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

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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. NH₂-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” (LBR³ or p58) (Worman et al., 1988; Foisner 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 doned protein, thymopoietin (Harris et al., 1994). Thymopoi- etin 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 nucleoaslastic (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 laminas 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 NH₂-terminal domain, eight potential membrane-spanning segments, and a hydrophilic COOH-terminal region (Worman et al., 1990; Ye and Worman, 1994; Schulier et al., 1994). Its NH₂-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; Chaudary 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; Shimaniuki et al., 1992). All three yeast proteins lack the NH₂-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 immunochmical and biochemical means (Georgatos et al., 1989), but it...
is not clear whether this polypeptide corresponds to one of the already doned proteins. LBR associates with B-type lamins both in vitro and in vivo (Warman et al., 1988; Simos and Georgatos, 1992; Ye and Warman, 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 polyepptides with molecular masses of 18 kDa (p18) and 34 kDa (p34) (Simos and Georgatos, 1992). Considering that the LBR complex might constitute a "functional" 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) (Kraimer 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 (Nikolakaki 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 MgCl2, 1 mM PMSF (buffer EB) and either 1% Triton X-100, or 1% Triton X-114 at room temperature, or 0.1 N HCl, 1 mM EDTA, 1 mM PMSF, and 1 mM DTT at 4 °C. The specific activity of 125I-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 described by Djaldeli et al. (1991) and Merdes et al. (1991). Binding assays in solution were done as follows. A sample of 125I-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 anti-LBR antibodies (7 μg in the presence or absence of 10 μg of non-specific peptide R1 IgG). In experiments involving labeled 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 anti-LBR or anti-A antibodies and protein A-Sepharose.

Immunological and Immunochimical Procedures—The polyclonal anti-lamin (al) and anti-LBR (aR1) antibodies have been developed in rabbits using as antigens the peptides L1 (Djaldeli 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 antibodies against the lamin B2 antibody E3 were a generous gift from E. A. Nigg et al. (ISPEC, Switzerland). For immunization, p18 purified by electrophoresis was injected into the thigh lymph nodes and subcutaneously in rabbits (50–100 μg) in complete Freund’s adjuvant at day 0. The animals were boosted at day 21 (same amount of protein in incomplete Freund’s adjuvant, subcutaneously), 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 Galfré 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 of turkey erythrocyte ghost. Hybridoma culture supernatants with antibody activities were isolated by 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 all the antibis were visualized with anti-mouse IgG and protein A-gold. Then, slides were fixed with 3.5% formaldehyde, treated with 0.5% Triton X-100 for 10 min at room temperature. After washing with 50 mM NH4Cl and permeabilization with 0.4% Triton X-100, the samples were incubated with monoclonal antibodies, 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 microelectron 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.

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 sequence. To determine whether this polypeptide corresponds to one of the already doned proteins, LBR associates with B-type lamins both in vitro and in vivo (Warman et al., 1988; Simos and Georgatos, 1992; Ye and Warman, 1994; Smith and Blobel, 1994), consistent with its presumed function as a "lamin receptor."
To explore the biochemical properties of p18, we did not contain proteins matching the molecular mass of p18 (Fig. 1). Plasma membrane preparations did establish methods (Georgatos and Blobel, 1987a) and analyzed the fractionated turkey erythrocytes according to previously established protocols (Tax et al., 1991). Upon extraction of the nuclear envelopes with 8 M urea (Fig. 1, 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 electrophoresis 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 NH₂-terminal sequences of the two proteins were identical (Fig. 1C).

Data base searches using the NH₂-terminal sequence of p18 (34 residues in the order MWAYTGTPVHPVGGFGLXFINR-RETPVXELYX; 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. 1C), which had been characterized previously as a component of the mitochondrial peripheral-type benzodiazepine receptors (Sprengel et al., 1989; Riond et al., 1988, 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 NH₂-terminal sequence with p18. Using Western blotting (Fig. 1B), 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. 1. Partitioning of p18 upon cell fractionation and determination of its NH₂-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 (U, lane 4), and urea-insoluble residue of nuclear envelopes (P, lane 5).

C

1. p18: MWAYTGTPVHPVGGFGLXFINR-RETPVXELYX

2. 18 kDa: MWAYTGTPVHPVGGFGLXFINR-RETPVXELYX

3. IBP: MAPVPAYGTPLPSLGFTGAQTYRTGRGFYMNAS...

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).
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 (-WAYTVGFTVPHVGGFLGFX-), 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. 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.
TritonX-100 (shown here). After this incubation, the cells were permeabilized with Triton X-114 and that the extracted material partitions exclusively with the detergent phase (Fig. 2). Immunoblotting of the various fractions showed that the bulk of the p18 is solubilized by Triton X-100 and that the extracted material partitions exclusively with the detergent phase (Fig. 2).

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.

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. 4c 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. 4d). 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 [35S]methionine/cysteine in the presence and absence of cycloheximide and performing...
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; Jamieison 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 immunofluorescence 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 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 ¹²⁵I, and examined its binding properties in vitro. Ligand blotting assays
revealed that $^{125}$I-p18 binds directly to lamin B and LBR (Fig. 7A). 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. 7B (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 by affinity-purified 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 (al) 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. 8C). 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 NH₂-terminal domain of LBR are both essential for proper nuclear localization (Smith and Blobel, 1993; Soullam and Worman, 1993, 1995). The NH₂-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 complements 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 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 NH₂-terminal regions. These include an invariant Trp residue at the beginning of the amino acid sequence, and two highly conserved peptide stretches (VGXT and GFGXG, 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 receptors (Sprengel et al., 1989).

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