THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 21, Issue of May 24, pp. 12617–12625, 1996

Printed in U.S.A.

Characterization of p18, a Component of the Lamin B Receptor Complex and a New Integral Membrane Protein of the Avian Erythrocyte Nuclear Envelope*

(Received for publication, December 26, 1995, and in revised form, February 14, 1996)

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Employing avian erythrocytes, we have previously isolated a multimeric complex consisting of the lamin B receptor (LBR, or p58), the nuclear lamins, an LBR-specific kinase, a 34-kDa protein, and an 18-kDa polypeptide termed p18. As the LBR kinase and the 34-kDa component have been recently characterized, we now proceed in the characterization of p18. We show here that p18 is an integral membrane protein specific to the erythrocyte nuclear envelope which binds to LBR and B-type lamins. NH_2-terminal sequencing indicates that p18 is distinct from other nuclear envelope components, but has similarity to the mitochondrial isoquinoline-binding protein. *In situ* analysis by immunoelectron microscopy and examination of digitonin-permeabilized cells by indirect immunofluorescence show that p18, unlike LBR and other lamin-binding proteins, is equally distributed between the inner and outer nuclear membrane. Furthermore, cycloheximide inhibition experiments reveal that the fraction of p18 that resides in the outer nuclear membrane does not represent nascent chains *en route* to the inner nuclear membrane, but rather material in equilibrium with the p18 that partitions with the inner nuclear membrane. The paradigm of p18 suggests that transmembrane complexes formed by the nuclear lamins and LBR provide potential docking sites for integral membrane proteins of the nuclear envelope that equilibrate between the rough endoplasmic reticulum and the inner nuclear membrane.

Relatively few integral membrane proteins of the nuclear envelope have been characterized so far (for reviews, see Gerace and Foisner (1994) and Georgatos *et al.* (1994)). These include the "lamin B receptor" (LBR[1] or p58) (Worman *et al.*, 1988; Bailer *et al.*, 1991), the lamina-associated polypeptides (LAPs) (Senior and Gerace, 1988; Foisner and Gerace, 1993), and a protein termed otefin (Padan *et al.*, 1990). Otefin has been identified in *Drosophila* cells, but its properties are not yet known. However, some molecular information concerning the LAPs and the LBR is available.

The LAP group of proteins includes the so-called LAP1 A, LAP1 B, LAP1 C, and LAP2. LAP1 C and LAP2 are typical intrinsic membrane proteins with a single (predicted) transmembrane domain and two hydrophilic end-regions (Martin *et al.*, 1995; Furukawa *et al.*, 1995). LAP1 A and B represent splicing variants of LAP1 C, but their exact amino acid sequence has not been determined yet (Martin *et al.*, 1995). Data base searches show that LAP2 is identical to a previously cloned protein, thymopoietin (Harris *et al.*, 1994). Thymopoietin is known to have important immunological functions (Goldstein, 1974; Goldstein *et al.*, 1979; Ranges *et al.*, 1982) and is expressed in many tissues, most abundantly in the thymus. It comprises three distinct variants (α, β, and γ) generated by differential splicing. β thymopoietin (which is identical to LAP2) and γ thymopoietin possess potential membrane-spanning domains and are located in the nuclear envelope; however, α thymopoietin lacks a hydrophobic region and seems to be nucleoplasmic (Harris *et al.*, 1994). The LAPs bind directly to lamin paracrystals under *in vitro* conditions; LAP1 A and 1B interact with all lamin types, while LAP2 associates exclusively with B-type lamins (Foisner and Gerace, 1993). LAP1 C does not show detectable binding to purified lamins *in vitro*, but clearly associates with A-type lamins under *in vivo* conditions (Powell and Burke, 1990). Interestingly, LAP2 also interacts with isolated chromosomes *in vitro* (Foisner and Gerace, 1993).

LBR possesses a long, charged NH_2-terminal domain, eight potential membrane-spanning segments, and a hydrophilic COOH-terminal region (Worman *et al.*, 1990; Ye and Worman, 1994; Schuler *et al.*, 1994). Its NH_2-terminal domain, which is exposed to the nucleoplasm, contains multiple phosphorylation sites (Simos and Georgatos, 1992; Courvalin *et al.*, 1992), DNA-binding motifs (Worman *et al.*, 1990; Ye and Worman 1994), as well as a stretch rich in serine/arginine motifs (Simos and Georgatos, 1994). LBR is widely expressed in human and avian cells (Bailer *et al.*, 1991; Chaudhary and Courvalin, 1993). In addition, three yeast proteins that exhibit significant similarity to the vertebrate LBR have been molecularly cloned (Chen *et al.*, 1991; Lorenz and Parks, 1992; Shimanuki *et al.*, 1992). All three yeast proteins lack the NH_2-terminal domain of vertebrate LBR, and one of them is an enzyme involved in ergosterol metabolism (Lorenz and Parks, 1992). (Ergosterol is a fungal sterol not found in higher eukaryotes.) Finally, a putative yeast homologue of vertebrate LBR has been identified by immunochemical and biochemical means (Georgatos *et al.*, 1989), but it

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This work is dedicated to Stavros and Adamantia Politis.

§ Supported by a fellowship from the BIOMED I program of the European Union.

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[1] The abbreviations used are: LBR, lamin B receptor; LAP, lamina-associated polypeptide; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; IBP, isoquinoline-binding protein; PBS, phosphate-buffered saline; TM, transmembrane domain.

is not clear whether this polypeptide corresponds to one of the already cloned proteins. 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."

In the terminally differentiated avian erythrocyte, LBR is known to form a multimeric complex, which includes the nuclear lamins, a specific kinase (LBR kinase), and two other polypeptides with molecular masses of 18 kDa (p18) and 34 kDa (p34) (Simos and Georgatos, 1992). Considering that the LBR complex might constitute a "junctional" assembly responsible for the coupling of the nuclear lamina to the inner nuclear membrane, we have undertaken a systematic effort to characterize the non-lamin nearest neighbors of LBR. As it turns out, p34 is the avian equivalent of a human nuclear protein known as p32 (Simos and Georgatos, 1994), which co-isolates with splicing factor 2 (SF2) (Krainer *et al.*, 1991) and interacts with the human immunodeficiency virus 1 product Rev *in vivo* (Luo *et al.*, 1994). Other data show that the LBR kinase is a resident protein of the nuclear envelope (Simos and Georgatos, 1992), which phosphorylates specifically serine/arginine dipeptide motifs present in LBR and in splicing factors (Nikolokaki *et al.*, 1996). Finally, observations described below indicate that p18, the last component of the LBR complex to be characterized, is itself an integral membrane protein of the erythrocyte nuclear envelope that interacts specifically with LBR and B-type lamins. The implications of these observations in nuclear envelope structure and dynamics are discussed below.

EXPERIMENTAL PROCEDURES

Cell Fractionation and Chemical Extraction—Turkey erythrocyte nuclear envelopes and plasma membranes were isolated as described previously (Georgatos and Blobel, 1987a). When required, the nuclear envelopes were further washed with 2 M KCl to remove the bulk of the histones. Extraction of nuclear envelopes with non-ionic detergents was done using 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM PMSF (buffer EB) and either 1% Triton X-100, or 1% Triton X-114 at 0 °C, for 10 min. Detergent-soluble and -insoluble fractions were separated by centrifugation at 12,000 × *g* for 15 min at 4 °C. When Triton X-114 was used, the soluble fraction was incubated for 2 min at 37 °C and the aqueous and detergent phases were separated by spinning for 5 min at 12,000 × *g* and at room temperature. Stripping of peripheral proteins was performed using 8 M urea, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM PMSF, and 1 mM DTT at room temperature, or 0.1 N NaOH at 0 °C, followed by ultracentrifugation for 30 min at 400,000 × *g* in a TLA 100.2 Beckman rotor. Metabolic labeling of turkey erythrocytes was done using a commercial kit, which contained [^{35}S]methionine and [^{35}S]cysteine at 10 mCi/ml.

Protein Chemical Procedures—LBR and p18 were purified by electroelution. The urea-insoluble fraction of the nuclear envelopes was resuspended in urea buffer by sonication, solubilized by the addition of appropriate volume of 4 × electrophoresis sample buffer (0.25 M Tris-HCl, pH 6.8, 9.2% SDS, 40% glycerol, 0.2% bromphenol blue, and 100 mM DTT) and analyzed by SDS-PAGE. After electrophoresis, unfixed gels were stained with copper (Lee *et al.*, 1987). The bands corresponding to LBR and p18 were excised and washed in 0.25 M EDTA, 0.25 M Tris-HCl, pH 9.0, to remove copper ions. The proteins were recovered from the gel pieces by electroelution in 25 mM Tris, 192 mM glycine, 0.025% (w/v) SDS using the Biotrap BT 1000 apparatus (Schleicher & Schuell), and dialyzed extensively against phosphate-buffered saline (PBS). The identity of the electroeluted proteins was routinely checked by Western blotting and confirmed several times by NH_2-terminal sequencing. The nuclear lamins were purified from urea extracts of turkey erythrocyte nuclear envelopes as described previously (Georgatos and Blobel, 1987b). To determine the NH_2-terminal sequence of the LBR-associated p18, the LBR-complex was isolated by immunoprecipitation using affinity-purified anti-LBR antibodies (for SDS-PAGE profiles of this material, see Simos and Georgatos (1992, 1994)). The LBR complex was resolved by preparative electrophoresis, and the proteins were transferred to ProBlott membranes (Applied Biosystems) and stained by Coomassie Blue. The band corresponding to p18 was excised from the blot, rinsed with distilled water, and used for microsequencing. To determine the sequence of nuclear envelope-bound p18 (initially referred to as "18-kDa protein"), samples of urea-extracted nuclear envelopes were analyzed by preparative gel electrophoresis and the 18-kDa band was electroeluted. Alternatively, samples of urea-extracted nuclear envelopes were analyzed by SDS-PAGE, the proteins transferred to ProBlott membranes, and the 18-kDa band was excised from the blot. Both isolation methods yielded the same results. Protein sequences were determined by Edman degradation employing the Applied Biosystems model 477A sequencing system. Electroeluted p18 (15 μg) was iodinated with $Na^{125}I$ by IODO-BEADS (Pierce) in a buffer containing 0.1 M sodium phosphate, pH 7.5, 0.1 mM EDTA, 1 mM PMSF for 2–10 min. After iodination, excess $Na^{125}I$ was removed by gel filtration through a Sephadex G-25 desalting column equilibrated at 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 4 mM DTT, 0.5 mM PMSF, and 0.1% Triton X-100. The specific activity of ^{125}I-p18 was 7,000–18,000 cpm/μg. Protein concentrations were determined using a Bio-Rad kit. Gel electrophoresis was performed according to Laemmli (1970).

Binding Assays—Ligand blotting assays were performed as specified by Djabali *et al.*(1991) and Merdes *et al.* (1991). Binding assays in solution were done as follows. A sample of ^{125}I-p18 (1 μg) was incubated with 1 or 2 μg of purified LBR for 1 h at room temperature and for 1 h at 4 °C. The incubation mixture was then diluted to 0.5 ml with the addition of 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM PMSF, 1% Triton X-100, and 1 mg/ml fish skin gelatin, and LBR was precipitated with affinity-purified aR1 antibodies (7 μg) in the presence or absence of the antigenic peptide R1 (100 μg). In experiments involving unlabeled p18, 1–2 μg of purified proteins (*i.e.* p18, LBR, and lamins A or B) were mixed in various combinations in 0.5 ml of 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5 mM PMSF, 1 mM DTT, 1% Triton X-100, and 0.2 mg/ml fish skin gelatin. The mixture was incubated for 2 h at room temperature before LBR or the lamins were immunoprecipitated with affinity-purified aR1 or aLI antibodies and protein A-Sepharose.

Immunological and Immunochemical Procedures—The polyclonal anti-lamin (aLI) and anti-LBR (aR1) antibodies have been developed in rabbits using as antigens the peptides LI (Djabali *et al.*, 1991) and R1 (Simos and Georgatos, 1992), respectively. These antibodies were used after affinity purification in peptide Affi-Gel 10/15 columns. The monoclonal anti-lamin B2 antibody E3 was a generous gift from E. A. Nigg (ISPEC, Switzerland). For immunization, p18 purified by electroelution was injected in the thigh lymph nodes and subcutaneously in rabbits (50–100 μg/animal, in complete Freund's adjuvant) at day 0. The animals were boosted at day 21 (same amount of protein in incomplete Freund's adjuvant, subscapularly), and sera were collected 1 week later. Subsequent boosts were administered at least 3 weeks after each bleed. The antisera were screened by immunoblotting and indirect immunofluorescence. To obtain monoclonal antibodies, electroeluted p18 was injected into mice as described by Galfre and Milstein (1981). Spleen cells from immunized mice were fused with the mouse myeloma cell line Ag8. Culture supernatants from fusion wells were screened by immunoblot analysis of turkey nuclear envelopes and immunofluorescence microscopy of turkey erythrocytes. Hybridomas that gave a positive reaction by both assays were subcloned two successive times by a limiting dilution procedure. The five monoclonal antibodies obtained are of the IgG1 subclass and were always mixed before use. Isolation of the LBR complex by immunoaffinity chromatography, immunoprecipitation, and immunoblotting were performed as described previously (Simos and Georgatos, 1992, 1994).

Microscopy—Indirect immunofluorescence microscopy on turkey or chicken blood cells and on various cultured cells was performed as described previously (Merdes *et al.*, 1991; Maison *et al.*, 1993; Meier and Georgatos, 1994). When needed, cells were permeabilized with digitonin (40 μg/ml) for 5 min at 4 °C. For indirect immunofluorescence assays on tissue cryosections, fresh chicken tissues (liver, spleen, intestine, and heart muscle) were embedded in Tissue-Tek (Miles Laboratories Inc., Elkhart, IN) and immediately frozen in liquid nitrogen. Eight-μm sections were cut, mounted on glass slides, and fixed with 3.5% formaldehyde for 10 min at room temperature. After quenching with 50 mM NH_4Cl and permeabilization with 0.4% Triton X-100, the sections were incubated with anti-p18 antibodies and fluorescein isothiocyanate-coupled secondary antibodies (Jackson). The sections were examined in a Zeiss Axiophot microscope. For pre-embedding immunoelectron microscopy, erythrocyte ghosts applied on coverslips were first fixed with 3.5% formaldehyde, treated with 0.5% Triton X-100, blocked with 0.5% gelatin in PBS, and incubated sequentially with polyclonal anti-p18 antibodies and protein A-gold, or with monoclonal antibodies, anti-mouse IgG and protein A-gold. Then, the samples were fixed in 1.5% glutaraldehyde, osmicated, embedded in Epon, sectioned, stained with uranyl acetate/lead citrate, and visualized in a Philips 400 micro-

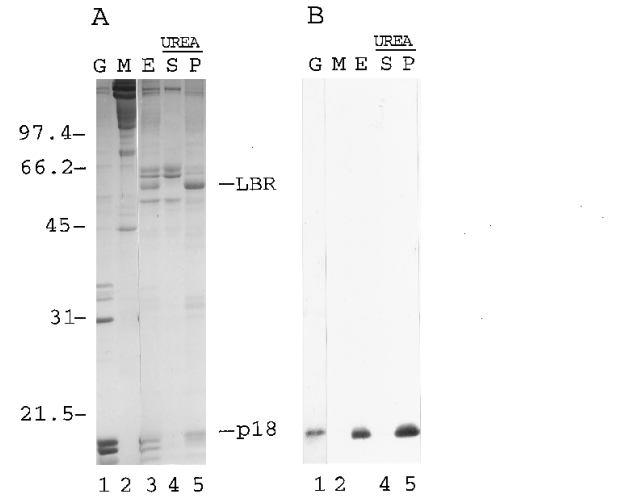

FIG. 1. **Partitioning of p18 upon cell fractionation and determination of its NH_2-terminal sequence.** Subcellular fractions and extracts of turkey erythrocytes were prepared as specified under "Experimental Procedures." Samples of these fractions were then analyzed by SDS-PAGE and either stained by Coomassie blue (*A*) or immunoblotted with a polyclonal antibody against p18 (*B*). Panels show total erythrocyte ghosts (*G*, *lane 1*), plasma membranes (*M*, *lane 2*), salt-washed nuclear envelopes (*E*, *lane 3*), urea extract of nuclear envelopes (*S*, *lane 4*), and urea-insoluble residue of nuclear envelopes (*P*, *lane 5*). *C*, NH_2-terminal sequencing of p18. "Authentic" p18 (*1*, *p18*) isolated by immunoprecipitation from whole cell lysates (Simos and Georgatos, 1992) and an 18-kDa protein purified from electrophoresed urea-extracted nuclear envelopes (*2*, *18 kDa*) were microsequenced in parallel. Comparison of the two sequences indicates identity. Data base searches also indicate that turkey p18 is similar to bovine IBP (*3*, *IBP*), an 18-kDa integral membrane protein known as the isoquinoline-binding protein subunit of the PBRs. Identical residues (*one-letter code*) between bovine IBP and turkey p18 are included in *boxes*. Conservative substitutions are indicated by *double dots*. *X* indicates unknown amino acid.

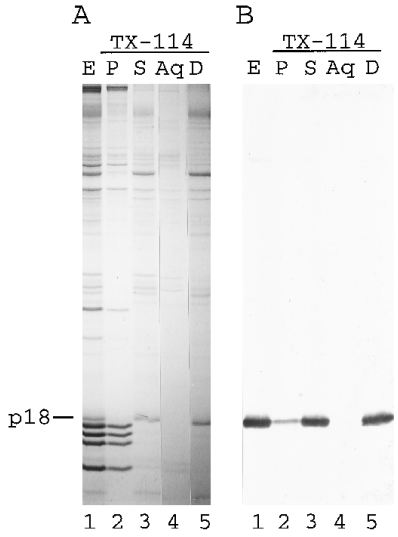

FIG. 2. **Extraction of p18 by Triton X-114.** Turkey erythrocyte nuclear envelopes were extracted by Triton X-114 at 4 °C, and the extract was warmed up to 37 °C to induce phase separation between the detergent phase and the aqueous phase. The resulting fractions were analyzed by SDS-PAGE and either stained by Coomassie Blue (*A*) or immunoblotted with an anti-p18 polyclonal antibody (*B*). Panels show salt-washed nuclear envelopes (*E*, *lane 1*), insoluble fraction after extraction of the nuclear envelopes with 1% Triton X-114 (*P*, *lane 2*), total soluble fraction after extraction with 1% Triton X-114 (*S*, *lane 3*), aqueous phase of soluble fraction (*Aq*, *lane 4*), and detergent phase of soluble fraction (*D*, *lane 5*).

scope operated at 80 kV. For immunoelectron microscopy on ultrathin frozen sections, erythrocyte ghosts or hypotonically swelled erythrocytes were fixed overnight in 8% paraformaldehyde in 250 mM Hepes, pH 7.4, and processed as described previously by Griffiths *et al.* (1984).

RESULTS

p18 Is a New Integral Membrane Protein of the Nuclear Envelope—To explore the biochemical properties of p18, we fractionated turkey erythrocytes according to previously established methods (Georgatos and Blobel, 1987a) and analyzed the fractions by SDS-PAGE. Plasma membrane preparations did not contain proteins matching the molecular mass of p18 (Fig. 1*A*, *lane 2*). On the other hand, nuclear envelope fractions did contain a polypeptide of 18 kDa, but this protein often comigrated with core histones and could not be easily discerned (Fig. 1*A*, *lane 3*). Upon extraction of the nuclear envelopes with 8 M urea (Fig. 1*A*, *lane 5*) or 0.1 M NaOH (data not shown), the 18-kDa band became clearly visible. The amount of the 18-kDa protein in nuclear envelope fractions varied slightly from preparation to preparation (the reason for this is explained below). However, in molar terms this polypeptide was nearly as abundant as LBR.

To find out whether the 18-kDa polypeptide corresponded to p18, urea-extracted nuclear envelopes were resolved by preparative SDS-PAGE and material was isolated either by electroelution of gel pieces, or by electrotransfer and excision of the corresponding band from blots (for technical details, see "Experimental Procedures"). The isolated 18-kDa protein was then microsequenced in parallel to "authentic" p18 co-immunoprecipitated with LBR from whole cell lysates (Simos and Georgatos, 1992) and purified in the same way from SDS gels. The NH_2-terminal sequences of the two proteins were identical (Fig. 1*C*).

Data base searches using the NH_2-terminal sequence of p18 (34 residues in the order MWAYTVGFTVPHVGGFLG*X*FINRRETPV*X*YE*X*L; *X* = unknown amino acid) confirmed that p18 was neither a degradation product of a known protein, nor a histone. The only sequenced protein that exhibited similarity to p18 was a 17–18-kDa polypeptide, the *isoquinoline-binding protein* (IBP) (Fig. 1*C*), which had been characterized previously as a component of the mitochondrial peripheral-type benzodiazepine receptors (Sprengel *et al.*, 1989; Riond *et al.*, 1989; Parola *et al.*, 1991; Riond *et al.*, 1991; for comments on this, see "Discussion").

To confirm the nuclear envelope localization of p18 in a more direct way, we immunized rabbits and mice with electrophoretically purified protein and raised one polyclonal and five monoclonal antibodies against it. The antibodies precipitated an 18-kDa, detergent-soluble polypeptide, which had the same NH_2-terminal sequence with p18. Using Western blotting (Fig. 1*B*), we could readily detect p18 in fractions of whole erythrocyte ghosts (*lane 1*), salt-washed nuclear envelopes (*lane 3*), and urea-extracted nuclear envelopes (*lane 5*). However, p18 could not be detected in the plasma membrane fraction (*lane 2*) or in urea extracts of nuclear envelopes (*lane 4*). The same results were obtained when turkey erythrocyte fractions were probed by the polyclonal or the monoclonal antibodies. However, whereas the latter reacted equally well with turkey and chicken red blood cells, the former reacted strongly with turkey erythrocyte p18 and less strongly with chicken erythrocyte p18. None of the antibodies reacted with material obtained from

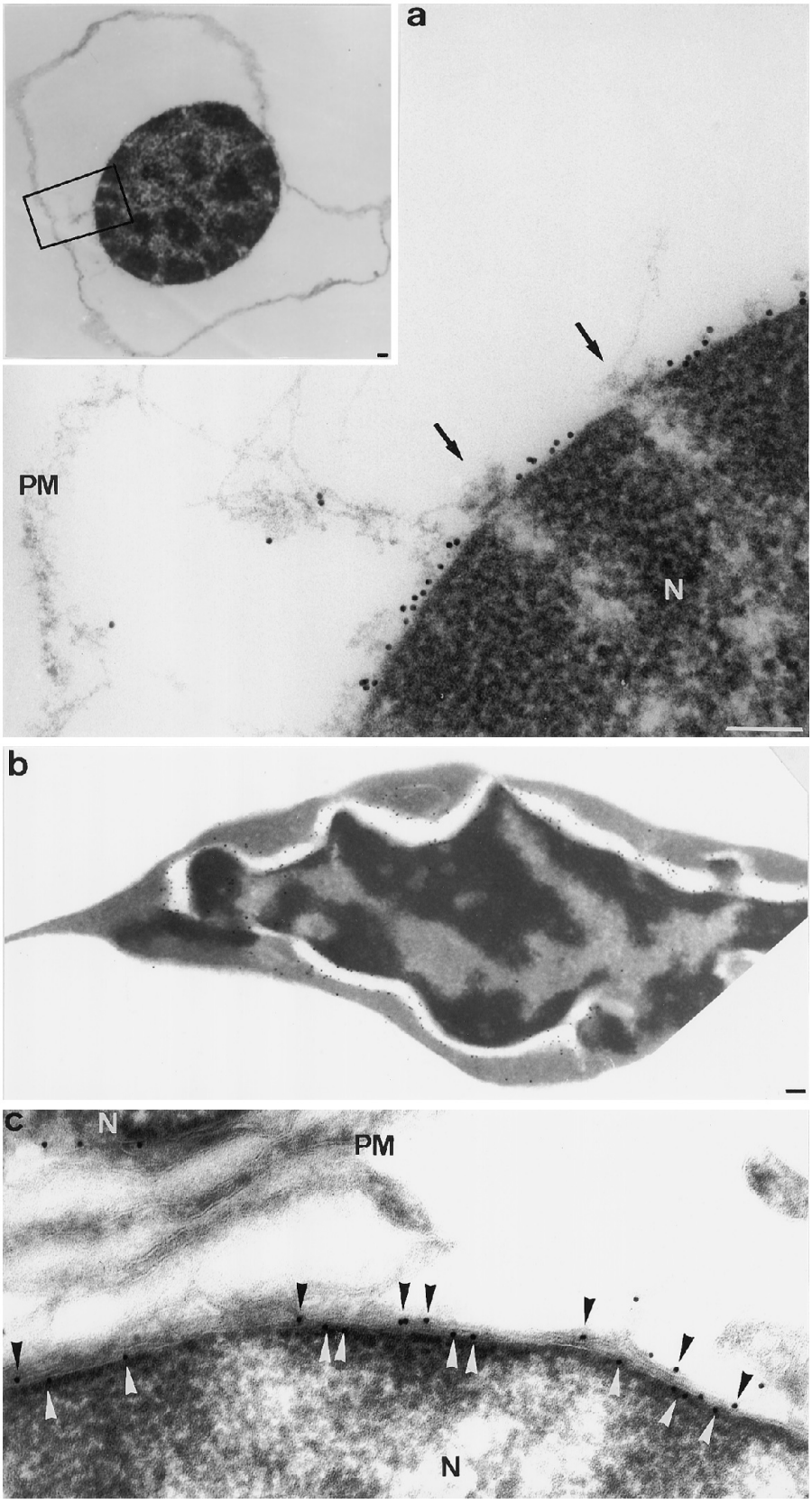

FIG. 3. **Subcellular distribution of p18 as detected by immunoelectron microscopy.** *a*, turkey erythrocytes were fixed with formaldehyde, permeabilized by Triton X-100, and incubated with a polyclonal anti-p18 antibody and protein A-gold. Notice the heavy decoration of the nuclear surface and the lack of labeling in the area of the nuclear pores (*arrows*). Similar results were obtained with the monoclonal antibodies against p18. "Unit membranes" do not appear in this image due to removal of the lipids by the detergent; however, the nuclear lamina is clearly discernible as a thick electron dense layer and so is the fibrillar membrane-skeleton, which underlies the plasma membrane. Also visible in *a* are long 10-nm filaments, which seem to extend from the nuclear pores to the plasma membrane. These filaments show the same level of background staining with anti-p18 antibodies and preimmune sera. *Inset* shows a low power view of the cell depicted at high magnification in *a*. *b*, low power view of an immunodecorated ultrathin frozen section of a non-lysed erythrocyte that has been exposed to hypotonic media (10 mM sodium phosphate). Observe the dilated perinuclear cisterna and the heavy staining with anti-p18 antibodies along the inner and outer nuclear membrane. *c*, immunodecorated ultrathin frozen section of an turkey erythrocyte ghost depicting at high magnification the region of the nuclear envelope. Membrane profiles are clearly visible. Heavy staining with the anti-p18 antibody is observed along the inner (*open arrowheads*) and the outer (*closed arrowheads*) nuclear membrane, whereas the plasma membranes (*PM*) are not decorated. *N* indicates the cell nucleus. *Bars* correspond to 100 nm.

mammalian cells (*e.g.* mouse erythroleukemia (MEL) cells, Chinese hamster ovary (CHO) cells, and normal rat kidney (NRK) cells; data not shown).

The NH_2-terminal sequence of p18 indicated the existence of a 20-amino acid stretch (-WAYTVGFTVPHVGGFLG*X*FI-), which had features (*i.e.* size and hydrophobicity) of a membrane-spanning region. This, combined with the fact that p18 could not be extracted from the nuclear envelopes by 8 M urea or alkali, suggested that it represents an intrinsic membrane protein. To substantiate this interpretation, we extracted the nuclear envelopes with the detergent Triton X-114 at low temperature and induced phase separation by warming up the

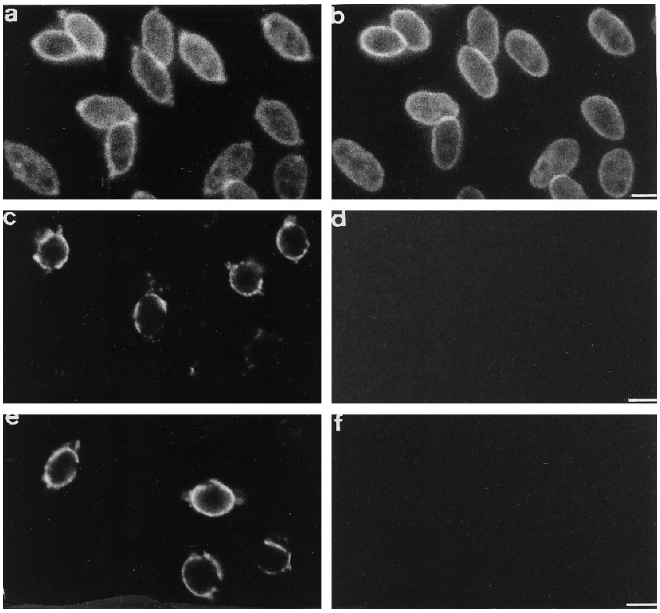

FIG. 4. **Localization of p18 in digitonin-permeabilized erythrocytes and in cells treated with cycloheximide.** Turkey red blood cells were incubated in the absence (*a–d*) or presence (*e* and *f*) of 100 μg/ml cycloheximide (10 min to 2 h, 37 °C; only the 30-min sample is shown here). After this incubation, the cells were permeabilized with Triton X-100 (*a* and *b*), or digitonin (*c–f*). *Panels* on the *left* show indirect immunofluorescence patterns after staining with a polyclonal antibody against p18. *Panels* on the *right* show the same specimens decorated with a monoclonal anti-lamin B2 antibody. (For further explanations see text.) *Bars* correspond to 1 μm.

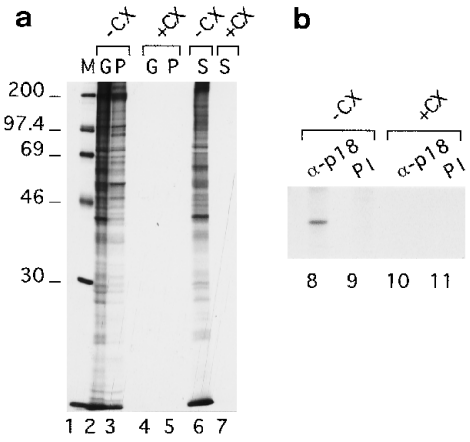

FIG. 5. **Cycloheximide inhibition experiment.** Washed erythrocytes were incubated in minimal essential medium containing [^{35}S]methionine and cysteine (10 mCi/ml) in the presence (+*CX*) or absence (−*CX*) of cycloheximide. The cells were extracted with PBS, 2 mM $MgCl_2$, 1% Triton X-100, 1 mM PMSF, and the lysates were either analyzed directly by SDS-PAGE (*a*) or used for immune precipitation experiments (*b*). Shown here is an autoradiogram of an SDS-polyacrylamide gel loaded with the following samples. *Lane 1*, sample of molecular weight markers; *lane 2*, total cell lysate of metabolically labeled turkey erythrocytes (*G*); *lane 3*, Triton X-100-insoluble pellet of metabolically labeled turkey erythrocytes (*P*); *lanes 4* and *5*, samples corresponding to *2* and *3* using erythrocytes that have been incubated in the presence of cycloheximide; *lanes 6* and *7*, Triton X-100-solubilized material from metabolically labeled erythrocytes in the absence or presence of cycloheximide, respectively (*S*); *lanes 8* and *10*, immune precipitation from the Triton X-soluble fraction using the specific anti-p18 polyclonal antibody; *lanes 9* and *11*, material precipitated from the same lysates by preimmune serum.

solubilized material at 37 °C. Immunoblotting of the various fractions showed that the bulk of the p18 is solubilized by Triton X-114 and that the extracted material partitions exclusively with the detergent phase (Fig. 2).

p18 Resides in Both the Inner and the Outer Nuclear Membrane—Using indirect immunofluorescence microscopy, we could localize p18 at the nuclear periphery of turkey and chicken erythrocytes (see below). However, to obtain morphological information at a higher level of resolution, Triton X-100-extracted erythrocyte ghosts were stained with polyclonal or monoclonal anti-p18 antibodies/protein A-gold and examined by electron microscopy. Preimmune sera did not decorate the erythrocyte ghosts (not shown), whereas specific anti-p18 antibodies labeled heavily the periphery of the nucleus. Immunogold particles were abundantly present in the region of the nuclear lamina, but were excluded from the region of the nuclear pores (Fig. 3*a*). Staining of the plasma membrane-skeleton was negligible as was the labeling of cytoskeletal elements (intermediate filaments) and occasionally seen mitochondria.

To differentiate between an inner or outer nuclear membrane localization, ultrathin frozen sections were prepared from non-extracted erythrocyte ghosts and processed for immunogold staining. Consistent with the previous data, the nuclear envelope was specifically decorated, whereas the plasma membrane was not. Upon closer examination, we noticed that both the inner and the outer nuclear membrane were stained by the anti-p18 antibodies (Fig. 3*c*). The partitioning of p18 with the inner and outer nuclear membrane could be better appreciated by examining intact (*i.e.* unlysed) erythrocytes in which the perinuclear cisternae were dilated by prior exposure to hypotonic media (Fig. 3*b*). Morphometric analysis of specimens such as the ones shown in Fig. 3 (*b* and *c*) confirmed that equal numbers of immunogold particles were present in the outer and the inner nuclear membrane.

The localization of p18 on both sides of the nuclear envelope was unanticipated, because all other integral membrane proteins of the nuclear envelope so far characterized (LAPs and LBR) reside exclusively in the inner nuclear membrane. To ensure that p18 was indeed present in the outer nuclear membrane by a different method, we examined digitonin-permeabilized cells. Digitonin is known to permeabilize the cholesterol-rich plasma membrane without affecting the integrity of the nuclear envelope. Thus, in digitonin-treated cells, antigens exposed on the outer nuclear membrane are accessible to exogenously added antibodies, whereas antigens located in the inner nuclear membrane are not. Digitonin-treated or Triton X-100-permeabilized erythrocytes (control) were doubly-stained with anti-p18 and anti-lamin B antibodies and examined by indirect immunofluorescence microscopy. Data depicted in Fig. 4*c* show that the anti-p18 antibodies labeled readily the surface of the nucleus, yielding a "patchy" staining pattern. In contrast, the nuclear lamina of digitonin-permeabilized cells was not decorated (Fig. 4*d*). In good agreement with previous studies (Soullam and Worman, 1995), the nuclei of digitonin-permeabilized erythrocytes were not stained by anti-LBR antibodies confirming the exclusively inner nuclear membrane localization of this protein (data not shown). Upon Triton X-100 permeabilization, staining of erythrocytes with anti-p18 or anti-lamin B antibodies yielded the same smooth rim fluorescence pattern typically observed with nuclear envelope antigens (Fig. 4, *a* and *b*). From these experiments, it seems reasonable to conclude that p18 is exposed on both sides of the nuclear envelope. In retrospect, the partitioning of p18 with the inner and outer nuclear membrane explains why nuclear envelope fractions, which during isolation lose parts of the outer nuclear membrane, contain variable amounts of p18.

Unlike their mammalian counterparts, avian erythrocytes are biosynthetically active. We could confirm that by incubating mature turkey erythrocytes with [^{35}S]methionine/cysteine in the presence and absence of cycloheximide and performing

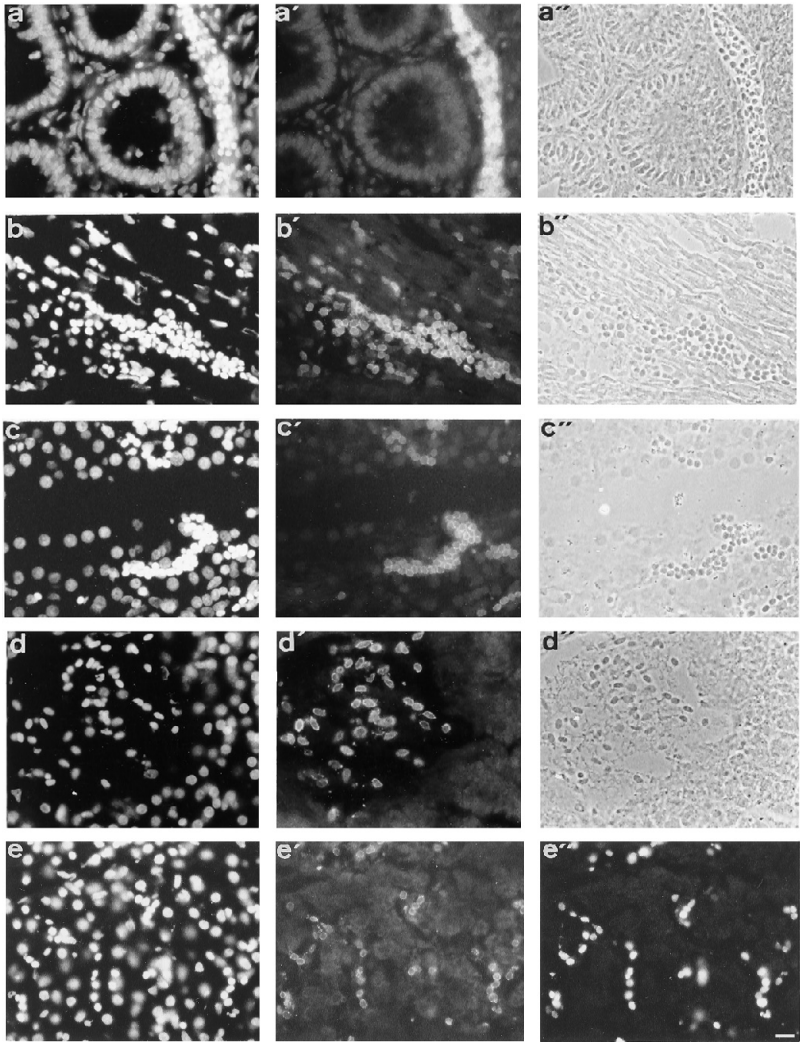

FIG. 6. **Tissue distribution of p18.** Frozen sections of chicken intestine (*a–a″*), chicken heart (*b–b″*), chicken liver (*c–c″* and *e–e″*), and turkey liver (*d–d″*) were probed with a mixture of monoclonal antibodies recognizing p18 (*a′*, *b′*, *c′*, and *e′*), a polyclonal antibody against p18 (*d′*), or a polyclonal antibody against histone H5 (*e″*). *Panels* (*a–e*) on the *left* show DAPI staining of the DNA, while *panels* (*a″-d″*) on the *right* show phase contrast images. Note that only erythrocytes are labeled. Apparent in *a″*, *b″*, and *c″* are blood vessels that contain packed red blood cells. *Bars*, 3 μm.

immune precipitation experiments with the anti-p18 antibodies (Fig. 5). To examine whether the pool of p18 in the outer nuclear membrane represents nascent chains *en route* to the inner nuclear membrane and whether the protein can be "chased" from one membrane compartment to the other (for pertinent information, see Bergmann and Singer (1981), Torrisi and Bonatti (1985), and Torrisi *et al.* (1987)), we performed the following experiment. Turkey red blood cells were incubated at 37 °C for 10–120 min in the presence or absence of cycloheximide, a protein synthesis inhibitor that does not interfere with intracellular transport (Green *et al.*, 1981; Jamieson and Palade, 1968). At the end of these incubations, the cells were permeabilized with digitonin and stained with anti-p18 and anti-lamin B antibodies.

As shown in Fig. 4 (*e* and *f*), cycloheximide treatment did not alter the immunostaining pattern observed before; p18 was still detectable on the outer nuclear membrane in digitonin-permeabilized cells. Validating this observation, cell counting showed that equal numbers of digitonin-permeabilized cells were decorated by the anti-p18 antibodies in untreated and cycloheximide-treated specimens. From these experiments, it can be inferred that p18 residing at the outer nuclear membrane does not represent nascent chains in transit.

p18 Is Predominantly Expressed in Erythrocytes—The next question we addressed was the tissue distribution of p18. SDS-PAGE and Western blotting analysis of organ homogenates did not prove informative because even perfused tissues contained numerous erythrocytes trapped in blood capillaries. For this reason, we decided to examine cryostat sections of different chicken organs by indirect immunofluoresence microscopy. In samples taken from intestine, heart, and liver (which contain dozens of different cell types), the five monoclonal anti-p18 antibodies labeled only a small subpopulation of cells (Fig. 6, *rows a–c*). To confirm these results, we used the polyclonal anti-p18 antibody and decorated turkey tissue sections (as explained before the polyclonal antibody against p18 did not react well with chicken material). Images depicted in Fig. 6 (*row d*) show that the polyclonal antibody reproduced the immunostaining pattern obtained previously using the monoclonal antibodies. Consistent with these data, none of the anti-p18 antibodies decorated chicken hepatoma (DU249) cells in culture (data not shown).

The appearance of the positively stained cells in the various tissue sections suggested that they were red blood cells. To substantiate this interpretation, liver sections were doubly stained with anti-p18 monoclonal antibodies and a polyclonal antibody against the erythrocyte-specific histone H5. Indeed, all anti-p18-positive cells were readily decorated with anti-H5 antibodies (Fig. 6, *row e*).

p18 Binds Directly to LBR and B-type Lamins—The nuclear envelope localization of p18 (this report) and its presence in the LBR complex (Simos and Georgatos, 1992) suggested that at least a subpopulation of this protein is physically bound to LBR. To distinguish between direct or indirect binding, we purified p18 by electroelution, labeled it with ^{125}I, and examined its binding properties *in vitro*. Ligand blotting assays

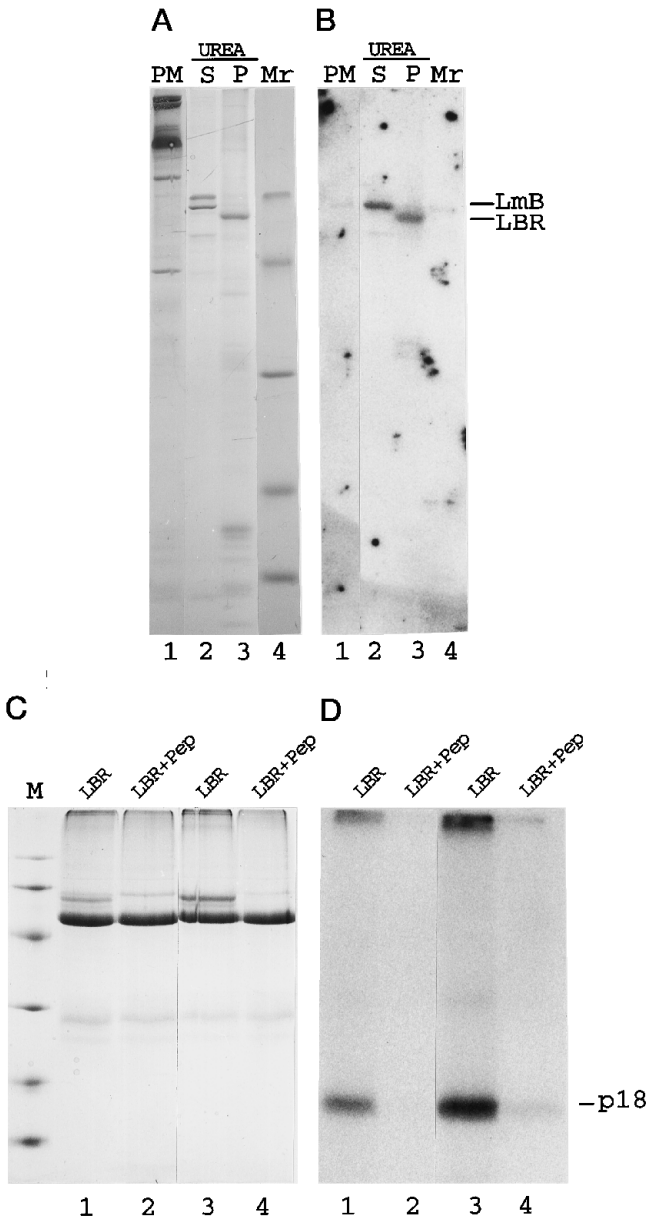

FIG. 7. **Binding of radiolabeled p18 to LBR and B-type lamins.** Turkey erythrocyte fractions (*PM*, plasma membranes; *S*, urea extract of nuclear envelopes; *P*, urea-insoluble residue of nuclear envelopes) and a sample of molecular weight markers (*Mr*; for molecular weights see Fig. 1) were run on SDS-polyacrylamide gels, transferred to nitrocellulose filters, and probed by ^{125}I-p18 as specified under "Experimental Procedures." After drying, the nitrocellulose filters were exposed to x-ray film and autoradiograms prepared. *A* shows Coomassie Blue-stained gel; *B* shows an autoradiogram of the nitrocellulose filter after ligand blotting. The positive signals in the area of lamin B (*LmB*) and LBR (*LBR*) are indicated. In another experiment, identical samples of I^{125}-labeled p18 were incubated with 1 μg (*lanes 1* and *2*) or 2 μg (*lanes 3* and *4*) of purified LBR and LBR was precipitated with affinity-purified aR1 antibodies in the absence (*lanes 1* and *3*) or the presence (*lanes 2* and *4*) of the antigenic peptide R1. The immunoprecipitates were analyzed by SDS-PAGE and either stained by Coomassie Blue (*C*) or autoradiographed (*D*).

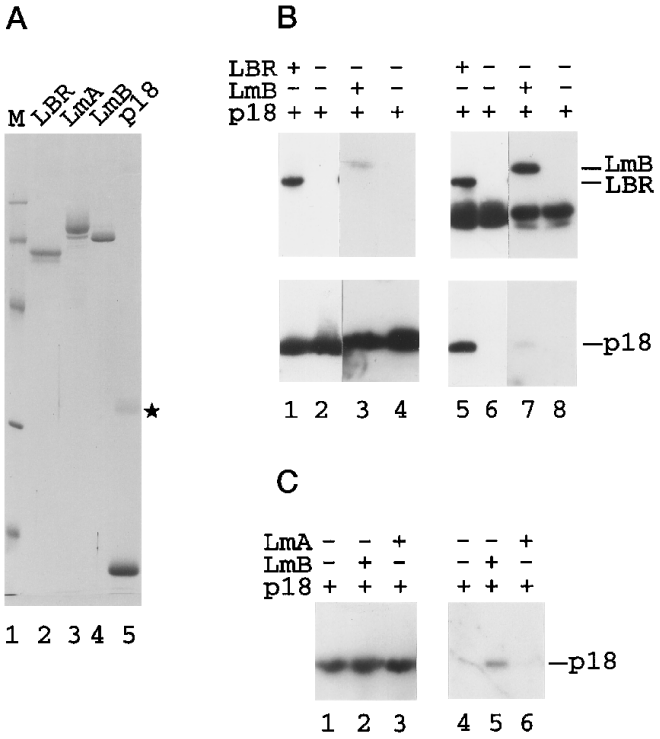

FIG. 8. **Binding of unlabeled p18 to LBR and nuclear lamin B.** *A*, Coomassie Blue-stained gel showing the purified proteins used in the binding experiments. Designations are as in previous figures. *M* is a sample of molecular weight markers with values as specified in Fig. 1. The band at 36 kDa in *lane 5* (*asterisk*) is a dimer of p18, which forms during electrophoresis and reacts with anti-p18 antibodies (G. Simos and S. D. Georgatos, unpublished results). *B*, binding assay in which p18 was incubated with LBR (*lanes 1* and *5*), lamin B (*lanes 3* and *7*), or buffer alone (*lanes 2*, *4*, *6*, and *8*). The incubation mixtures were used for immune precipitation with affinity-purified aR1 or aLI antibodies, which recognize LBR and A/B lamins, respectively. The supernatants (after trichloroacetic acid precipitation, *lanes 1–4*) and the immune pellets (*lanes 5–8*) were analyzed by SDS-PAGE. LBR, lamin B, and p18 were identified by cutting the blots in half and performing Western blotting with the corresponding antibodies. *C*, binding assay in which p18 was incubated with buffer alone (*lanes 1* and *4*), with lamin B (*lanes 2* and *5*) or with lamin A (*lanes 3* and *6*). The reaction mixtures were immunoprecipitated with affinity-purified aLI antibodies. The supernatants (after trichloroacetic acid precipitation, *lanes 1–3*) and the immune pellets (*lanes 4–6*) were analyzed by immunoblotting using anti-p18 antibodies. Only the relevant parts of the blots are shown.

revealed that ^{125}I-p18 binds directly to lamin B and LBR (Fig. 7*A*). This binding was specific because the radioactive probe did not decorate other erythrocyte proteins (*e.g.* spectrin, band 3, and lamin A) or molecular weight markers.

To confirm these results by another method, we performed binding experiments in solution using ^{125}I-p18 and purified LBR. As illustrated in Fig. 7*B* (*lanes 1* and *3*), ^{125}I-p18 bound to LBR and the binary complex of the two proteins was readily precipitated by affinity-purified anti-LBR antibodies (aR1). The specificity of this interaction was demonstrated by performing the same experiment in the presence of the antigenic peptide R1, against which the anti-LBR antibodies were raised. Under these conditions, neither LBR nor ^{125}I-p18 were precipitated with aR1 IgG (Fig. 7*B*, *lanes 2* and *4*). Consistent with a concentration-dependent binding, about twice as much ^{125}I-p18 was co-precipitated with LBR when the input LBR was doubled (Fig. 7*B*, compare *lanes 1* and *3*).

To rule out the possibility that binding to LBR and lamin B was due to a minor ^{125}I-labeled contaminant (*e.g.* a core histone), we repeated the binding experiments employing a different approach. In this version of the binding assay, purified nuclear envelope proteins (for SDS-PAGE profiles of the preparations used, see Fig. 8*A*) were mixed in various combinations and the complexes formed were subsequently precipitated with affinity-purified anti-LBR or anti-lamin antibodies. The immune pellets were run on SDS gels, blotted, and probed with anti-p18, anti-LBR, or anti-lamin antibodies. As shown in Fig. 8*B* (*lanes 1* and *5*), unlabeled p18 and LBR were co-precipitated by aR1. However, p18 could not be detected in the immune pellet when LBR was omitted from the reaction (*lanes 2* and *6*).

Co-incubation of p18 with lamin B and precipitation with anti-lamin antibodies (aLI) yielded a small but detectable amount of p18 in the immune pellet (*lanes 3* and *7*). This weak binding appeared to be specific, as no p18 was seen in the corresponding control (*lanes 4* and *8*). The specificity of the p18-lamin B interaction could be further demonstrated by repeating the binding assay with equivalent quantities of lamin B and lamin A (Fig. 8*C*). No binding of p18 to nuclear lamin A was seen (*lanes 3* and *6*), whereas binding of p18 to lamin B was readily detectable (*lanes 2* and *5*). Taken together, these data show that p18 binds specifically to LBR and B-type lamins. Apparently, binding of p18 to LBR is stronger than binding to the B-type lamins.

DISCUSSION

Interactions of p18 with Components of the Nuclear Envelope—Using a combination of approaches, we have characterized a new integral membrane protein of the nuclear envelope, p18. The co-isolation of p18 and LBR in a native complex (Simos and Georgatos, 1992) and the direct binding of p18 to LBR and B-type lamins *in vitro* (this report) indicate that these proteins form a transmembrane assembly at the level of the inner nuclear membrane. It is likely that the targeting of LBR and the assembly of the LBR complex are facilitated by cooperative interactions between LBR, p18, and B-type lamins. Transfection studies show that the first transmembrane region and the NH_2-terminal domain of LBR are both essential for proper nuclear localization (Smith and Blobel, 1993; Soullam and Worman, 1993, 1995). The NH_2-terminal domain of LBR may anchor this protein to the lamina and the chromatin network, whereas the first transmembrane domain may laterally link LBR to p18 and other membrane proteins (including pre-existing LBR).

Membrane Partitioning of p18—In situ studies documented that p18 is present in both the outer and the inner nuclear membrane. In other words, p18 seems to partition with two distinct membrane compartments: the rough endoplasmic reticulum (represented by the outer nuclear membrane in mature erythrocytes) and the inner nuclear membrane. A similar type of partitioning has been observed previously with vesicular stomatitis and Sindbis virus glycoproteins when mammalian cells were infected with these agents (Bergmann and Singer, 1981; Torrisi and Bonatti, 1985; Torrisi *et al.*, 1987). However, it should be noted that the free diffusion of viral proteins along the endomembranes and the nuclear envelope is clearly due to overexpression and does not reflect a physiological condition.

That about half of the p18 complement does not co-localize (and thus does not interact) with LBR and the lamins appears somewhat paradoxical. However, precedent for this exists in the case of the carbonate/chloride exchanger (band 3 protein), an abundant integral membrane protein of the erythrocyte plasma membrane. At any one instance, only ~ 10% of band 3 is associated with the spectrin-actin membrane skeleton (via ankyrin), whereas ~ 90% of it is uncoupled (for a discussion, see Pinder *et al.* (1995)). At this point we do not know whether the two subpopulations of p18 are structurally identical, or whether this protein is post-translationally modified in a compartment-specific manner.

As indicated by the cycloheximide inhibition experiments, the fraction of p18 that resides in the outer nuclear membrane does not represent nascent chains *en route* to the inner nuclear membrane. Instead, p18 equilibrates between the inner and the outer nuclear membrane. One explanation for this may be that, at some point, the abundance of p18 exceeds the binding sites provided by the the LBR complex. However, an alternative interpretation that need to be further investigated could be that resident proteins of the rough endoplasmic reticulum provide alternative binding sites for p18 and actively anchor this protein at the outer nuclear membrane.

Relation of p18 to IBP—As mentioned above, p18 shows some similarity to IBP, a component of the mitochondrial peripheral-type benzodiazepine receptors that possesses five potential transmembrane regions. The two proteins have the same molecular mass and share sequence features at their NH_2-terminal regions. These include an invariant Trp residue at the beginning of the amino acid sequence, and two highly conserved peptide stretches (VG*X*T and GGF*X*G, where *X* indicates hydrophobic residue) (for sequences, see Riond *et al.* (1989, 1991), Sprengel *et al.* (1989), and Parola *et al.* (1991)). Although more work is necessary to assess the significance of this sequence similarity, based on the available information we would argue that p18 is structurally distinct from IBP. First, p18 is specific to the mature erythrocytes, which contain very few mitochondria (Harris *et al.*, 1971; Zentgraf *et al.*, 1971), and is not detectable in other cell types known to contain IBP; second, antibodies against erythrocyte p18 label heavily the nuclear envelope but do not stain the mitochondria of the red blood cells. Thus, the simplest interpretation would be that p18 and mitochondrial IBP belong to a larger family, which includes structurally related cytoplasmic as well as nuclear membrane proteins. Favoring this interpretation is also the fact that IBP shows significant similarity in three of the transmembrane domains (TM1, TM2, and TM4) with the subunits of the $GABA_A$ receptors (Sprengel *et al.*, 1989).

Acknowledgments—We are indebted to A. Sawyer for expert assistance in hybridoma work, A. Haberman and A. Pyrpasopoulou for help in electron microscopy, and T. Graf for providing the anti-H5 antibody and other materials. We acknowledge Klaus Weber (Max Planck Institute for Biophysical Chemistry, Goettingen, Federal Republic of Germany) and R. Kellner and T. Houthaeve (EMBL Protein and Peptide Group) for help in protein sequencing. Finally, we thank K. Simons, E. Hurt, A. Pyrpasopoulou, and J. Meier for commenting on the manuscript.

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