Mitotic Phosphorylation of the Lamin B Receptor by a Serine/Arginine Kinase and p34\(^{cd2}\)‡*

(Received for publication, September 15, 1996, and in revised form, November 5, 1996)

Eleni Nikolakaki‡, Juergen Meiers§, George Simos§, Spyros D. Georgatos‡‡, and Thomas Giannakouros‡‡‡

From the 3Biochemistry School of Chemistry, The Aristotelian University of Thessaloniki, Thessaloniki
54 006, Greece, 4Programme of Cell Biology, European Molecular Biology Laboratory, 69 017 Heidelberg, Germany, and
5Department of Basic Sciences, Faculty of Medicine, The University of Crete, Heraklion 71 110, Crete, Greece

The lamin B receptor (LBR) is an integral protein of the inner nuclear membrane that is modified at interphase by a nuclear envelope-bound protein kinase. This enzyme (RS kinase) specifically phosphorylates arginine-serine dipeptide motifs located at the NH2-terminal domain of LBR and regulates its interactions with other nuclear envelope proteins. To compare the phosphorylation state of LBR during interphase and mitosis, we performed phosphopeptide mapping of in vitro and in vivo -labeled LBR and analyzed a series of recombinant proteins and synthetic peptides. Our results show that LBR undergoes two types of mitotic phosphorylation mediated by the RS and the p34\(^{cd2}\) protein kinases, respectively. The RS kinase modifies similar sites at interphase and mitosis (i.e. Ser\(^{56}\), Ser\(^{70}\), Ser\(^{80}\), Ser\(^{82}\) and Ser\(^{84}\)), whereas p34\(^{cd2}\) mainly phosphorylates Ser\(^{71}\). These findings clarify the phosphorylation state of LBR during the cell cycle and provide new information for understanding the mechanisms responsible for nuclear envelope assembly and disassembly.

The nuclear lamina is a filamentous meshwork underlying the inner nuclear membrane (1, 2). In most cells this structure is a heteropolymer of type A and B lamins (3) linked to the inner nuclear membrane through integral membrane proteins. These lamin-binding proteins include the lamin B receptor (LBR) (4) and the lamina-associated polypeptides (5).

LBR possesses a long, hydrophilic NH2-terminal domain protruding into the nucleoplasm, eight hydrophobic segments that are predicted to span the membrane, and a hydrophilic COOH-terminal domain (6, 7). The NH2-terminal domain of LBR contains distinct sites for protein kinase A and p34\(^{cd2}\) kinase phosphorylation (8, 9) as well as a stretch rich in arginine (RS) motifs (10). The RS motifs are specifically modified by a protein kinase that co-isolates with LBR and is part of a multimorphic complex (8, 10). This LBR complex also includes the nuclear lamins and three polypeptides with molecular masses of 18 (p18), 150 (p150), and 34 (p34/p32) kDa, respectively (for pertinent information see Refs. 8, 10, and 12). The latter protein has been shown to interact with the splicing factor 2 (SF2) as well as with the HIV-1 proteins Rev and Tat (13–15). Phosphorylation of LBR by the RS kinase completely abolishes binding of p34/p32, suggesting that this enzyme regulates interactions among the components of the LBR complex (11).

At the onset of mitosis, the structure of the nuclear envelope is dramatically altered. The nuclear lamina depolymerizes as a result of hyperphosphorylation of the nuclear lamins at specific sites involved in lamin-lamin (16), lamin-chromatin (17), and lamin-membrane (5) interactions. Following depolymerization, the bulk of type A lamins disperse in the cytoplasm, whereas type B lamins remain bound to remnants of the nuclear envelope. At the same time, the nuclear envelope membranes break down into vesicular structures (1). Apart from lamin hyperphosphorylation, Courvalin et al. (9) also reported that LBR is phosphorylated on serine and threonine residues during mitosis.

As the events responsible for nuclear membrane breakdown are not completely understood and in light of the fact that LBR is phosphorylated by the RS kinase during interphase, we found it important to examine the specific modifications of LBR during mitosis. Results presented below reveal that during mitosis LBR is phosphorylated by both RS and p34\(^{cd2}\) protein kinases.

**EXPERIMENTAL PROCEDURES**

Materials—Phosphocellulose and Affi-Gel 10 were purchased from Whatman Biosystems Ltd., United Kingdom, and Bio-Rad, respectively. Peptides R\(_{8}\) (80KRSR80KSRSR80KSRS80), R\(_{15}\) (15KQRSKS-15KRSR15KRSR15KRSR15), and R\(_{18}\) (18KIFE-AKTPKPSKS18) were made at the Protein Sequencing and Peptide Synthesis Facility of the European Molecular Biology Laboratory, Heidelberg, Germany. R\(_{18}\) peptide was coupled to Affi-Gel 10 as described previously (11). Recombinant p34\(^{cd2}\)-cyclin B was purchased from New England Biolabs Ltd., United Kingdom. Histone H\(_{1}\) was obtained from Boehringer Mannheim GmbH, Germany. [\(^{32}\)P]ATP (6000 Ci/mmol) as well as [\(^{32}\)P]phosphate (10 mCi/ml) were purchased from ICN Pharmaceuticals Ltd., United Kingdom. Histone H\(_{1}\) was obtained from Hoffmann-La Roche (Germany Cancer Research Center, Heidelberg, Germany). All other chemicals were purchased from Sigma.

Construction of Plasmids and Expression of Fusion Proteins—The pGEX-2T bacterial expression vector (Pharmacia Biotech Inc.) was used to construct plasmids that encode the wild type NH2-terminal (wtNt) and three mutated forms (wtNt\(^{18}\), wtNt\(^{34}\), and wtNt\(^{32}\)) of the NH2-terminal domain of chicken LBR (6) fused with glutathione S-transferase (GST). To generate the cDNA coding for wtNt (amino acids 1-84) fused with GST, a partial cDNA library of chicken breast muscle was purchased from Amer-
Mitotic Phosphorylation of LBR

1–20), 30 cycles of the polymerase chain reaction were performed as described (11). Full-length LBR cloned to the EcoRI site of Bluescript SK" was used as a template. The LBR-SK clone was a generous gift of J. H. Warren (Columbia University, New York). The sense primer contained nucleotides 1–156 of the LBR sequence, while the CAGTA was added 5′ to the BamHI site. The antisense primer was complementary to nucleotides +598 +165 of LBR. A complementary stop codon was added 5′ to this sequence, preceded by an EcoRI site. GC was added 5′ to the EcoRI site. The polymerase chain reaction product was purified using the QIAEX gel extraction kit (QIAGEN Inc., Chatsworth, CA). Purified DNA was digested with EcoRI and BamHI, purified, and recloned into a BamHI/EcoRI site of the pGEX-2T. Escherichia coli strains XL-1 Blue were transformed by standard methods.

An oligonucleotide-directed in vitro mutagenesis system (Altered SitesII In vitro Mutagenesis system, Promega, Corp., Madison, WI) was used to mutate the sites that are potentially phosphorylated by p34cdc2/cyclin B protein kinase. Using the oligonucleotides 5′TCATGGGACCTTGGAGGA-3′, 5′GACCGAGGCTCGCTAGC-3′; and 5′TTTCTCGGGATTTATATTTGCG-3′, the regions of the cDNA corresponding to residues 14–21 of LBR preceded by a BamHI site were amplified with PCR using the primers containing the restriction sites. The PCR product was cloned into pGEX-2T and sequenced to ensure the integrity of the wild-type sequence.

Purification of LBR Kinase—LBR kinase was isolated from turkey erythrocyte nuclear envelopes as described previously (11). Briefly, the 1 × NaCl extract of nuclear envelopes (following dilution to 0.3 M and clarification by centrifugation) was applied to a phosphocellulose column containing the R0 peptide. The column was subsequently washed with saline (155 mM NaCl, 20 mM sodium phosphate, pH 7.4), and bound proteins were eluted by a linear (0.3–1 M) NaCl gradient. Kinase activity was measured using [γ-33P]ATP (600 Ci/mmol) in a reaction volume of 25 μl.

For RS kinase phosphorylation, 6 μl of the enzyme preparation (activity, 2000 units/ml; 1 unit is the amount of p34cdc2/cyclin B required to catalyze the transfer of 1 pmol of phosphate to histone H4 in 1 min at 30°C) was incubated with 6 μg of GST-wtNt or with 1.5 μg of electrophoretically purified LBR in a buffer composed of 25 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM dithiothreitol, 1 mM EGTA, and 50 μM γ-32P[ATP (600 Ci/mmol) in a reaction volume of 25 μl.

Samples were incubated for 30 min at 30°C, and the reaction was stopped by adding the appropriate volume of 5 × Laemmli buffer (21) and heating at 95°C for 3 min. Electrophoretically purified LBR was obtained from urea-insoluble nuclear envelopes as described previously (11).

Phosphopeptide Mapping and Phosphoamino Acid Analysis—Proteolytic digestion was performed as described by Luo et al. (22) and Simos and Georgatos (8). Briefly, immunoprecipitates of in vivo phosphorylated LBR or in vitro phosphorylated GST-wtNt were run on SDS-PAGE and then transferred to a nitrocellulose sheet. The radiolabeled nitrocellulose sheet was then incubated with p34cdc2/cyclin B antibody (24). To assess the ability of p34cdc2/cyclin B to phosphorylate LBR and the best substrate for p34cdc2/cyclin B, the phosphorylated LBR or GST-wtNt in a buffer composed of 25 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 200 mM NaCl, 1 mM dithiothreitol, and 50 μM γ-32P[ATP (600 Ci/mmol) in a reaction volume of 25 μl.

RESULTS

To determine whether LBR is a substrate for p34cdc2/cyclin B protein kinase, electrophoretically purified LBR as well as salt-washed nuclear envelopes preheated at 60°C for 10 min (to inactivate the endogenous RS kinase) were used as substrates for in vitro phosphorylation assays. Fig. 1 shows that p34cdc2 modifies both the envelope-associated and the purified LBR protein. Under these conditions p34cdc2 also phosphorylated lamin A, lamin B, and residual histones H4 and H2B left behind after salt extraction of the nuclear envelopes.

Inspection of the amino acid sequence of chicken LBR revealed the presence of three potential p34cdc2 phosphorylation sites (Ser171, Ser185, and Thr188; see Fig. 2A) conforming to the consensus Ser/Thr-Pro-X (24). To assess the ability of p34cdc2 to modify these sites, we synthesized three peptides (R₁, R₂, and R₃) modeled after the published sequence, each one containing one potential phosphorylation site (Fig. 2A). As shown in Fig. 2B, the phosphorylation of purified LBR was inhibited by peptides R₁ and R₂, which acted as substrates for the kinase. In contrast, peptide R₃ was poorly phosphorylated by p34cdc2 and did not significantly inhibit the phosphorylation of purified LBR. To express these results quantitatively, the same type of in vitro phosphorylation assays were performed using a range of peptide concentrations. Data were plotted in Table I, and the table shows that R₁ was the strongest inhibitor of p34cdc2-mediated phosphorylation of LBR and the best substrate for p34cdc2, whereas R₃ was the weakest inhibitor and the poorest substrate for the kinase.

In agreement with these observations, only phosphoserine could be detected when in vitro phosphorylated LBR was ana-
Phosphorylated GST-wtNtA71, GST-wtNtA84, and GST-wtNtA188 yielded one major phosphopeptide (peptide not shown). Of the three mutated proteins were used as substrates in in vitro phosphorylation assays. p34<sup>cdc2</sup> could efficiently phosphorylate GST-wtNt, GST-wtNtA84, and GST-wtNtA188, whereas the phosphorylation of GST-wtNtA<sup>D</sup> was significantly impaired (Fig. 3D). This was not due to a global misfolding of the polypeptide chain induced by the replacement of Ser<sup>71</sup> because both GST-wtNt and GST-wtNtA<sup>71</sup> were efficiently modified by the RS kinase (Fig. 5A).

To confirm these results we performed two-dimensional proteolytic peptide mapping. Fig. 4 shows that p34<sup>cdc2</sup>-phosphorylated GST-wtNt yielded one major phosphopeptide (peptide designated b), one phosphopeptide of moderate intensity (peptide designated c), and two minor phosphopeptides (peptides designated a and d). Phosphopeptide mapping of in vitro phosphorylated GST-wtNtA<sup>71</sup>, GST-wtNtA<sup>84</sup>, and GST-wtNtA<sup>188</sup> by p34<sup>cdc2</sup> revealed that the major phosphopeptide (b) corresponds to phosphorylation of Ser<sup>71</sup>, peptides a and c correspond to phosphorylation of Ser<sup>84</sup>, and peptide d corresponds to phosphorylation of Thr<sup>188</sup>. From the sum of all these observations it can be concluded that Ser<sup>71</sup> of avian LBR is the major site phosphorylated by p34<sup>cdc2</sup>-protein kinase under in vitro conditions, whereas Ser<sup>84</sup> is weakly modified by the enzyme. It is noteworthy that in some of our experiments we have been unable to detect the spot corresponding to Thr<sup>188</sup>. The very low extent of Thr phosphorylation could explain our inability to detect it when we performed phosphoamino acid analysis of in vitro phosphorylated LBR by p34<sup>cdc2</sup> (see Fig. 3A).

That LBR can be directly phosphorylated by p34<sup>cdc2</sup>-protein kinase implies that the lamin B receptor protein has the potential of being an in vitro substrate for mitotic kinases. To explore this idea, we performed experiments using mitotic cell extracts. Fig. 5C shows that membrane-free cytosol prepared from nocodazole-arrested chicken hepatoma (DU249) cells contained both p34<sup>cdc2</sup> and RS protein kinase activities. This can be deduced from the fact that mitotic extracts phosphorylated GST-ARSNt (which lacks the RS region of LBR and is not a substrate for the RS kinase), GST-wtNtA<sup>71</sup> (which is not phosphorylated by p34<sup>cdc2</sup>), and GST-BS (a fusion protein consisting of GST and five RS repeats but missing the putative p34<sup>cdc2</sup> site of LBR). The same results were obtained with mitotic extracts from HeLa cells (Fig. 5D). To confirm that p34<sup>cdc2</sup>-cyc1n B is truly the kinase responsible for mitotic LBR phosphorylation, we immunodepleted HeLa cell extracts with an anti-cyclin B polyclonal antibody. Fig. 5E shows that extracts pre-treated with the anti-cyclin B antibody had lost their ability to...
phosphorylate histone H1, and GST-ΔRSNt and contained only the RS kinase activity, whereas a typical p34^cdc2 pattern was obtained with the immunoprecipitated activity (Fig. 5F).

Pursuing this point further, interphase DU249 cells and cells arrested at prometaphase were labeled metabolically with [32P]orthophosphate, and the in vitro phosphorylated LBR was immunoprecipitated by aR\textsubscript{1} antibodies (see “Experimental Procedures”). The level of phosphorylation was similar in interphase and mitotic cells (data not shown; see also Ref. 9). The 58-kDa bands corresponding to immunoprecipitated LBR were excised and processed for phosphoamino acid analysis and two-dimensional tryptic phosphopeptide mapping. Only phosphoserine could be detected, irrespective of whether phosphorylation occurred during the interphase or the prometaphase (data

**Fig. 3. Identification of the site on LBR that is phosphorylated in vitro by p34^{cdcl2} protein kinase.** A, phosphoamino acid analysis following in vitro phosphorylation of electroeluted LBR by p34^{cdcl2} protein kinase. \[^{32}P\]-Labeled LBR was analyzed by SDS-PAGE, blotted onto nitrocellulose filters, and subsequently excised and subjected to hydrolysis and phosphoamino acid analysis as described under “Experimental Procedures.” Migration of phosphoserine (PS), phosphothreonine (PT), and phosphotyrosine (PY) standards is shown at left. B, SDS-PAGE analysis and Coomassie Blue staining of GST, GST fusion protein containing the NH\textsubscript{t}-terminal domain of LBR (GST-wtNt, amino acids 1–205), and similar fusion proteins except that Ser\textsuperscript{71}, Ser\textsuperscript{84}, and Thr\textsuperscript{188} were mutated to Ala (constructs termed GST-wtNtA\textsubscript{71}, GST-wtNtA\textsubscript{84}, and GST-wtNtA\textsubscript{188}, respectively). The full-length fusion protein migrates with an apparent molecular mass of approximately 51 kDa. The lower bands represent degradation products (see also Ye and Worman (7) and Nikolakaki et al. (11)). C, immunoblotting of bacterially expressed proteins using an affinity-purified anti-LBR antibody raised against peptide P\textsubscript{r} (aR\textsubscript{1}). The blots were stained using an alkaline phosphatase-conjugated goat anti-rabbit antibody. Note that in addition to full-length fusion protein, aR\textsubscript{1} also reacts with degradation products. D, in vitro phosphorylation of bacterially expressed proteins by p34^{cdcl2} protein kinase. The samples were analyzed by SDS-PAGE and autoradiographed. Molecular mass standards are shown at left (in kDa).

**Fig. 4. Tryptic phosphopeptide analysis of GST-wtNt and alanine mutants of GST-wtNt (for nomenclature, see text and legend to Fig. 3).** In vitro phosphorylated fusion proteins, by p34^{cdcl2} protein kinase, were transferred to nitrocellulose and digested with trypsin. The eluted phosphopeptides were separated by electrophoresis at pH 8.9 (horizontal direction; cathode to the right) and by ascending chromatography. Origins of sample application are marked by \(\bigcirc\).

**Fig. 5. Mitotic cell extracts contain both p34^{cdcl2} and RS protein kinase activities.** Autoradiograms of in vitro-phosphorylated histone H\textsubscript{1}, GST-wtNt, GST-wtNtA\textsubscript{71}, GST-RS, and GST-ΔRSNt (for nomenclature, see text and legend to Fig. 3) by partially purified RS kinase (A), p34^{cdcl2} protein kinase (B), cell extracts made from DU249 cells arrested in mitosis (C), mitotic cell extracts made from HeLa cells (D), mitotic cell extracts from HeLa cells pretreated with an anti-cyclin B antibody (E), and immunoprecipitates of mitotic HeLa cell extracts with the anti-cyclin B antibody (F). Additional bands represent phosphorylated proteins associated with protein A-Sepharose/anti-cyclin B beads. Molecular mass standards are shown at left (in kDa).
not shown; for a typical TLC profile see Fig. 3A). The maps of mitotically phosphorylated LBR and LBR modified at interphase were qualitatively similar (some residues phosphorylated at interphase were phosphorylated to a lower extent at mitosis, i.e. phosphopeptides 1, 3, 8, and 9) except for one spot that was present in the former but absent in the latter (Fig. 6, compare panels C and D). This spot represented the major phosphopeptide (phosphopeptide b) corresponding to Ser71 as shown by mixing equal counts/min of the tryptic digests, in vitro phosphorylated GST-wtNt by p34cdc2, and LBR modified in vivo at interphase (Fig. 6, compare panels B and C with panel E). This mix reproduced the phosphopeptide pattern of LBR that had been modified by mitotic kinases in vivo. The same mitotic pattern was also obtained by mixing equal counts of in vitro phosphorylated GST-wtNt by p34cdc2 and LBR modified in vivo at mitosis (data not shown). To confirm that Ser71 is the additional site phosphorylated at mitosis by p34cdc2, we performed the following experiment. Mitotic extracts (prepared from DU249 cells as described under “Experimental Procedures”) were used to phosphorylate either GST-wtNt or GST-wtNTA71. The phosphorylated proteins were then analyzed by two-dimensional tryptic phosphopeptide mapping. The phosphopeptide map of in vitro phosphorylated GST-wtNt was identical to the map derived from mitotic LBR in vivo (data not shown), whereas the map of in vitro phosphorylated GST-wtNTA71 was similar to the map derived from interphase LBR phosphorylated in vivo; that is, phosphopeptide b was conspicuously missing (compare panels F and C). From the sum of these observations two major conclusions can be drawn. First, the RS and the p34cdc2 protein kinases are both responsible for the mitotic phosphorylation of LBR and second, Ser71 is the major site phosphorylated in vivo by p34cdc2.

**DISCUSSION**

In this study we demonstrated that LBR undergoes mitotic phosphorylation and that the RS protein kinase is the main protein kinase responsible for this modification. Comparison of tryptic phosphopeptide maps of in vivo 32P-labeled LBR immunoprecipitated from chicken cells indicates that the enzyme modifies similar sites at interphase and mitosis. Some serine residues of the RS motif phosphorylated at interphase are phosphorylated to a lower extent at mitosis. Furthermore, we demonstrated that LBR is also a substrate for p34cdc2 protein kinase during mitosis. Using recombinant proteins produced in bacteria, phosphoamino acid analysis and two-dimensional phosphopeptide mapping of in vitro and in vivo 32P-labeled LBR, we have been able to demonstrate that Ser71 is the major site phosphorylated by p34cdc2 at mitosis. Courvalin et al. (9) reported that Thr188 is likely to be phosphorylated by this enzyme during mitosis. According to our results the extent of Thr phosphorylation is very low and most probably Thr188 represents a minor site modified by p34cdc2. In line with our observations is the fact that the phosphoamino acid analysis presented by Courvalin et al. (9) demonstrated that mitotic LBR contained mainly phosphoserine, whereas phosphothreonine was hardly detectable.

Previous reports have shown that the RS protein kinase is strongly associated with LBR, participating in a subassembly of nuclear envelope proteins termed “the LBR complex” (8, 11). The enzyme phosphorylates LBR in a constitutive fashion during interphase (8, 11) and belongs to a novel class of protein kinases that specifically modify arginine-serine (RS) dipeptide motifs. Other members of this novel class of enzymes include a kinase associated with small nuclear ribonucleoprotein particles, which phosphorylates the U1 small nuclear ribonucleoprotein 70-kDa protein and ASF/SF2 (25), and a cell cycle-regulated serine kinase (SRPK1, SR Protein Kinase 1) that can

**TABLE II**

Existence of a p34cdc2 phosphorylation motif in RS-containing proteins

| Predicted p34cdc2 phosphorylation sites are shown in bold and RS motifs are underlined. Numbers in parentheses correspond to the appropriate references. |
|---------------------------------|---------------------------------|
| SSPSRSSRSRSRSRSPG              | Chicken LBR (6)                |
| RSPRSSRSRSRSRSRSRRSRY          | Human splicing factor SC35 (42) |
| RSPSSRSRSSRSSRSSRSSRSSNS        | Human splicing factor SF2 (13)  |
| RSPSSRSSRSSRSSRSSRSSRSSKS       | Mussel sperm-specific protein PH-2B (43) |
| RSPTRRSSRSSRSSRSSRSAS          | Mussel sperm-specific protein PH-1 (43) |
| KSPRSSRSSRSSRSSKSA             | Yeast cytoskeleton assembly control protein SLA1 (44) |
| RSPNRCRGGSSGPTRRQSSRSRSRSRSRSPG | Human papillomavirus E2 protein (45) |
| RTPCSFADQLISTFIANNYLCYFYRRR    | Nuclear polyhedrosis virus polyhedral envelope protein (46) |
phosphorylation of LBR, mediated by p34\(^{cd2}\), might consider that there is some cross-talk between these two mechanisms by which p34\(^{cd2}\) induces the profound structural changes characteristic of mitotic cells remain quite obscure.

It is interesting to note here that, at least at the end of mitosis, the first step in nuclear envelope reformation appears to be the binding of mitochromic vesicles to the surfaces of chromosomes, followed by fusion of these vesicles and assembly of an envelope structure around chromatin (reviewed in Ref. 41). Taking into account that LBR is phosphorylated by the RS kinase and by p34\(^{cd2}\) protein kinase and that the major phosphorylation target of p34\(^{cd2}\) is Ser\(^{71}\) (which is located near the RS repeats), one might consider that there is some cross-talk between these two phosphorylation events. An intriguing possibility would be that phosphorylation of LBR, mediated by p34\(^{cd2}\) protein kinase, together with RS phosphorylation function as a switch preventing premature membrane assembly around chromosomes. This idea is consistent with the previously reported observation that phosphorylation of Lap2 by mitotic cytosol inhibits its binding to chromosomes (5). Along these lines, it is also noteworthy that at least part of p34\(^{cd2}\) and RS protein kinase activities is associated with chromosomes.\(^2\)

Finally, we need to note the existence of a p34\(^{cd2}\) phospho-

---

\(^2\) G. Simos and S. D. Georgatos, unpublished observations.

---

**Mitotic Phosphorylation of LBR**

6213

---

G. Simos and S. D. Georgatos, unpublished observations.