A Nuclear Envelope-associated Kinase Phosphorylates Arginine-Serine Motifs and Modulates Interactions between the Lamin B Receptor and Other Nuclear Proteins*

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Several integral proteins of the inner nuclear membrane have been characterized recently (for recent reviews, see Gerace and Foisner (1994) and Georgatos et al. (1994)). One such protein, originally identified in nucleated avian erythrocytes is the "lamin B receptor" (LBR) or "p58." cDNA sequencing of this protein, originally identified in nucleated avian erythrocytes, has revealed that the protein possesses a long, hydrophilic NH2-terminal domain protruding into the nucleoplasm, eight hydrophobic segments which are predicted to span the membrane, and a hydrophilic COOH-terminal domain. The NH2-terminal domain of LBR binds to p34/p32, whereas a mutated domain lacking the RS region does not. 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.

Several integral proteins of the inner nuclear membrane have been characterized recently (for recent reviews, see Gerace and Foisner (1994) and Georgatos et al. (1994)). One such protein, originally identified in nucleated avian erythrocytes is the "lamin B receptor" (LBR) or "p58." cDNA sequencing of this protein, originally identified in nucleated avian erythrocytes, has revealed that the protein possesses a long, hydrophilic NH2-terminal domain protruding into the nucleoplasm, eight hydrophobic segments which are predicted to span the membrane, and a hydrophilic COOH-terminal domain. The NH2-terminal domain of LBR binds to p34/p32, whereas a mutated domain lacking the RS region does not. 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.

LBR is widely expressed in cells of higher eukaryotes, and the human gene has been recently characterized (Schuler et al., 1994). In addition, three yeast proteins have been identified that are homologous to the hydrophobic regions and the COOH-terminal domain of LBR, but lack most of the NH2-terminal domain (Chen et al., 1991; Lorenz and Parks, 1992; Shimamukai et al., 1992). One of these LBR-related polypeptides (ERG24) is involved in sterol metabolism, and its function in yeast is not complemented by higher eukaryotic LBR (Smith and Blobel, 1994). On the basis of this evidence, it has been previously proposed that "full-length" and NH2-truncated forms of LBR may represent distinct members of a multigene family which includes nuclear envelope and ER1 proteins (Georgatos et al., 1994).

LBR associates with B-type lamins both in vitro and in vivo (Worman et al., 1988; Simos and Georgatos, 1992; Ye and Worman, 1994; Smith and Blobel, 1994), consistent with its presumed function as a lamin receptor. Although the NH2-terminal domain of LBR is probably responsible for lamin B binding (Ye and Worman, 1994), interactions between the far-nuclear group of lamin B and the transmembrane regions of LBR also seem likely (Georgatos et al., 1994; Smith and Blobel, 1994). The association of B-type lamins with LBR is not disrupted during mitosis, when the nuclear envelope is fragmented and the nuclear lamina depolymerized (Meier and Georgatos, 1994).

Recent work has shown that during interphase LBR forms a multimeric complex which includes the nuclear lamins A and B, a specific LBR kinase, and three other polypeptides with molecular masses of 18 (p18), 34 (p34), and 150 (p150) kDa, respectively (Simos and Georgatos, 1992). p18 has been characterized recently as a new integral membrane protein of the bird erythrocyte nuclear envelope. Furthermore, p34 has been identified as the avian equivalent of a human nuclear protein known as p32 (Simos and Georgatos, 1994). p32 has been characterized previously and found to co-isolate with splicing factor 2 (SF2) (Krainer et al., 1991). Recently, Luo et al. (1994) have shown that p32 also interacts with the viral trans-activator Rev, which is required for the replication of human immunodeficiency virus type 1 (HIV-1). Another interaction of p32 seems to involve the HIV-1 protein Tat (Fridell et al., 1995).

The LBR kinase was previously shown to cofractionate with LBR and to phosphorylate LBR in vivo and in vitro, exclusively.
at serine residues. The enzyme is clearly distinct from protein kinase A and Cdc2 kinase, for both of which LBR is a substrate (Simos and Georgatos, 1992). Reasoning that the LBR kinase may regulate interactions between LBR and its partners, we decided to characterize this activity in detail. Results presented below show that the LBR kinase belongs to a novel class of protein kinases which modify specifically RS motifs (Woppmann et al., 1993; Gui et al., 1994). The LBR kinase regulates, through phosphorylation of the RS region, the binding of p34/p32 to the NH2-terminal domain of LBR.

MATERIALS AND METHODS

Reagents—Affi-Gel 10, Protein A-Sepharose, and phosphocellulose were purchased from Bio-Rad, Pharmacia (Pharmacia Uppsala, Sweden), and Whatman (Whatman Biosystems Ltd., United Kingdom), respectively. Histones were obtained from Boehringer Mannheim (Boehringer Mannheim GmbH, Germany). [γ-32P]ATP (6,000 Ci/mmol) was obtained from Amersham (Amersham, Buckinghamshire, UK). A preparation of RS phospho-sites, and the peptide R8 (RSRRSSRSGPRAKG150), corresponding to the RS domain of phospho-sites, were kindly provided by C. Calvio and A. Lammont (EMBL, Heidelberg, Germany). Peptides R1 (RKQRSSQSSSSSRSSSGS150), R5 (KSSSSRSSRSGPRAKG150), R6 (KGRRSRSSSHRE105), R7 (KIEFAIKTPESKPSST140), R8 (ANSQKNNRPNADPK511), R9 (C130TERNDSSKLEQOKLPDVE150), and R10 (RPEDEHCKKKGNLAYWAL569)569, representing different regions of chicken LBR (Warman et al., 1990), were made at the Protein Sequencing and Peptide Synthesis Facility of EMBL. A cysteine residue (*C) was added to the sequences for coupling purposes. R5 peptide was coupled to Affi-Gel 10 by incubating 30 mg of the peptide with 3 ml of the column as described previously (Georgatos and Blobel, 1987b). The anti-LBR antibody aR1, raised against the peptide R6, as well as an anti-p34 antibody (ap34-C), raised against the COOH-terminal residues (COOH-Prp2) of p34, were kindly provided by G. Blobel (Yale, New Haven, CT). p34 was purified by applying the Triton X-100 extract of turkey erythrocyte ghosts to an immunoaffinity column (Schleicher & Schuell GmbH, Germany). p34 was purified by applying the Triton X-100 extract of turkey erythrocyte ghosts to an immunoaffinity column containing 1 mg of affinity-purified aR1 IgG bound to Protein A-Sepharose (Pharmacia Uppsala, Sweden) and ligated into the expression vector (Pharmacia Biotech Inc.) was used to construct plasmids expressing the enzyme. p34 was applied to a phosphocellulose column previously equilibrated with 20 mM Tris-HCl, pH 7.5, 0.3% Triton X-100, 1.5 M NaCl, and elution of the protein kinase was accomplished by a linear (10%–20%) NaCl gradient. Protein concentration was determined by the method of Bradford (1976). For the determination of Km, the amount of substrate in the reaction mixture was varied between 0.1 and 1 μg, and incorporation of radioactivity was measured by excising the radioactive bands from an SDS-PAGE gel and scintillation counting. The Km values were calculated using the MicroCal Origin (version 2.94) program. Determination of stoichiometry, phosphorylation was carried out using a concentration of substrate at the end of the linear range of the reaction, and the incorporation of radioactivity was measured by scintillation counting of excised radioactive bands from an SDS-PAGE gel.

In situ kinase assays were performed according to Kameshita and Fujisawa (1989). LBR was added to the separating gel, at a concentration of 0.1 mg/ml, prior to polymerization. For control experiments, LBR was omitted or replaced with 0.1 mg/ml bovine serum albumin. Electrophoresis was carried out at 25 mA for approximately 1 h, using 12% acrylamide minigels. SDS was removed from the gel by equilibration in 20% 2-propanol, 50 mM Tris-HCl, pH 8.0. The kinase was then fully denatured by incubating in 6 M guanidinium hydrochloride for 1 h at 30°C and then renatured and allowed to renature overnight at 4 °C in 50 mM Tris-HCl, 14 mM 2-mercaptoethanol, and 0.04% Tween 40, pH 8.0. Gels were incubated in 4 ml of assay buffer (10 mM MgCl2, 200 mM NaCl, 25 mM Tris-HCl) for 20 min, at room temperature. The kinase assay was then initiated by the addition of 50 μl cold ATP and 50 μCi of [γ-32P]ATP (6,000 Ci/mmol). Incubation was carried out for an additional 60 min. The reaction was terminated by extensive washing with 5% trichloroacetic acid containing 10 mM sodium pyrophosphate. The gels were dried and exposed to Kodak X-Omat film.

Proteolytic peptide mapping was performed essentially as described by Luo et al. (1991). Briefly, phosphorylated LBR was run on a SDS-PAGE gel and then transferred to a nitrocellulose sheet. The radiolabeled bands were excised in 0.5% PVP (polyvinylpyrrolidone) in 100 mM acetic acid, for 1 h, at 37 °C and washed extensively with water. Samples were then digested to completion with trypsin. The digestion products were subsequently applied to thin layer cellulose plates (Kodak) and run in a solvent system consisting of 500 V. Ascending chromatography (in the second dimension) was performed using as a solvent a mixture of 1-butanol:pyridine:acetic acid:water in ratios of 750:500:150:600.

Expression of GST Fusion Proteins—The pGEX-2T bacterial expression vector (Pharmacia Biotech Inc.) was used to construct plasmids expressing GST fusion proteins. LBR (1976) was used as template. The LBR-5′-coding sequence was amplified using primers designed on the basis of mRNA sequences, and cloned into the BamHI and EcoRI sites of pGEX-2T. The polymerase chain reaction was performed using the QIAEX Gel extraction kit (QIA-GEN Inc.). Purified DNA was digested with EcoRI and BamHI, and ligated into the BamHI/EcoRI site of pGEX-2T. Escherichia coli DH5α was transformed with the kinase-coding plasmids. An oligonucleotide-directed in vitro mutagenesis system (Alteredsites11, in Vivo Mutagenesis System, Promega) was used to delete five serine and arginine repeats in the NH2-terminal domain of LBR. Using the oligonucleotide 5′-TGCTGCGCCGACCTGTCGCGAGAGAATC-3′, the codons for amino acids 75 to 84 were deleted (ΔRS75). In addition, the following oligonucleotides were used to delete the oligonucleotide 5′-CTGCTGCGCCGACCTGTCGCGAGAGAATC-3′, the codons for amino acids 5 to 14 were deleted (ΔRS14). Digestion products were analyzed on an agarose gel and purified using the QIAEX Gel extraction kit (QIA-GEN Inc.). Purified DNA was digested with EcoRI and BamHI, and ligated into the BamHI/EcoRI site of pGEX-2T. Escherichia coli DH5α was transformed with the kinase-coding plasmids. An oligonucleotide-directed in vitro mutagenesis system (Alteredsites11, in Vivo Mutagenesis System, Promega) was used to delete five serine and arginine repeats in the NH2-terminal domain of LBR. Using the oligonucleotide 5′-TGCTGCGCCGACCTGTCGCGAGAGAATC-3′, the codons for amino acids 75 to 84 were deleted (ΔRS75). In addition, the following oligonucleotides were used to delete the oligonucleotide 5′-CTGCTGCGCCGACCTGTCGCGAGAGAATC-3′, the codons for amino acids 5 to 14 were deleted (ΔRS14). Digestion products were analyzed on an agarose gel and purified using the QIAEX Gel extraction kit (QIA-GEN Inc.). Purified DNA was digested with EcoRI and BamHI, and ligated into the BamHI/EcoRI site of pGEX-2T. Escherichia coli DH5α was transformed with the kinase-coding plasmids.
RESULTS

The LBR Kinase Modifies Specifically RS Motifs—We have reported previously that turkey erythrocyte nuclear envelopes contain substantial levels of LBR kinase activity and that the envelope-bound enzyme can be partially solubilized by extraction with the nonionic detergent Triton X-100 (Simos and Georgatos, 1992). To examine more systematically the distribution of the LBR kinase and obtain a preparation enriched in this enzyme, we fractionated turkey erythrocytes using established methods (Georgatos and Blobel, 1987a, 1987b). The different subcellular fractions were assayed for kinase activity employing as a substrate exogenous LBR that had been purified by electrophoresis. Cytosolic and plasma membrane fractions together contained approximately 20% of the total LBR kinase activity, whereas a significant portion of the kinase was released in the nuclear fraction (Fig. 1). High levels of the enzyme were present in the nuclear content, released from the nuclei after treatment with DNase I, and then centrifuged for 3 min in an Eppendorf centrifuge. The beads were resuspended in 25 μl of electrophoresis sample buffer, and bound material was analyzed on 12% SDS-polyacrylamide gels. Phosphorylation of GST-wtNt was achieved by incubating glutathione-Sepharose beads with purified LBR kinase in the presence of 100 μM ATP. After 30 min, the beads were pelleted, washed with PBST, and used for the binding assays, as described. Blots were processed as described by Harlow and Lane (1988), using the ap34-C antibody. For detection of immunoreactive proteins, alkaline phosphatase-conjugated goat anti-rabbit antibodies were used.

As a first step to characterize the enzyme we were interested in, we used salt or Triton X-114 extracts which contained LBR-free kinase to phosphorylate a set of peptides representing different regions of the NH2 and COOH-terminal domains of LBR (Fig. 3B, for details see "Materials and Methods"). Two NH2-terminal peptides (R2 and R0) which contained three or five arginine-serine (RS) motifs, respectively, could be phosphorylated by the LBR kinase (Fig. 3A). However, a third NH2-terminal peptide (R0) which contained three RS motifs but no downstream flanking sequence could not serve as a substrate and affected LBR phosphorylation marginally (Fig. 3A). Interestingly, the synthetic derivative R0 (which contained the highest number of RS motifs) inhibited completely the phosphorylation of purified LBR by the corresponding kinase, while the other two peptides inhibited LBR phosphorylation to a lower extent (Fig. 3A). These data strongly suggested that the RS dipeptide motifs represent the major phosphorylation sites of LBR modified by the LBR kinase.

To probe this point further, we expressed in E. coli a fusion protein consisting of GST and the NH2-terminal domain of LBR (residues 1–205; construct termed GST-wtNt). For control purposes, we also expressed in bacteria a similar fusion protein missing the RS motifs (deletion of residues 75–84; construct termed GST-ΔRSNt), as well as a protein consisting of GST and the RS region of LBR (residues 75–84; construct termed GST-RS). The three recombinant proteins were used as substrates for in vitro phosphorylation assays.

Data depicted in Fig. 4 reveal that LBR kinase present in...
salt or Triton X-114 extracts could efficiently phosphorylate GST-wtNt, whereas GST-DRSNt was not phosphorylated. This was not due to a global misfolding of the polypeptide chain induced by the deletion of the RS region, because both GST-DRSNt and GST-wtNt (both of which contain a consensus protein kinase A site) were efficiently modified by protein kinase A and exhibited the same solubility and ligand-binding properties.3 Finally, GST-RS could serve as a substrate for the LBR kinase, but was phosphorylated at a lower stoichiometry than GST-wtNt. The relatively lower extent of phosphorylation in the latter case might be due to the lack of “context” information normally provided by sequences flanking the RS region. This idea is further supported by the fact that the synthetic peptide R₀, which includes the RS region but lacks long neighboring sequences, was also phosphorylated substoichiometrically in comparison to intact LBR or GST-wtNt (data not shown).

Exploiting this information, we proceeded with the purifica-

3 E. Nikolakaki, J. Meier, G. Simos, and S. D. Georgatos, unpublished observations.

FIG. 2. Extraction of LBR kinase activity from nuclear envelopes by Triton X-114 or 1 M NaCl. A, equivalent fractions of whole turkey erythrocyte nuclear envelopes (NE), extracted nuclear envelopes with 1% Triton X-114 (TX-Pel), the Triton X-114 extract of nuclear envelopes (TX-Sup), the aqueous phase of the Triton X-114 extract (TX-Aq), and the detergent phase of the Triton X-114 extract (TX-Det) were incubated with [γ-32P]ATP in the absence (-) or presence (+) of exogenous LBR. Samples were analyzed by SDS-PAGE and stained with Coomassie Blue (left panel) or autoradiographed (right panel). B, equivalent fractions of turkey erythrocyte nuclear envelopes and 1 M NaCl extracts of nuclear envelopes were incubated with [γ-32P]ATP in the absence (-) or presence (+) of exogenous LBR. Samples were analyzed by SDS-PAGE and stained with Coomassie Blue (left panel) or autoradiographed (right panel). LBR is indicated by an arrow. Bars on the left indicate molecular masses (in kDa).

FIG. 3. Phosphorylation of purified LBR by protein kinases present in the aqueous phase of the Triton X-114 extract of nuclear envelopes in the presence of various synthetic peptides. A, 1.5 μg of electroeluted LBR were incubated with 0.5 μM each peptide and the aqueous phase of the Triton X-114 extract in the presence of 50 μM [γ-32P]ATP in a total reaction volume of 25 μl. Samples were subsequently analyzed by SDS-PAGE on 15% gels and autoradiographed. An autoradiogram of the SDS-gel is shown. B, amino acid sequences of the peptides used. The relative position of the peptides in the LBR molecule is schematically indicated. Black boxes along the LBR sequence numbered with Roman numerals, represent potential transmembrane domains.

FIG. 4. Phosphorylation of GST, GST fusion protein containing the NH₂-terminal domain of LBR (GST-wtNt, amino acids 1-205), GST fusion protein containing the NH₂-terminal domain of LBR, but missing the RS motifs (GST-ΔRSNt, amino acids deleted 75-84), and GST fusion protein containing five RS dipeptide repeat (GST-RS) by LBR kinase present in the aqueous phase of the Triton X-114 extract of turkey erythrocyte nuclear envelopes. 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 (1994)). A, SDS-PAGE analysis and Coomassie Blue staining of GST, GST-wtNt, GST-ΔRSNt, and GST-RS. B, immunoblotting of bacterially expressed proteins using an affinity-purified antiserum against GST (aR₁). The blots were stained using an alkaline phosphatase-conjugated rabbit anti-rabbit antibody. Note that in addition to full-length fusion protein, aR₁ also reacts with degradation products. C, Phosphorylation of bacterially expressed proteins by the LBR kinase. The samples were analyzed by SDS-PAGE on 12% gels and autoradiographed. Molecular mass standards are shown at left (in kDa).
tion of the LBR kinase from nuclear envelope extracts. To this end, we first chromatographed the salt extract or the aqueous phase of the Triton X-114 extract through phosphocellulose and loaded the pool of the fractions possessing LBR kinase activity onto an agarose column containing immobilized R0 peptide (for details see "Materials and Methods"). Analysis of the eluted fractions by SDS-PAGE and staining of the corresponding gels with silver nitrate revealed the presence of two bands, a major one at 54 kDa and a minor one at 110 kDa (Fig. 5A). In situ phosphorylation assays in polyacrylamide gels to which 0.1 mg/ml purified LBR were incorporated revealed that a protein with a molecular mass of 110 kDa could modify LBR (Fig. 5B, lane 2). This was specific because no labeling was detected when LBR was omitted from the gel or replaced by bovine serum albumin (data not shown). In addition, the same 110-kDa polypeptide appeared to phosphorylate LBR when, instead of the column-purified preparation, LBR kinase co-immunoprecipitated with LBR from a Triton X-100 lysate of nuclear envelopes was used in an in situ gel assay (Fig. 5B, lane 1). These data suggest that the 110-kDa band corresponds to the catalytic subunit of the LBR kinase. The nature of the 54-kDa protein which copurifies with the kinase but contains no LBR-phosphorylating activity is presently unknown.

The column-purified enzyme was also able to bind to LBR in solution and could be co-immunoprecipitated with LBR using affinity-purified antibodies (Fig. 5C, lanes 1 and 2). Binding involved the RS dipeptide motifs of the LBR, since the kinase was able to associate with the GST-wtNt immobilized on glutathione-Sepharose beads, whereas no interaction with GST-\(A\)RSNt was observed (Fig. 5C, lanes 3–5).

The partially purified LBR kinase did not modify histones, casein, and myelin basic protein, but did phosphorylate intact LBR and GST-wtNt (Fig. 6B). Interestingly, when a well-characterized subcellular fraction containing SR proteins (Zailer et al., 1992) was incubated with column-purified kinase, we found that the enzyme could efficiently phosphorylate the 30-kDa major component (Fig. 6B). The other proteins present in the SR fraction were not phosphorylated to a significant extent suggesting that the LBR kinase may show substrate selectivity. The 30-kDa band contains two distinct polypeptides RSP30a and RSP30b, which have also been described as SF2 and SC35, respectively. The phosphorylation of LBR and RSP30 was inhibited by an excess of the synthetic peptide R0, as well as by a peptide containing six arginine-serine (RS) repeats (R0sp, Fig. 6C). From the sum of these observations it can be inferred that the LBR kinase belongs to a novel class of enzymes which can also modify SR proteins (Gui et al. 1994).

To determine more specifically the serine residues of LBR that are phosphorylated by the LBR kinase, we expressed in E. coli fusion proteins identical with GST-wtNt except that in each case one of Ser\(^{76}\), Ser\(^{78}\), Ser\(^{80}\), Ser\(^{82}\), and Ser\(^{84}\) of the RS motif was mutated to glycine or alanine (Table I). Mutation of Ser\(^{76}\) to Gly resulted in a construct that could not be expressed in E. coli, even though the sequence and the proper subcloning of the mutated cDNA into the pGEX-2T expression vector were confirmed. However, the other four recombinant proteins were appropriately expressed, purified, and used as substrates for in vitro phosphorylation assays with the partially purified LBR kinase. Results presented in Table I and Fig. 7 reveal that all four fusion proteins could be phosphorylated similarly to wtNt. The apparent \(K_m\) of the kinase for the recombinant proteins was in the range of 1.7–2.4 \(\mu M\). Taking into consideration that

![Fig. 5. Partial purification and characterization of the LBR kinase.](http://www.jbc.org/)

![Fig. 6. Substrate specificity of LBR kinase.](http://www.jbc.org/)
The stoichiometry of the phosphorylation reaction for both wtNt and the mutants was close to 1, any one of the serines of the RS motif, but only one per molecule, should be phosphorylated at steady state. That several spots have been observed previously in two-dimensional phosphopeptide maps of in vivo or in vitro phosphorylated LBR (for relevant information, see Simos and Georgatos, 1992, 1994) is consistent with this interpretation. Similar phosphopeptide mapping confirm that the peptides phosphorylated by the partially purified LBR kinase correspond to the peptides phosphorylated in vivo.

The RS-containing Region at the NH2-terminal Domain of LBR. Harbors a p34/p32 Binding Site—We have previously shown that p34/p32 is co-immunoprecipitated with LBR when Triton X-100 extracts of whole erythrocytes or erythrocyte ghosts are incubated with affinity-purified anti-LBR antibodies (Simos and Georgatos, 1992, 1994). To find out whether the p34/p32-LBR interaction involves the RS motifs of the latter, GST-wtNt immobilized on glutathione-Sepharose beads was incubated with a fraction highly enriched in p34/p32 (for details on purification, see “Materials and Methods”). A and B show that p34/p32 and GST-wtNt formed a binary complex. This complex could be detected by probing blots of the material co-sedimenting with GST-wtNt-glutathione-Sepharose beads with affinity-purified anti-p34/p32 antibodies (Simos and Georgatos, 1994). Similar results were obtained when GST-wtNt immobilized on glutathione-Sepharose beads, or electroleuted LBR bound to anti-LBR antibodies/protein A-Sepharose beads, were incubated with a Triton X-100 extract of turkey erythrocyte nuclear envelopes, and the material bound to the beads was analyzed by immunoblotting (Fig. 8B, lane 1, and data not shown).

The specificity of this binding was assessed by performing the same type of experiment with the deletion mutant GST-ΔRSNt. Under these conditions, the binding of p34/p32 to the glutathione beads carrying the mutant protein was greatly inhibited (Fig. 8A, lane 4) or completely abolished (Fig. 8B, lane 3). Finally, repetition of the assay in the presence of an excess of R0 peptide also abolished binding between GST-wtNt and p34/p32 (Fig. 8B, lane 2). From these results it can be concluded that p34/p32 binds to LBR by interacting with the RS-containing region.

Phosphorylation by the LBR Kinase Modulates Binding of p34/p32 to LBR—Knowing that p34/p32 and LBR interact through the RS motifs and considering that these motifs are specifically modified by the LBR kinase, we set to examine the stoichiometry of the phosphorylation reaction for both wtNt and the mutants was close to 1, any one of the serines of the RS motif, but only one per molecule, should be phosphorylated at steady state. That several spots have been observed previously in two-dimensional phosphopeptide maps of in vivo or in vitro phosphorylated LBR (for relevant information, see Simos and Georgatos, 1992, 1994) is consistent with this interpretation. Similar phosphopeptide mapping confirm that the peptides phosphorylated by the partially purified LBR kinase correspond to the peptides phosphorylated in vivo.

### Table I

| Construct          | Stoichiometry | $K_m$ a |
|--------------------|---------------|---------|
| GST-wtNt (75RSRSRSRSRS84) | 0.7            | 2.1     |
| GST-NtI (75RSRSGRSGSRS84) | 0.7            | 2.3     |
| GST-NtA80 (75RSRSRSRARS84) | 0.6            | 1.8     |
| GST-NtA82 (75RSRSRSRARS84) | 0.7            | 1.7     |
| GST-NtI (75RSRSRSRARS84) | 0.8            | 2.4     |
| GST-ΔRSNt         | 0.0           |         |

a All recombinant proteins showed the same pattern in SDS-PAGE as GST-wtNt (see also Fig. 4). Due to the presence of the degradation products, an average molecular mass of 40 kDa was assumed for all fusion proteins.

### Discussion

Characterization of the LBR-associated Kinase—We report here the characterization of the LBR-associated kinase. The partially purified enzyme is able to bind to the NH$_2$-terminal domain of LBR, phosphorylating the same sites as those modified by the LBR kinase, we set to examine the stoichiometry of the phosphorylation reaction for both wtNt and the mutants was close to 1, any one of the serines of the RS motif, but only one per molecule, should be phosphorylated at steady state. That several spots have been observed previously in two-dimensional phosphopeptide maps of in vivo or in vitro phosphorylated LBR (for relevant information, see Simos and Georgatos, 1992, 1994) is consistent with this interpretation. Similar phosphopeptide mapping confirm that the peptides phosphorylated by the partially purified LBR kinase correspond to the peptides phosphorylated in vivo.

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#### DISCUSSION

Characterization of the LBR-associated Kinase—We report here the characterization of the LBR-associated kinase. The partially purified enzyme is able to bind to the NH$_2$-terminal domain of LBR, phosphorylating the same sites as those...
LBR-associated Kinase Modifies RS Motifs

Fig. 9. Phosphorylation by the LBR kinase inhibits binding of p34 to the NH2-terminal domain of LBR. Immunoblot showing binding of p34 when a Triton X-100 lysate of turkey erythrocyte nuclear envelopes was incubated with GST-wtNt immobilized on glutathione-Sepharose beads (lane 1). In lanes 2, 3, and 4, immobilized GST-wtNt was incubated with buffer and 100 μM ATP (lane 2), purified LBR kinase and ATP (lane 3), or purified LBR kinase, in the absence of ATP (lane 4), prior to incubation with the Triton X-100 lysate. The blots were processed as described in Fig. 8.

Regulation of LBR Interactions by Phosphorylation—Previous studies have shown that the poly peptides participating in the multimeric LBR complex is p34 (Simos and Georgatos, 1994), a homologue of a previously identified protein (p32) associated with splicing factor 2 (Krainer et al., 1991). This protein does not have a splicing activity itself and does not affect the splicing activity of SF2 (Krainer et al., 1991). However, the fact that p32 and SF2 co-purify through several chromatographic steps suggests a specific and probably functional interaction between the two proteins. As p32/p34 interacts with the HIV-1 products Rev and Tat (Luo et al., 1994; Fridell et al., 1995), it is also possible that p32/p34 may affect the properties of viral transcriptional and splicing regulators.

Because p34 does not co-isolate with LBR when extraction and purification are performed under conditions that favor phosphorylation (e.g., in the presence of ATP and phosphatase inhibitors; Simos and Georgatos, 1992, 1994), we suspected that p34 may interact with the RS domain of LBR in a phosphorylation-dependent manner. Results obtained by in vitro binding assays clearly show that p34 binds tightly to the RS motifs of LBR when the latter is unphosphorylated, but dissociates from it upon phosphorylation mediated by the LBR kinase.

Recent studies have shown that the RS domains mediate protein-protein interactions between components of the splicing machinery (Wu and Maniatis, 1993; Kohz et al., 1994), probably in a phosphorylation-dependent manner (Woppmann et al., 1993; Memoud et al., 1994). The existence of RS motifs in the LBR molecule and the occurrence of a splicing factor-associated protein among the constituents of the LBR complex raise the possibility that LBR, alone or in combination with p34, may interact with components of the splicing machinery. Taking into account earlier observations (Spector et al., 1991), it can be speculated that LBR and its partners act as transient docking sites for nuclear “speckles,” in the nuclear envelope. Such a possibility is further supported by the fact that snRNPs migrate to the nuclear periphery when murine erythroleukemia cells (MEL) are induced to differentiate in vitro (Antoniou et al., 1993).

Based on the fact that the lamins are peripheral membrane proteins, whereas LBR traverses the inner nuclear membrane, LBR was considered to function as a lamina receptor. On the other hand, LBR, together with the integral membrane protein LAP2 (Fischler and Gerace, 1993), are the most obvious candidates to mediate the association of interphase nuclear membranes to chromatin (Maison et al., 1995). The data presented here expand the possible functions of LBR, raising the possibility that the LBR complex is a molecular device that may couple the karyoskeleton (nuclear lamina) to regulatory factors involved in different aspects of gene expression.

It is also noteworthy that protein kinase A and Cdc2, which can be induced by hormones and mitotic factors, modify sites which are close, but distinct, from those phosphorylated by the RS kinase. Given that this segment of the molecule is exposed to the nu cleoplasm and is charged (Worman et al., 1990), these modifications may as well participate in the regulation of LBR-protein and/or LBR-DNA interactions during interphase or mitosis. Such potential interactions remain to be addressed in future studies.

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