuclear import of lamin B₂ was monitored by either indirect immunofluorescence microscopy (A) or immunoblotting (B), using a MAb specific for chicken lamin B₂ (L-20, Kitten and Nigg, 1991). Nuclear envelopes remained intact in the vast majority of permeabilized cells, as indicated by the inaccessibility of endogenous lamins to appropriate antibodies (not shown). (A) Immunofluorescent staining reveals that chicken lamin B₂ is specifically imported into HeLa cell nuclei after incubation for 60 min at 37°C (b). Little import is observed at time 0 (a) or if the import reaction is carried out for 60 min at 4°C (c). d-f correspond to a-c, respectively, and show staining of nuclear

DNA with Hoechst dye. Bar in c, 10 μ m. (B) Lanes 2-6 show a time course of the in vitro nuclear uptake of chicken lamin B₂ into HeLa nuclei. All samples (about 200,000 cells each) were incubated in parallel with 200 ng of purified lamin B₂ (lane 1); after the times indicated, cells were washed, lysed in gel sample buffer and the retention of lamin B₂ (reflecting import into nuclei) was monitored by immunoblotting. For control, transport reactions were carried out for 60 min either without addition of ATP (lane 7), or at 4°C (lane 8), or in the absence of lamin B₂ (lane 9). Note that the partial nuclear uptake of lamin B₂ seen in lane 7 is readily explained by the presence of ATP in the reticulocyte lysate component of the transport mix.

phosphorylated in vivo. One of these (phosphopeptide 1) is phosphorylated constitutively during interphase of the cell cycle, while the other (phosphopeptide 2) is phosphorylated specifically in response to treatment of cells with PMA. Both peptides were mapped to the COOH-terminal end domain of lamin B₂ (Fig. 4). Phosphopeptide 1 most probably spans residues 396-409 and contains a string of serines. Of these, residues 400 and 404 appear to be the preferred targets of PKC in vitro. Whether or not the exact same residues are phosphorylated in the corresponding peptide in vivo remains to be determined. Phosphopeptide 2 comprises residues 410-412. Both serines 410 and 411 are phosphorylated by PKC in vitro, and it is plausible that the same kinase is responsible for phosphorylating these residues in response to PMA in vivo. Alternatively, these sites might be phosphorylated by a kinase that is activated by PKC; however, such an enzyme would have to display indistinguishable substrate specificity.

No information on PKC phosphorylation sites has previously been reported for B-type lamins. PKC sites have been mapped in human and murine lamin C, a member of the A-type lamin family (Ward and Kirschner, 1990; Eggert et al., 1991); lamin C is essentially a truncated version of lamin A and only found in mammals (for review, see McKeon, 1991). The functional significance of lamin C phosphoryla-

tion by PKC is not known, and the relationship between the phosphorylation sites determined in lamin C and those reported here for lamin B₂ remains to be determined.

Phosphorylation by Protein Kinase C Does Not Disassemble In Vitro Formed Lamin Head-to-Tail Polymers

The finding that the major PKC phosphoacceptor sites map to the COOH-terminal end domain of lamin B₂ contrasts with results obtained previously with cytoplasmic intermediate filament proteins. In the case of vimentin, for example, several PKC phosphorylation sites were mapped to the NH₂ terminus; concomitantly, PKC was shown to induce disassembly of in vitro formed vimentin filaments (for references, see Ando et al., 1989). Disassembly of lamin polymers by p34^{cdc2} also requires phosphorylation of a critical residue within the NH₂-terminal end domain (Heald and McKeon, 1990; Peter et al., 1991, 1992). It appears likely, therefore, that the absence of major PKC phosphorylation sites in the lamin B₂ NH₂ terminus explains why PKC does not disrupt longitudinal interactions within lamin B₂ head-to-tail polymers (Peter et al., 1991; this study), nor confer detergent solubility to lamins when incubated with isolated nuclei (Peter et al., 1990a).

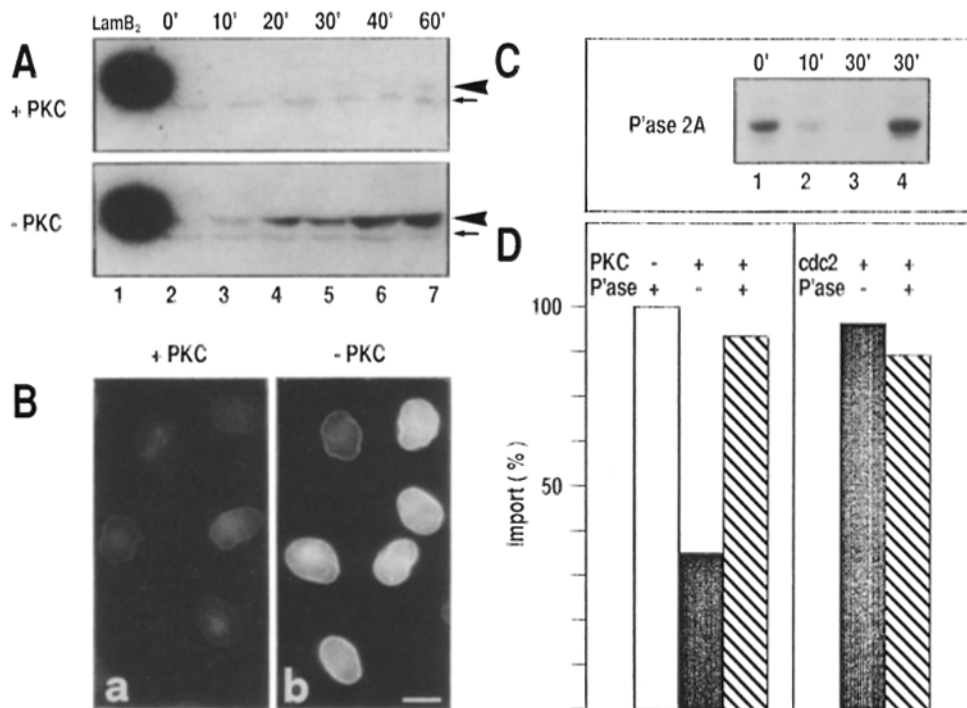


Figure 7. Nuclear uptake of lamin B₂ in vitro is inhibited by phosphorylation by PKC. (A and B) Before being used for in vitro transport assays, lamin B₂ was phosphorylated with PKC or, for control, incubated with kinase buffer alone. (A) Time course of nuclear import of phosphorylated (+PKC, upper) and unphosphorylated lamin B₂ (-PKC, lower), as determined by immunoblotting (see legend to Fig. 6). Note that only unphosphorylated lamin B₂ (arrowheads) was imported to a significant level. (Arrows mark the position of an unidentified cross-reactive cellular protein). (B) A transport experiment was carried out at 37°C for 60 min using either phosphorylated (+PKC, left) or unphosphorylated (-PKC, right) lamin B₂, and imported lamin B₂ was visualized by indirect immunofluorescence microscopy.

copy. Bar in *b*, 10 μm. (C and D) Lamin B₂ was phosphorylated by PKC in the presence of [γ-³²P]-ATP; then aliquots were incubated for the times indicated in the presence (lanes 1-3) or absence (lane 4) of purified phosphatase 2A, and dephosphorylation was monitored by SDS-PAGE and autoradiography. Only the relevant part of the gel is shown. (D, left) Phosphorylated (+PKC) or unphosphorylated (-PKC) lamin B₂ was incubated in the presence (+Pase) or absence (-Pase) of purified phosphatase 2A for 30 min. Transport assays were then carried out at 37°C for 60 min, and nuclear import of lamin B₂ was quantified by densitometric scanning of the immunoblots. Histograms show the amounts of transported lamin B₂, normalized to the amount of transported unphosphorylated lamin B₂ (set to 100%). Transport of unphosphorylated lamin B₂ was not influenced by the addition of phosphatase 2A. For control (D, right), parallel experiments were carried out, using p34^{cdc2}/cyclin B instead of PKC for the phosphorylation of lamin B₂. Neither phosphorylation by p34^{cdc2}/cyclin B nor subsequent dephosphorylation significantly affected lamin B₂ transport.

Phosphorylation on Protein Kinase C Sites Inhibits Nuclear Transport of Lamin B₂

We found that phosphorylation by PKC virtually abolished nuclear import of lamin B₂ in an in vitro system, and that activation of PKC in intact cells reduced the rate of nuclear transport of newly synthesized lamin B₂ in vivo. Based on the results of the in vitro studies which provide a direct link between phosphorylation of serines 410/411 and inhibition of nuclear transport, it is attractive to speculate that the transport inhibition observed in vivo is also due to phosphorylation of newly synthesized lamin B₂ on the corresponding residues. However, we emphasize that this has not been demonstrated directly, and alternative explanations for the transport inhibition observed in vivo are not excluded. In particular, activated PKC might influence nuclear transport by phosphorylating proteins involved in the transport process per se (e.g., components of the nuclear pore complex).

What Are the Functional Consequences of Lamin Phosphorylation by PKC?

In vitro, PKC phosphorylation of lamin B₂ almost completely inhibited nuclear transport. In PMA-treated cells in vivo, however, nuclear import of newly synthesized lamin B₂ was delayed but not abolished. Because lamin B₂ has a half life of ~24 h (Borer et al., 1989), it is unlikely that such a transient reduction in the nuclear import of newly synthe-

sized protein would produce a significant quantitative effect on the nuclear lamina. However, although cellular pools of unassembled lamin subunits are very small, recent studies indicate that new subunits undergo continuous exchange with proteins already present in polymeric structures (Goldman et al., 1992). One might speculate, therefore, that a transiently reduced flow of lamin proteins to the nucleus and/or the appearance of phosphorylated versions of the protein might lead to profound rearrangements in the structure of the nuclear lamina. Also, assuming that a competition for polymer binding might exist between newly synthesized lamins and other proteins (e.g., chromosome-associated factors), it is conceivable that a transient shortage within the nucleus of newly synthesized lamins might favor changes in lamin-chromatin interactions.

PMA-induced phosphorylation of lamin B₂ was not limited to newly synthesized protein, because incorporation of phosphate into peptides 1 and 2 could still be detected when cells were treated with PMA in the presence of cycloheximide, an inhibitor of protein synthesis (H. Hennekes and E. A. Nigg, unpublished results). This indicates that phosphorylation occurred also on lamins which had already entered the nucleus at the time of PKC activation, and thus argues in favor of an additional role of phosphorylation at PKC sites, besides the observed inhibitory effect on nuclear import. Although the available evidence argues against a role for PKC in causing lamina depolymerization during mitosis

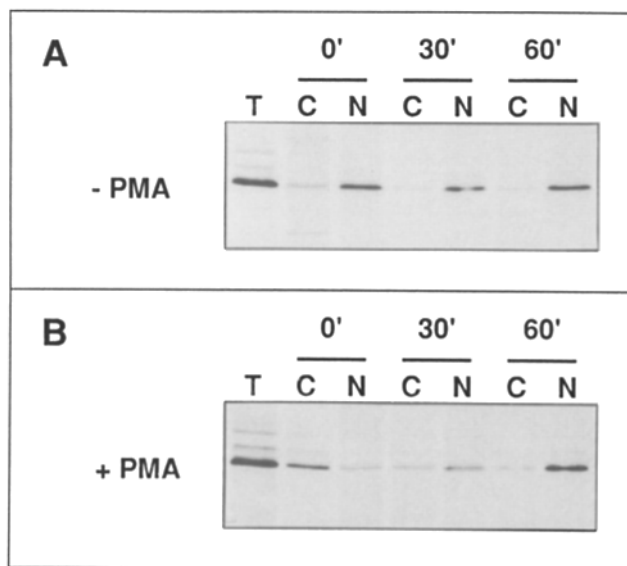


Figure 8. Stimulation of PKC in vivo leads to retarded nuclear uptake of newly synthesized lamin B₂. DU249 cells were labeled for 15 min with ³⁵S-methionine in the absence (A) or presence (B) of 0.32 μM PMA followed by a chase with 5 mM unlabeled methionine for the times indicated. Then cells were enucleated as described in Methods and lamin B₂ was immunoprecipitated from the cytoplasmic (C) and nuclear (N) fractions. Lanes T show the total of labeled lamin B₂ precipitated from nonenucleated cells. Immunoprecipitated proteins were separated by SDS-PAGE and detected by fluorography.

(Peter et al., 1990a, 1991; this study), it would be premature to exclude that PKC-dependent phosphorylation may have significant effects on the structure of the nuclear lamina. In particular, phosphorylations on PKC sites might control lateral interactions among lamin polymers, the branching of lamin filaments, or interactions between lamins and other proteins. Clearly, more needs to be learned about the principles governing lamina assembly, before the functional consequences of phosphorylation at PKC sites can be fully understood.

We note that serines (or threonines) at the positions corresponding to 410 and 411 in chicken lamin B₂ have been conserved also in avian and mammalian lamin B₁ (Peter et al., 1989; Höger et al., 1988). They were not conserved in murine lamin B₂ (Höger et al., 1990), but this may reflect the fact that lamin B₂ is a comparatively minor species in most mammalian tissues (Lehner et al., 1986a).

The Role of Phosphorylation in Regulating Nuclear Transport

Our present findings have implications extending beyond the structure and function of the nuclear lamina. In particular, they suggest that the hormonally controlled activation of PKC might reduce the import of many newly synthesized proteins into the nucleus, either via phosphorylation of the proteins themselves and/or via phosphorylation of as yet unidentified components of the nuclear transport machinery. In the case of short-lived regulatory proteins (e.g., proteins involved in controlling transcription or replication), even a moderate reduction in the rate of nuclear transport might cause a significant reduction in the nuclear concentration of the corresponding proteins.

In conclusion, our results demonstrate that nuclear transport rates are sensitive to changes in the state of activity of cytoplasmic protein kinases. These findings add to a growing body of evidence implicating protein phosphorylation in both positive and negative control of nuclear transport rates (Rihs and Peters, 1989; Rihs et al., 1991; Addison et al., 1990; Jans et al., 1991; Moll et al., 1991). Specific examples include the yeast transcription factor SWI5, which is phosphorylated in a cell cycle-dependent manner by the CDC28 protein kinase (Moll et al., 1991), and Simian Virus 40 large T antigen, which is phosphorylated by both casein kinase II and p34^{cdc2} (for references see Rihs et al., 1991; Jans et al., 1991). Taken together, these studies suggest that the regulation of nuclear transport processes by phosphorylation may represent an important element in relaying signals from the cell surface to the nucleus.

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