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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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Previous studies have identified a subassembly of nuclear envelope proteins, termed "the LBR complex." This complex includes the lamin B receptor protein (LBR or p58), a kinase which phosphorylates LBR in a constitutive fashion (LBR kinase), the nuclear lamins A and B, an 18-kDa polypeptide (p18), and a 34-kDa protein (p34/p32). The latter polypeptide has been shown to interact with the HIV-1 proteins Rev and Tat and with the splicing factor 2 (SF2). Using recombinant proteins produced in bacteria and synthetic peptides representing different regions of LBR, we now show that the LBR kinase modifies specifically arginine-serine (RS) dipeptide motifs located at the nucleoplasmic, NH_2-terminal domain of LBR and in members of the SR family of splicing factors. Furthermore, we show that the NH_2-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 chicken and human LBR has revealed that the protein possesses a long, hydrophilic NH_2-terminal domain protruding into the nucleoplasm, eight hyrdophobic segments which are predicted to span the membrane, and a hydrophilic COOH-terminal domain (Worman *et al.*, 1990; Ye and Worman, 1994). The NH_2-terminal domain of LBR contains distinct sites for protein kinase A and Cdc2 kinase phosphorylation (Simos and Georgatos, 1992; Courvalin *et al.*, 1992), as well as a stretch rich in arginine-serine (RS) motifs (Simos and Georgatos, 1994). Such dipeptide motifs have been identified in a variety of splicing factors and have been shown to mediate protein-protein interactions between components of the splicing machinery (Wu and Maniatis, 1993; Kohtz *et al.*, 1994).

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 NH_2-terminal domain (Chen *et al.*, 1991; Lorenz and Parks, 1992; Shimanuki *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 "NH_2-truncated" forms of LBR may represent distinct members of a multigene family which includes nuclear envelope and ER[1] 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 NH_2-terminal domain of LBR is probably responsible for lamin B binding (Ye and Worman, 1994), interactions between the farnesyl 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.[2] 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

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[1] The abbreviations used are: ER, endoplasmic reticulum; HIV, human immunodeficiency virus; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; GST, glutathione *S*-transferase.

[2] G. Simos, C. Maison, and S. D. Georgatos, unpublished observations.

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 NH_2-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-^{32}P]ATP (6,000 Ci/mmol) was obtained from Amersham (Amersham, Bacacos SA, Greece). A preparation of RS splicing factors, and the peptide R_{SF} (RSRSRSRSRSRS) corresponding to the RS domain of splicing factors, were kindly provided by C. Calvio and A. Lamond (EMBL, Heidelberg, Germany).

Peptides R_1 (^{61}KQRKSQSSSSSPSRRSRSRS^{80}), R_2 (^{78}SRSRSRSPGRPAKG^{91}), R_0 (^{70}SSPSRRSRSRSRSRSPGRPAKG^{91}), R_3 (^{90}KGRRRSSSHSRE^{101}), R_4 (^{182}KIFEAIKTPEKPSSKT^{197}), R_5 (*C^{497}ANSQKNNFRRNPADPK^{511}), R_6 (*C^{119}KPSENNTRRYNGEPDSTERND^{139}), R_7 (*C^{135}TERNDTSSKLLEQQKLKPDVE^{155}), and R_8 (^{580}DEHHCKKKYGLAWERY^{595}*C), representing different regions of chicken LBR (Worman *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. R_0 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 aR_1, raised against the peptide R_1, as well as an anti-p34 antibody (ap34-C), raised against the COOH-terminal residues (CGG^{141}TGESEWKDTNYTLNTDS^{157}) of HeLa p32 (Honore *et al.*, 1993), were prepared and affinity-purified as described previously (Simos and Georgatos, 1992, 1994). The tripeptide CGG was added to the sequences for coupling purposes. All other chemicals were purchased from Sigma (Sigma, Deisenhofen, Germany).

Cell Fractionation—Turkey erythrocyte nuclear envelopes and plasma membranes were isolated as described previously (Georgatos and Blobel, 1987a, 1987b). Nuclear content was isolated by extensive digestion of nuclei with DNase I.

Protein Chemical Methods—LBR was purified by electroelution from urea-insoluble nuclear envelope fractions. For these purposes, SDS-polyacrylamide gels were initially stained with copper sulfate, and the appropriate bands were excised. After washing in 0.25 M EDTA, 0.25 M Tris-HCl, pH 9.0, to remove copper ions, LBR was recovered in 25 mM Tris, 192 mM glycine, 0.025% SDS using the Biotrap BT 1000 apparatus (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 aR_1 IgG bound to Protein A-Sepharose. p34 was eluted by 1 M NaCl and was further purified by passing the 1 M NaCl eluate of the immunoaffinity column through a Sephacryl S-100 gel filtration column (Simos and Georgatos, 1994).

To isolate the LBR kinase, turkey erythrocyte nuclear envelopes were extracted either with 50 mM Tris-HCl, pH 7.5, 1 M NaCl, 1 mM dithiothreitol and 1 mM PMSF, or with 20 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 150 mM NaCl, 1 mM dithiothreitol, 1 mM PMSF, and 1% Triton X-114. The aqueous phase of the Triton X-114 extract (Bordier, 1981) or the 1 M NaCl extract (following dilution to 0.3 M and clarification by centrifugation at 15,000 $\times$ *g* for 20 min) were applied to a phosphocellulose column previously equilibrated with 20 mM Tris-HCl, pH 7.5, 0.3 M NaCl, 1 mM dithiothreitol, and 1 mM PMSF. The bound proteins were eluted by a linear (0.3–1 M) NaCl gradient. Kinase-containing fractions were pooled, the salt concentration was adjusted to 0.35 M, and the material was further chromatographed through an Affi-Gel 10 column containing the R_0 peptide. Analysis of the flow-through fractions showed that all the kinase activity was bound to the column. The column was subsequently washed with 0.9 M NaCl, and elution of the kinase activity was accomplished by a linear (0.9–2.2 M) NaCl gradient. The active fractions were pooled, concentrated with an Amicon device, and used in subsequent experiments. RS kinase activity was determined by measuring the incorporation of PO_4^{3-} from [γ-^{32}P]ATP to electroeluted LBR. Routine assays were carried out at 30 °C in a total volume of 25 μl containing 25 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 200 mM NaCl, 50 μM [γ-^{32}P]ATP (6,000 Ci/mmol), 0.3% Triton X-100, 1.5 μg of LBR, and an aliquot of the enzyme. After 30 min, the reaction was terminated by the addition of 5 μl of 5 $\times$ electrophoresis sample buffer (Laemmli, 1970) and analyzed by SDS-PAGE. SDS-PAGE was performed according to Laemmli (1970) using 12% polyacrylamide gels. Protein concentration was determined by the method of Bradford (1976). For the determination of K_m, the amount of substrate in the reaction mixture was varied between 0.1 and 5 μg, and incorporation of radioactivity was measured by excising the radioactive bands from an SDS-PAGE gel and scintillation counting. The K_m values were calculated using the MicroCal Origin (version 2.94) program. For 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 room temperature 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 $MgCl_2$, 200 mM NaCl, 25 mM Tris-HCl) for 20 min, at room temperature. The kinase assay was then initiated by the addition of 50 μM cold ATP and 50 μCi of [γ-^{32}P]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 radioactive LBR bands were excised, soaked in 0.5% PVP 360 (polyvinylpyrrolidone) in 100 mM acetic acid, for 1 h, at 37 °C and washed extensively with water. Samples were then digested to completion with tosylphenylalanyl chloromethyl ketone-treated trypsin in 50 mM NH_4HCO_3, at 37 °C, overnight. Residual salt was removed by repeated lyophilization, and the digests were subsequently applied to thin layer cellulose plates (Kodak) for two-dimensional peptide mapping. Electrophoresis (in the first dimension) was run at pH 8.9 (1% ammonium carbonate) for 1 h at 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 that encode various regions of chicken LBR (Worman *et al.*, 1990) fused with glutathione *S*-transferase (GST) (Smith and Johnson, 1988). To generate the cDNA coding for wtNt, 30 cycles of the polymerase chain reaction were performed as described by Saiki *et al.* (1987), using the GeneAmp kit (Perkin-Elmer Corp., Norwalk, CT). Full-length LBR cloned to the *Eco*RI site of Bluescript SK$^-$ was used as template. The LBR-SK$^-$ clone was a generous gift of G. Blobel (Rockefeller University, New York) and H. J. Worman (Columbia University, New York). Parameters for polymerase chain reaction were as follows: denaturation at 92 °C for 1 min, annealing at 57 °C for 1 min, and extension at 72 °C for 40 s. The sense primer contained nucleotides +1 to +21 of LBR preceded by a *Bam*HI site. CAGTA was added 5′ to the *Bam*HI site. The antisense primer was complementary to nucleotides +598 to +615 of LBR. 5′ to this sequence was a complementary stop codon preceded by an *Eco*RI site. GC was added 5′ to the *Eco*RI site. The polymerase chain reaction product was purified using the QIAEX Gel extraction kit (QIAGEN Inc.). Purified DNA was digested with *Eco*RI and *Bam*HI, repurified, and ligated into the *Bam*HI/*Eco*RI site of pGEX-2T. *Escherichia coli* strains XL-1 Blue were transformed by standard methods.

An oligonucleotide-directed *in vitro* mutagenesis system (Altered Sites^{R}II, *In Vitro* Mutagenesis System, Promega) was used to delete five serine and arginine repeats in the NH_2-terminal domain of LBR. Using the oligonucleotide 5′-TGCTGGCCGACCAGGTCTGGAAGAGAACT-3′, the codons for amino acids 75 to 84 were deleted (ΔRSwtNt). In addition, using the oligonucleotides 5′-TCTGCTTCTACCTCTTCTGGA-3′, 5′-TCGAGATCTGCCTCTACTTCT-3′, 5′-TCTGGATCGAGCTCTGCTTCT-3′, 5′-AGGAGATCTGGCTCGAGATCT-3′, and 5′-GACCAGGAGCTCTGGATCG-3′, Ser^{76}, Ser^{78}, Ser^{80}, Ser^{82}, and Ser^{84} were

mutated to Gly (GST-NtG^{76}), Gly (GST-NtG^{78}), Ala (GST-NtA^{80}), Ala (GST-NtA^{82}), and Ala (GST-NtA^{84}), respectively. The mutated cDNAs were sequenced and subcloned into the pGEX-2T expression vector as described previously for wtNt.

A fragment containing all five arginine-serine repeats was generated using the following two complementary oligonucleotides 5′-GATCCA-GAAGTAGAAGCAGATCTCGATCCAGATCTAG-3′ and 5′-AATTCTA-GATCTGGATCGAGATCTGCTTCTACTTCTG-3′ and inserted into the *Bam*HI/*Eco*RI site of pGEX-2T expression vector.

GST fusion proteins were produced in bacteria and purified using glutathione-Sepharose (Pharmacia), as described by Smith and Johnson (1988). When used in phosphorylation experiments, the fusion proteins were dialyzed previously against 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM PMSF, and 1 mM 2-mercaptoethanol.

Binding Assays—The standard binding assays involved 3 μg of GST-wtNt or GST-ΔRSwtNt immobilized on glutathione-Sepharose beads and ~1 μg of partially purified p34, resuspended in PBST (20 mM phosphate buffer, pH 7.4, 150 mM NaCl, 1% Triton X-100, and 0.5 mM PMSF) in a total volume of 1 ml. In other experiments, purified p34 was replaced by a Triton X-100 extract of turkey erythrocyte nuclear envelopes. The mixtures were incubated for 60 min at room temperature 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.

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 activity, 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 electroelution. Cytosolic and plasma membrane fractions together contained approximately 20% of the total LBR kinase activity, while 80% of this was recovered 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, as well as in the 1 M NaCl extract of the nuclear envelopes. However, the salt-washed nuclear envelopes retained a significant portion of the kinase and represented the fraction with the highest specific activity. Taking this information into account, we chose as a source for the kinase the whole nuclear envelope fraction which contained about 50% of the total LBR kinase activity.

To selectively extract the LBR kinase we used two methods. First, we treated nuclear envelopes at 4 °C with the detergent Triton X-114 and then induced phase separation by raising the temperature of the extracted material to 37 °C. Under these conditions, the bulk of solubilized LBR partitioned with the detergent phase, whereas a significant portion of the kinase activity was released in the aqueous phase (Fig. 2*A*). Alternatively, we treated the nuclear envelopes with 1 M NaCl which extracted considerable amounts of the enzyme, but did not release significant amounts of LBR or other integral membrane proteins (Fig. 2*B*). The detergent extract of nuclear envelopes was practically devoid of histone kinase activity and contained low levels of casein kinase; however, the salt extract of nuclear envelopes contained significant amounts of both histone and casein kinase activity (Fig. 2, *A* and *B*, and data not shown).

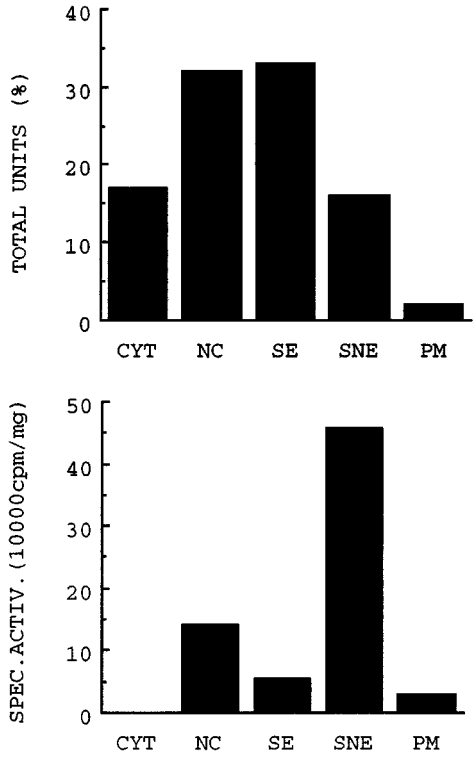

FIG. 1. **Subcellular distribution of LBR kinase.** Fractions of turkey erythrocyte cytosol (*CYT*), nuclear content (*NC*) released after DNase I digestion of nuclei, high salt extract (*SE*) of nuclear envelopes, salt-washed nuclear envelopes (*SNE*), and plasma membranes (*PM*) were incubated with 1.5 μg of purified LBR and 50 μM [γ-^{32}P]ATP. Samples were analyzed by SDS-PAGE and autoradiographed. The radioactive bands corresponding to LBR were excised, and the radioactivity was determined by Cerenkov counting. LBR kinase activity of the different fractions is expressed either as total units (%) or as specific activity (cpm/mg of protein). The protein concentration of the cytosol is much higher than the other fractions due to the abundance of hemoglobin, and, therefore, the specific activity in this fraction appears close to zero.

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 NH_2- and the COOH-terminal domains of LBR (Fig. 3*B*, for details see "Materials and Methods"). Two NH_2-terminal peptides (R_2 and R_0) which contained three or five arginine-serine (RS) motifs, respectively, could be phosphorylated by the LBR kinase (Fig. 3*A*). However, a third NH_2-terminal peptide (R_1) which contained three RS motifs but no downstream flanking sequence could not serve as a substrate and affected LBR phosphorylation marginally (Fig. 3*A*). Interestingly, the synthetic derivative R_0 (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. 3*A*). These data strongly suggested that the RS dipeptide motifs represent the major phosphorylation sites of LBR modified by the LBR kinase.

To explore this point further, we expressed in *E. coli* a fusion protein consisting of GST and the NH_2-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

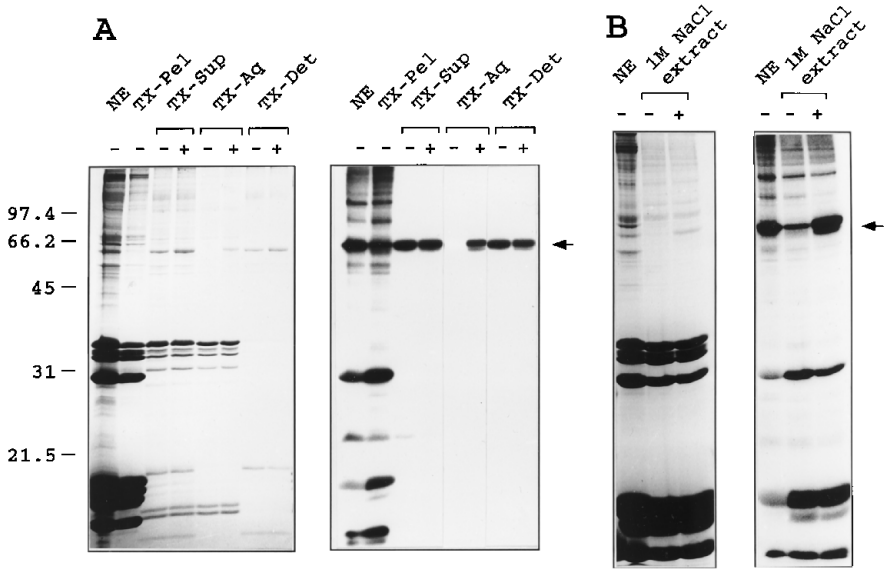

FIG. 2. **Extraction of LBR kinase activity from nuclear envelopes by Triton X-114 or 1 M NaCl.** *A*, equivalent fractions of whole turkey erythrocyte nuclear envelopes (*NE*), extracted nuclear envelopes with 1% Triton X-114 (*TX-Pel*), the Triton X-114 extract of nuclear envelopes (*TX-Sup*), the 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-^{32}P]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-^{32}P]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).

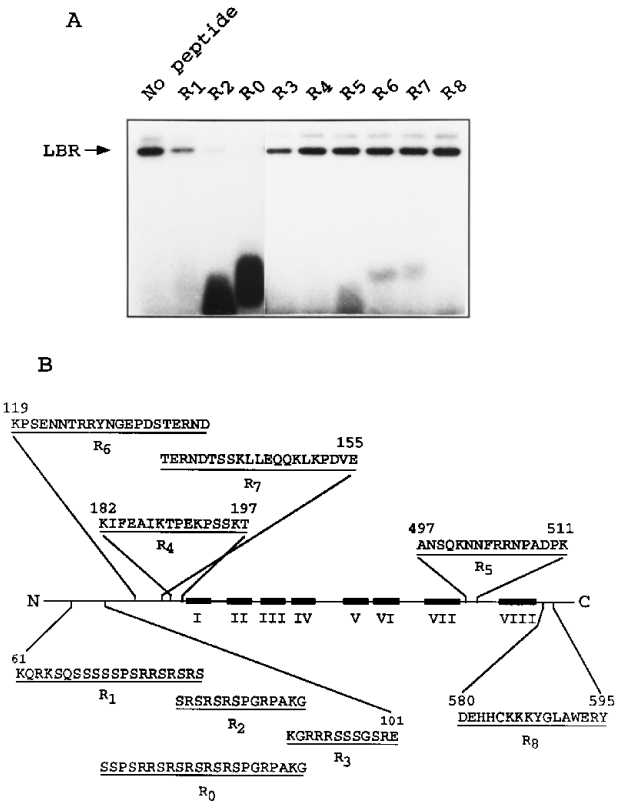

FIG. 3. **Phosphorylation of purified LBR by protein kinases present in the aqueous phase of the Triton X-114 extract of nuclear envelopes in the presence of various synthetic peptides.** *A*, 1.5 μg of electroeluted LBR were incubated with 0.5 mM each peptide and the aqueous phase of the Triton X-114 extract in the presence of 50 μM [γ-^{32}P]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.

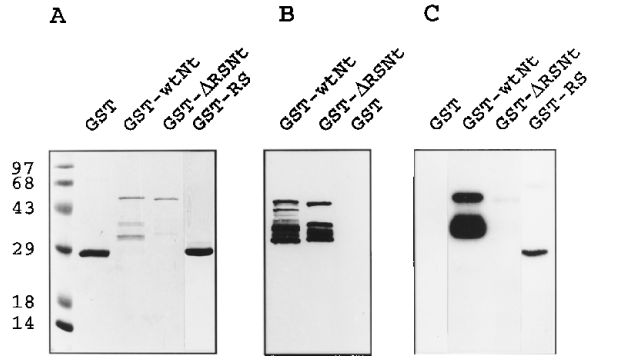

FIG. 4. **Phosphorylation of GST, GST fusion protein containing the NH_2-terminal domain of LBR (*GST-wtNt*, amino acids 1–205), GST fusion protein containing the NH_2-terminal domain of LBR, but missing the RS motifs (*GST-ΔRSNt*, amino acids deleted 75–84), and GST fusion protein containing five RS 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 anti-LBR antibody, raised against peptide R_1 (aR_1). The blots were stained using an alkaline phosphatase-conjugated rabbit goat anti-rabbit antibody. Note that in addition to full-length fusion protein, aR_1 also reacts with degradation products. *C*, *in vitro* 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).

salt or Triton X-114 extracts could efficiently phosphorylate GST-wtNt, whereas GST-ΔRSNt 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-ΔRSNt 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 R_0, 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.

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 R_0 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. 5*A*). *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. 5*B*, *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 the *in situ* gel assay (Fig. 5*B*, *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.

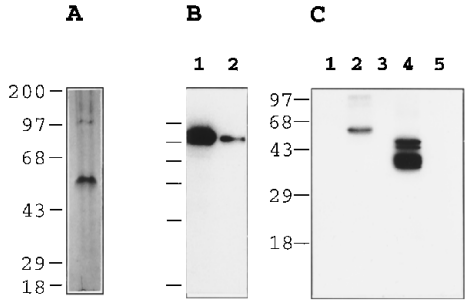

FIG. 5. **Partial purification and characterization of the LBR kinase.** *A*, SDS-PAGE analysis on 8% gel and silver staining of the material eluted from the R_0-agarose affinity column (for details see text). *Bars* on the *left* indicate molecular masses (in kDa). *B*, *in situ* kinase assay of column-purified LBR kinase (*lane 2*) and of the kinase co-immunoprecipitated with LBR by affinity-purified aR_1 antibody from a Triton X-100 extract of nuclear envelopes (*lane 1*). Samples were electrophoresed on a 12% SDS-polyacrylamide gel containing 0.1 mg/ml electroeluted LBR, renatured *in situ*, incubated with [γ-^{32}P]ATP, and subjected to autoradiography. *Bars* on the *left* indicate the same molecular masses as in *A*. *C*, binding of column-purified LBR kinase to the NH_2-terminal domain of LBR. Purified LBR kinase was incubated with Protein A-Sepharose beads (*lane 1*), electroeluted LBR bound to aR_1 antibody/Protein A-Sepharose beads (*lane 2*), glutathione-Sepharose beads (*lane 3*), GST-wtNt immobilized on glutathione-Sepharose beads (*lane 4*), and GST-ΔRSNt immobilized on glutathione-Sepharose beads (*lane 5*). The co-sedimenting material was incubated with [γ-^{32}P]ATP and analyzed by SDS-PAGE and autoradiographed. In control assays (*lanes 1* and *3*) and in the case of GST-ΔRSNt-glutathione-Sepharose beads (*lane 5*), 2.5 μg of GST-wtNt were added in the reaction mixtures to provide a substrate for the LBR kinase. *Bars* on the *left* indicate molecular masses (in kDa).

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. 5*C*, *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-ΔRSNt was observed (Fig. 5*C*, *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. 6*B*). Interestingly, when a well-characterized subcellular fraction containing SR proteins (Zhaler *et al.*, 1992) was incubated with column-purified kinase, we found that the enzyme could efficiently phosphorylate the 30-kDa major component (Fig. 6*B*). 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 SRp30a and SRp30b, which have also been described as SF2 and SC35, respectively. The phosphorylation of LBR and SRp30 was inhibited by an excess of the synthetic peptide R_0 as well as by a peptide containing six arginine-serine (RS) repeats (R_{SF}, Fig. 6*C*). 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 μM. Taking into consideration that

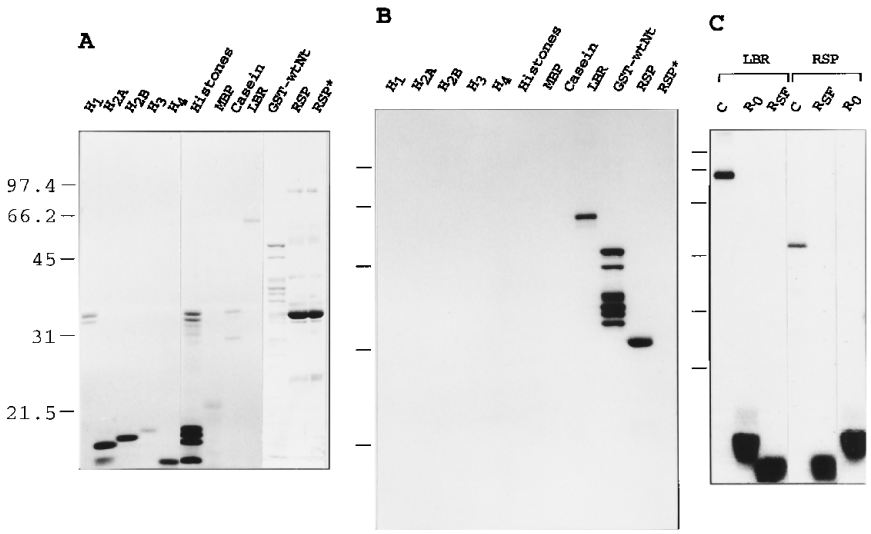

FIG. 6. **Substrate specificity of LBR kinase.** Phosphorylation of H_1, H_{2A}, H_{2B}, H_3, H_4, myelin basic protein, casein, electroeluted LBR, GST-wtNt, and RS proteins (*RSP*), previously heated to 70 °C for 10 min, by purified LBR kinase. RSP* shows the phosphorylation of heated RS proteins in the absence of LBR kinase. All substrates were added to the assay mixture at a final concentration of 0.15 mg/ml except for LBR, the final concentration of which was 0.08 mg/ml. The samples were analyzed by SDS-PAGE on 12% gels and stained with Coomassie Blue (*A*) or autoradiographed (*B*). *Bars* on the *left side* of *A* and *B* indicate molecular masses (in kDa). *C*, inhibition of phosphorylation of LBR and SR proteins by 0.5 mM R_0 and R_{SF} peptide. Autoradiograms of the gels are shown. The *five top bars* on the *left* indicate the same molecular masses as in *A*, and the *sixth bar* corresponds to 14 kDa.

TABLE I

Determination of the site(s) phosphorylated by the LBR kinase

Phosphorylation of the various recombinant proteins (0.1 mg/ml for the calculation of stoichiometry, 0.004–0.2 mg/ml for the calculation of K_m) by the partially purified LBR kinase (0.012 μg) was measured as described under "Materials and Methods." The concentration of ATP was 100 μM.

Construct	Stoichiometry	K_m[a]
	mole P_i/mol protein	μM
GST-wtNt (^{75}RSRSRSRSRS^{84})	0.7	2.1
GST-NtG^{78} (^{75}RSR<u>G</u>RSRSRS^{84})	0.7	2.3
GST-NtA^{80} (^{75}RSRSR<u>A</u>RSRS^{84})	0.6	1.8
GST-NtA^{82} (^{75}RSRSRSR<u>A</u>RS^{84})	0.7	1.7
GST-NtA^{84} (^{75}RSRSRSRSR<u>A</u>^{84})	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 existence of the degradation products, an average molecular mass of 40 kDa was assumed for all fusion proteins.

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)) 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*.[4]

The RS-containing Region at the NH_2-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"). Fig. 8*A* shows 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 electroeluted 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. 8*B*, *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. 8*A*, *lane 4*) or completely abolished (Fig. 8*B*, *lane 3*). Finally, repetition of the assay in the presence of an excess of R_0 peptide also abolished binding between GST-wtNt and p34/p32 (Fig. 8*B*, *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

[4] E. Nikolakaki, G. Simos, S. D. Georgatos, and T. Giannakouros, unpublished observations.

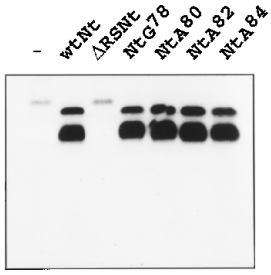

FIG. 7. **Inhibition of LBR phosphorylation by GST-wtNt and serine mutants of GST-Nt (for nomenclature see text and Table I).** One μg of purified LBR was incubated with radiolabeled ATP and the LBR kinase in the presence of buffer (−), GST-wtNt, GST-ΔRSNt, or mutated forms of GST-Nt (10 μg), as indicated. The reaction products were run on SDS-polyacrylamide gels and autoradiographed. Note that LBR phosphorylation is competed off by GST-wtNt and all serine mutants, but not by GST-ΔRS. Also notice that GST-Nt and the serine mutants are all phosphorylated by the enzyme, whereas GST-ΔRS is not.

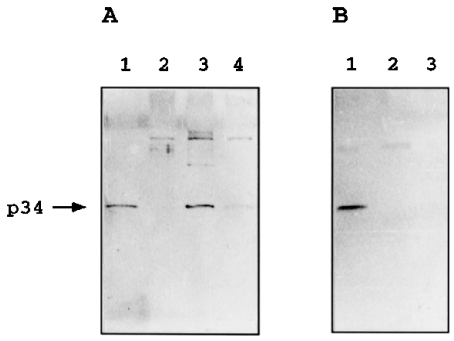

FIG. 8. **The NH_2-terminal domain of LBR binds to p34/p32 *in vitro*.** *A*, immunoblot showing binding of p34 when a fraction highly enriched in p34 was incubated with GST (*lane 2*), GST-wtNt (*lane 3*), or GST-ΔRSNt (*lane 4*) immobilized on glutathione-Sepharose beads. *Lane 1* shows a reference sample containing purified p34. *B*, GST-wtNt (*lane 1*) or GST-ΔRSNt (*lane 3*) immobilized on glutathione-Sepharose beads were incubated with a Triton X-100 lysate of nuclear envelopes, and the material bound to the beads was analyzed by immunoblotting. In *lane 2*, binding to GST-wtNt was assessed in the presence of 0.25 mM R_0 peptide. The blots were probed with affinity-purified ap34-C antibodies and stained using an alkaline phosphatase-conjugated rabbit goat anti-rabbit antibody.

whether binding of p34/p32 to LBR is affected by RS phosphorylation. For this purpose, GST-wtNt immobilized on glutathione-Sepharose beads was incubated either with buffer and ATP or with purified LBR kinase and ATP (see above) to allow phosphorylation to occur. Afterwards, the beads were further incubated with a detergent extract of nuclear envelopes containing p34/p32. Examination of the material that co-sedimented with the beads by immunoblotting showed that p34/p32 does not bind to phosphorylated GST-wtNt (Fig. 9, *lane 3*), but does bind to unphosphorylated GST-wtNt (Fig. 9, *lane 1*).

To distinguish whether the binding is indeed inhibited by phosphorylation, or whether the kinase and p34/p32 compete for the same binding site on LBR, we repeated the same type of experiment in the presence of the LBR kinase but adding or omitting ATP in the course of the assay. Fig. 9, *lane 4*, shows that binding of p34/p32 to the fusion protein was inhibited only when ATP was present in the reaction mixture. Thus, binding of p34/p32 to LBR seems to be inhibited after phosphorylation of the RS motifs.

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 mod-

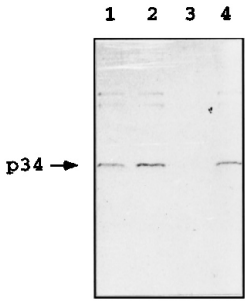

FIG. 9. **Phosphorylation by the LBR kinase inhibits binding of p34 to the NH_2-terminal domain of LBR.** Immunoblot showing binding of p34 when a Triton X-100 lysate of turkey erythrocyte nuclear envelopes was incubated with GST-wtNt immobilized on glutathione-Sepharose beads (*lane 1*). In *lanes 2*, *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.

ified *in vivo* (Simos and Georgatos, 1992). Using synthetic peptides representing different regions of LBR and recombinant proteins produced in bacteria, we have shown that LBR kinase modifies specifically arginine-serine (RS) dipeptide motifs occurring in LBR and in splicing factors. Our results suggest that any one of the serine residues in the RS region of LBR can serve as a phosphorylation site for the LBR kinase.

The family of SR proteins includes essential splicing factors that commit precursor mRNA to splicing and mediate spliceosome assembly (Fu, 1993). Although their role in the splicing mechanism is not yet clear, mutational studies have shown that the RS domains in $U2AF^{65}$ and in ASF/SF2 are required for splicing activity (Zamore *et al.*, 1992; Caceres and Krainer, 1993; Zuo and Manley, 1993). Recent data suggest that phosphorylation promotes spliceosome assembly but blocks the catalytic steps of splicing, and the prime candidates for the targets of phosphorylation are the RS domain-containing splicing factors (Mermoud *et al.*, 1994; Gui *et al.*, 1994).

There have been two reports on protein kinases which phosphorylate specifically RS motifs (Woppmann *et al.*, 1993; Gui *et al.*, 1994). One such activity is associated with snRNP particles and phosphorylates the U1 snRNP 70-kDa protein at a subset of the sites phosphorylated *in vivo* (Woppmann *et al.*, 1993). This activity also phosphorylates the COOH-terminal, RS-rich domain of ASF/SF2. In addition, Gui *et al.* (1994) identified a cell cycle-regulated serine kinase (SRPK1 = SR Protein Kinase 1), with an apparent molecular mass of 92 kDa, which can phosphorylate splicing factors of the RS family. Purified SRPK1 can induce disassembly of speckled intranuclear snRNP structures in interphase nuclei. The exact location of the kinase in the nucleus is not known.

At present, we do not know if the LBR kinase is the same with the enzyme purified by Gui *et al.* (1994). SDS-PAGE analysis together with *in situ* gel assays predict a molecular mass of 110 kDa for the catalytic subunit of the LBR kinase, which is significantly higher than that of SRPK1. This observation indicates that the two kinases are distinct; however, species-specific differences between birds and mammals may also be the reason for this difference in apparent M_r.

Regulation of LBR Interactions by Phosphorylation—Previous studies have shown that one of the polypeptides 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; Kohtz *et al.*, 1994), probably in a phosphorylation-dependent manner (Woppmann *et al.*, 1993; Mermoud *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 lamin receptor. On the other hand, LBR, together with the integral membrane protein LAP2 (Foisner and Gerace, 1993), are the most obvious candidates to mediate the association of interphase nuclear membranes to chromatin (Maison *et al.*, 1995).[5] 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 nucleoplasm 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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