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A lamin B receptor in the nuclear envelope

(membrane proteins/protein–protein interactions/binding assays/intermediate filaments)

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ABSTRACT **Using a solution binding assay, we show that purified ^{125}I-labeled lamin B binds in a saturable and specific fashion to lamin-depleted avian erythrocyte nuclear membranes with a K_d of $\approx$0.2 μM. This binding is significantly greater than the binding of ^{125}I-labeled lamin A and is competitively inhibited by unlabeled ligand. We demonstrate that a 58-kDa integral membrane protein (p58) is a lamin B receptor by virtue of its abundance in the nuclear envelope and association with ^{125}I-labeled lamin B in ligand blotting assays. Specific antibodies raised against p58 recognize one protein in isolated nuclei and partially block ^{125}I-labeled lamin B binding to lamin-depleted nuclear membranes. Cell fractionation and indirect immunofluorescence microscopy show that p58 is located in the periphery of the nucleus. This protein may serve as a membrane attachment site for the nuclear lamina by acting as a specific receptor for lamin B.**

The nuclear lamina is a filamentous meshwork situated between the inner nuclear membrane and heterochromatin (1–3). This structure provides support for the adjacent inner nuclear membrane (4) and possible attachment sites for chromatin (2, 5, 6) and cytoplasmic intermediate filaments (7). The nuclear lamins, a family of interrelated polypeptides structurally homologous to intermediate filament proteins, are the building blocks of the nuclear lamina (3, 8–10). Two major types of lamins have been recognized in higher eukaryotic cells: the acidic B-lamins and the neutral A-lamins (10–12).

During mitosis and lamina depolymerization, the B-lamins remain associated with intracellular membrane vesicles thought to be remnants of the inner nuclear membrane, whereas the A-lamins become soluble (12). The B-lamins are also more resistant to extraction from the nuclear envelope compared to the A-lamins (13). Because of these findings, the B-lamins are thought to mediate the coupling of the lamina to the inner nuclear membrane (5, 13). It has been proposed that lamin B associates with the membrane either by a hydrophobic interaction with components of the lipid bilayer (14) or by an attachment to a transmembrane receptor protein (2, 13). These questions and controversies prompted the present investigations.

MATERIALS AND METHODS

Cell Fractionation and Protein Purification. Nuclear envelopes and plasma membranes from turkey erythrocytes were prepared as previously described (7). Lamin-depleted envelopes were prepared by extraction with 8 M urea/10 mM Tris·HCl, pH 8.0/1 mM EDTA (see below). Lamins A and B were purified from the urea extract of turkey or rat nuclear envelopes by DEAE-cellulose chromatography and were ^{125}I-labeled with Bolton–Hunter reagent as described (7).

Extractions with (*i*) 8 M urea/10 mM Tris·HCl, pH 8.0/1 mM EDTA, (*ii*) 0.10 M Na_2CO_3 (pH 11.5), and (*iii*) 2% Triton X-100/2 M KCl/10 mM Tris·HCl, pH 8.0, were performed on nuclear envelopes at room temperature. All extraction solutions also contained 1 mM dithiothreitol and 0.2 mM phenylmethylsulfonyl fluoride. Bath sonication was used to suspend nuclear envelope pellets in the extraction solutions. Supernatants were separated from extracted pellets by centrifugation at 365,000 $\times$ *g* for 20 min.

One-dimensional electrophoresis on sodium dodecyl sulfate (SDS)/polyacrylamide slab gels (10.5% acrylamide) was performed according to Laemmli (15). Two-dimensional electrophoresis was done as described by O'Farrell (16). Protein concentrations were determined by the method of Smith *et al.* (17). Antisera against p58 and turkey lamin B were produced by injecting electrophoretically purified antigens into guinea pigs (Pocono Rabbit Farm, Canadensis, PA). Anti-ankyrin antibodies from rabbit were supplied by V. T. Marchesi (Yale University). Western blotting was performed as previously described (7). A 1:400 dilution of anti-p58 antibodies was used in each blot.

^{125}I-Labeled Lamin (^{125}I-Lamin) Binding Assays. A detailed description of the binding assay used has been previously published (7). Briefly, ^{125}I-lamins were added to assay mixtures containing 19 μg (protein) of urea-extracted (lamin-depleted) envelopes or 77 μg (protein) of plasma membranes suspended in 100–180 μl of 155 mM NaCl/20 mM Tris·HCl, pH 7.3/0.2 mM phenylmethylsulfonyl fluoride/0.2 mg of bovine serum albumin per ml. Samples were then incubated at 25°C for 1 hr. ^{125}I-lamins bound to lamin-depleted nuclear envelopes were separated from free ^{125}I-lamins by centrifugation as previously described for vimentin (7) except that the sucrose cushion was omitted. Supernatants were carefully separated from the pellets, and both were counted in a γ counter. Specific activities of ^{125}I-lamin A or ^{125}I-lamin B used ranged from 2 to 152 $\times$ 10^3 cpm/μg. Amounts of self-sedimenting probes (determined from assays in which no membranes were added to the assay mixtures) were less than 14% of total binding.

In the antibody inhibition studies, lamin-depleted envelopes were incubated on ice for 1 hr either with a 1:3 dilution of anti-p58 antiserum or non-immune serum, and envelopes were then pelleted at 4°C and resuspended in assay buffer. Binding assays were performed as described above except that incubation with ^{125}I-lamin B was for 30 min.

Ligand Blotting Assays. Electrophoresis, transfer to nitrocellulose sheets, incubations with ^{125}I-lamin B, and washes were performed as described (18, 19), except that the transfer buffer contained 20 mM Tris acetate (pH 7.0), 2 mM EDTA, and 0.01% SDS. The specific activity of ^{125}I-lamin B used to probe the blots was 5 $\times$ 10^3 cpm/μg.

Indirect Immunofluorescence Microscopy. Peripheral turkey erythrocytes, washed three times with ice-cold 155 mM NaCl/20 mM sodium phosphate, pH 7.4 (PBS), and resuspended to a hematocrit of 10%, were allowed to settle on glass

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Abbreviation: ^{125}I-lamin, ^{125}I-labeled lamin.

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slides coated with 0.1% alcian blue. Adherent cells, washed once in ice-cold PBS, were fixed with 4% formaldehyde/2 mM $MgCl_2$ in PBS at room temperature for 4 min. Cells were then washed three times with ice-cold PBS containing 2 mM $MgCl_2$ and 100 mM glycine. Adherent fixed cells were lysed with 10 mM sodium phosphate, pH 8.0/2 mM $MgCl_2$/0.5% Triton X-100/1 mM phenylmethylsulfonyl fluoride on ice for 5 min and then washed three times with ice-cold 0.5% Triton X-100 in PBS (PBS/Triton). Anti-p58 and anti-lamin B antisera from guinea pigs and anti-ankyrin antisera from rabbits were diluted 1:10 in PBS/Triton. Cells were incubated with diluted antisera for 1 hr at room temperature and then washed five times with PBS/Triton at room temperature. Next, cells were incubated with secondary fluorescent antibody (fluorescein-conjugated rabbit anti-guinea pig IgG or fluorescein-conjugated goat anti-rabbit IgG diluted 1:150 in PBS/Triton) for 1 hr at room temperature and washed five times with PBS/Triton, and slides were mounted in a solution of *p*-phenylenediamine at 1 mg/ml in 90% glycerol (pH 8.0).

RESULTS

Lamin B Binding to Lamin-Depleted Nuclear Envelopes. When turkey erythrocyte nuclear envelopes are extracted with 8 M urea in the presence of EDTA, the lamins and other peripheral proteins are almost completely removed (Fig. 1*a*; see also ref. 7). These lamin-depleted preparations can be used to assess the binding of purified ^{125}I-lamins (Fig. 1*b*) to the nuclear membrane. Urea-extracted envelopes bind appreciable quantities of rat liver ^{125}I-lamin B in a saturable manner (Fig. 1*c*), whereas binding to plasma membranes, used as a control, is significantly less. The corresponding Scatchard plot (20) (Fig. 1*c Inset*) is linear, indicating the absence of cooperativity or receptor heterogeneity, and extends to >75% receptor occupancy. The estimated K_d, assuming that lamin B is in the form of a dimer (3), is 0.2 μM, and the estimated receptor concentration is about 500 μg per mg of envelope protein. When the binding of turkey erythrocyte ^{125}I-lamin B is examined (Fig. 1*d*), the results are comparable to those seen with rat liver lamin B (Fig. 1*c*), indicating that the membrane binding properties of lamin B are conserved. The membrane binding of turkey erythrocyte ^{125}I-lamin A is substantially less than that of ^{125}I-lamin B (Fig. 1*d*), and an excess of unlabeled lamin A only minimally inhibits the binding of ^{125}I-lamin B compared to a similar amount of unlabeled lamin B, which inhibits 76% of the binding (Fig. 1*e*). These data suggest that a specific receptor for lamin B exists in the nuclear envelope. Furthermore, the existence of a receptor for lamin A of similar affinity cannot be demonstrated.

Identification of a Candidate for a Lamin B Receptor by Ligand Blotting. To identify a candidate for a lamin B receptor, we examined the binding of ^{125}I-lamin B to proteins of urea-extracted nuclear envelopes electrophoretically separated and transferred to nitrocellulose sheets (10, 18, 19, 21, 22). Fig. 2 shows that as previously demonstrated (18, 19) ^{125}I-lamin B binds to lamin A, endogenous lamin B, and vimentin in urea extracts of nuclear envelopes (Fig. 2, lane 1). ^{125}I-lamin B also binds to a protein migrating at 58 kDa (p58) that is not seen in urea extracts of the envelopes and is exclusively present in the urea-extracted, lamin-depleted envelopes (Fig. 2, lane 2; also see below). Little or no binding to control proteins is detected in the same blots (Fig. 2, lane

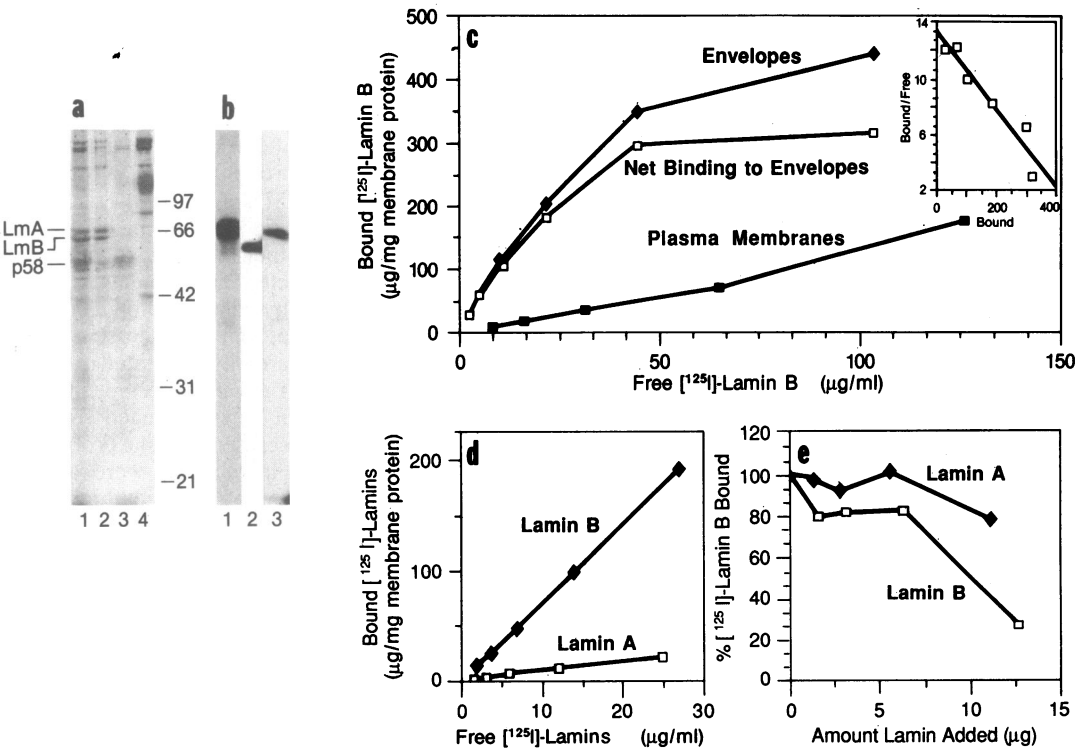

FIG. 1. (*a*) Coomassie blue-stained electrophoretic profiles of turkey erythrocyte nuclear envelopes (lane 1), urea extract of nuclear envelopes (lane 2), urea-extracted (lamin-depleted) nuclear envelopes (lane 3), and plasma membranes (lane 4). Bands corresponding to lamin A (LmA), lamin B (LmB), and a major 58-kDa polypeptide of nuclear envelopes (p58) are indicated. Migration of molecular mass standards (in kDa) are shown at right. (*b*) Autoradiographic profiles of turkey ^{125}I-lamin A (lane 1), turkey ^{125}I-lamin B (lane 2), and rat ^{125}I-lamin B (lane 3). (*c*) Total binding of rat ^{125}I-lamin B to lamin-depleted turkey erythrocyte nuclear envelopes (◆), total binding of the same probe to erythrocyte plasma membranes (■), and net binding (total binding minus binding to plasma membranes) to lamin-depleted envelopes (□). (*Inset*) Scatchard plot of net binding to lamin-depleted envelopes. (*d*) Net binding of turkey ^{125}I-lamin B to lamin-depleted envelopes (◆) and net binding of turkey ^{125}I-lamin A to lamin-depleted envelopes (□). (*e*) Inhibition of ^{125}I-lamin B binding to lamin-depleted nuclear envelopes by various concentrations of unlabeled lamin A (◆) and lamin B (□). The amount of ^{125}I-lamin B added was 2 μg. Experiments shown in *c*, *d*, and *e* were executed in duplicate, and the variation was always <10%.

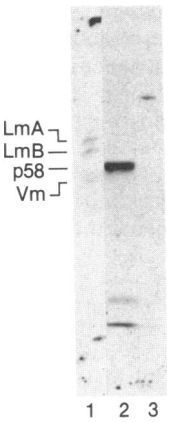

FIG. 2. Ligand blotting assays showing binding of ^{125}I-lamin B to electrophoretically separated proteins of urea extracts of nuclear envelopes (lane 1), urea-extracted envelopes (lane 2), and control proteins (lane 3). Bands corresponding to lamin A (LmA), lamin B (LmB), vimentin (Vm), and p58 are indicated. Control proteins were phosphorylase B (showing some background binding), bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor. The blot shown was performed with ^{125}I-lamin B from rat liver.

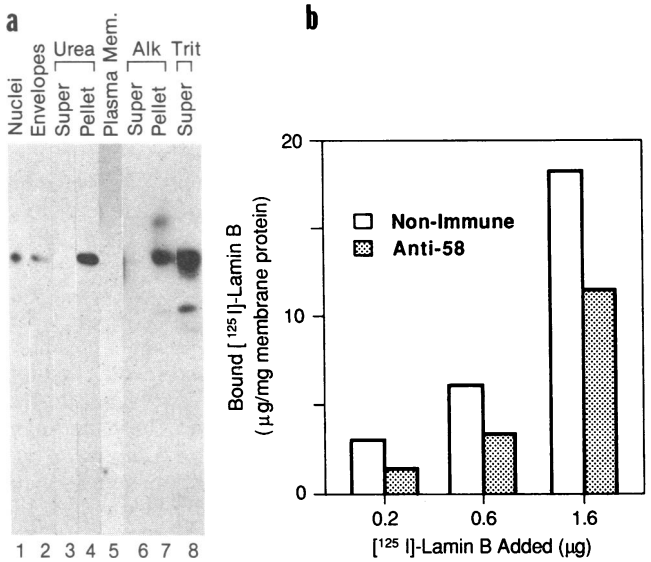

FIG. 3. (*a*) Western blotting with anti-p58 antibodies of fractions of turkey erythrocytes indicated above the corresponding lanes. Urea, 8 M urea/10 mM Tris·HCl, pH 8.0/1 mM EDTA; Alk, 0.10 M Na_2CO_3 (pH 11.5); Trit, 2% Triton X-100/2 M KCl; Super, supernatant. (*b*) Inhibition of ^{125}I-lamin B binding to urea-extracted nuclear envelopes by anti-p58 antibodies. Total binding of turkey erythrocyte ^{125}I-lamin B to envelopes preincubated with nonimmune and anti-p58 antisera is shown for three different amounts of ^{125}I-lamin B used in each assay.

3). At the same specific activity, ^{125}I-lamin A associates with lamins A and B but binds to the 58-kDa component to a much lower degree, and identical blots are obtained whether the ^{125}I-lamin B used is from either rat or turkey (data not shown). These data make p58 a likely candidate for a lamin B receptor.

Characterization of p58 with Anti-p58 Antibodies. Antibodies obtained by immunizing guinea pigs with electrophoretically purified p58 (Fig. 1*a*) were used to further characterize this lamin B-binding protein. Fig. 3*a* shows that in Western blots these antibodies recognize only one band in whole nuclei (lane 1), salt-washed nuclear envelopes (lane 2), and urea-extracted envelopes (lane 4). No proteins are recognized by anti-p58 antibodies in urea extracts of nuclear envelopes (Fig. 3*a*, lane 3) or plasma membranes (Fig. 3*a*, lane 5). Western blotting also demonstrates that p58 is solubilized with a mixture of 2% Triton X-100 and 2 M KCl (Fig. 3*a*, lane 8) but is not extracted from nuclear envelopes with 0.10 M Na_2CO_3 (pH 11.5) (Fig. 3*a*, lanes 6 and 7), indicating that p58 is an integral membrane protein. Blots of two-dimensional gels (data not shown) show that p58 has a pI of ≈6.2.

We also used anti-p58 antibodies to further demonstrate that p58 is a lamin B receptor. Urea-extracted nuclear envelopes were incubated with anti-p58 antibodies and ^{125}I-lamin B binding was examined in a fashion similar to that shown in Fig. 1. Anti-p58 antibodies blocked a significant percentage of ^{125}I-lamin B binding to urea-extracted nuclear envelopes over a range of ^{125}I-lamin B concentrations (Fig. 3*b*). As the ^{125}I-lamin B concentration in the binding assays was increased, the percent inhibition of binding correspondingly decreased. This suggests that ^{125}I-lamin B and anti-p58 antibodies competed for the same site on the receptor molecule. Inhibition of ^{125}I-lamin B binding to lamin-depleted nuclear envelopes further supports the hypothesis that p58 is indeed a lamin B receptor.

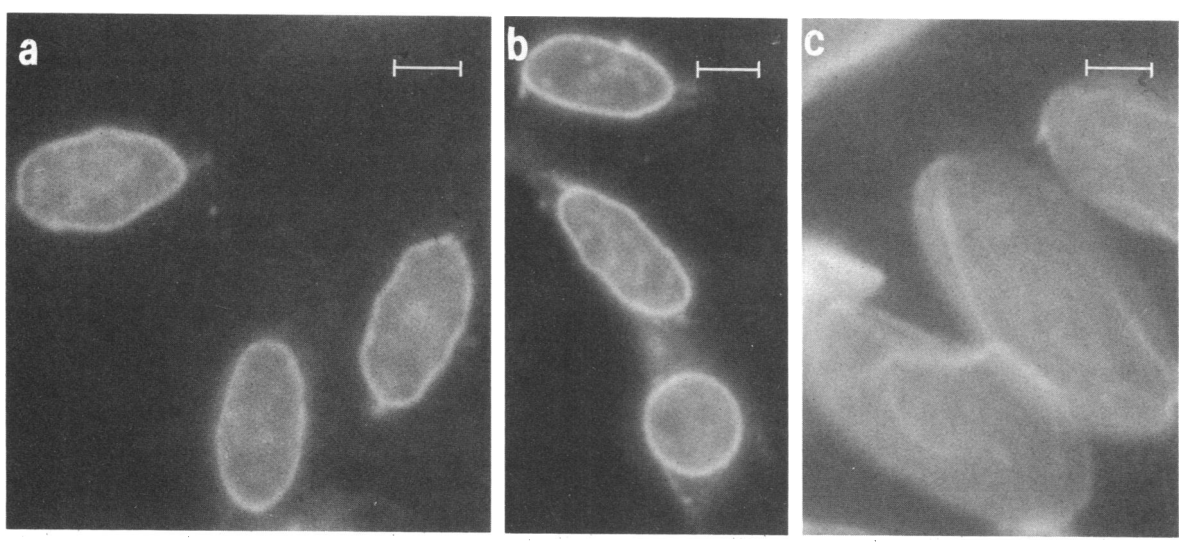

FIG. 4. Subcellular localization of p58 by using specific antibodies and indirect immunofluorescence microscopy. Circulating turkey erythrocytes decorated with anti-p58 antibodies (*a*), anti-lamin B antibodies (*b*), and anti-ankyrin antibodies (*c*) are shown. (Bars = 5 μm.)

Immunolocalization of p58. Indirect immunofluorescence microscopy with anti-p58 antibodies (Fig. 4) was performed to confirm the subcellular localization of p58. These antibodies gave a nuclear rim fluorescence pattern (Fig. 4*a*) identical to the fluorescence pattern seen with anti-lamin B antibodies (Fig. 4*b*) and different than that obtained with antibodies against ankyrin, a plasma membrane protein (Fig. 4*c*). This fluorescence pattern is consistent with a nuclear envelope localization of p58 (2).

DISCUSSION

The present data demonstrate that lamin B binds to lamin-depleted nuclear envelopes in a saturable and specific fashion and support the conclusion that lamin B is attached to the nuclear membrane by a receptor protein. These findings, along with the finding that a B-lamin from *Xenopus laevis* does not contain a hydrophobic transmembrane domain in its primary structure (10), make unlikely the previous hypothesis that lamin B can exist as an intrinsic membrane protein (14). We have identified p58, a major integral membrane protein in avian erythrocyte nuclear envelopes, as a candidate for such a receptor. The amount of this protein in urea-extracted envelopes is consistent with the concentration of a lamin B receptor as estimated by Scatchard analysis. The lamin B receptor function of p58 is further supported by the findings that ^{125}I-lamin B associates directly with p58 on nitrocellulose blots and that the binding of lamin B to lamin-depleted nuclear envelopes is partially inhibited by specific antibodies against p58.

The *in vitro* solution binding studies show that the estimated K_d for lamin B binding to lamin-depleted nuclear envelopes is 0.2 μM. It should be emphasized that this number is only an estimate and that several uncontrollable factors may influence its calculation to some degree. First, we have assumed that lamin B is in the form of a dimer (3), but it is possible that higher-order polymers may exist in solution and still bind to the receptor. Second, it is possible that lamin B may further self-associate on the nuclear envelope after the attachment of a single lamin B dimer to p58. Although the association constants for lamin B with itself have not been quantitated, recent evidence suggests that native lamin B has only a low propensity to self-associate unless it is hyperphosphorylated with protein kinase A (19).

The finding of a specific receptor for lamin B and the lack of a lamin A receptor of similar affinity may explain why all cells examined to date contain lamin B but not necessarily lamin A (or C) (23, 24). The presence of lamin B may be essential for the attachment of the lamina to the nuclear membrane by way of a receptor such as p58 in all cells. In preliminary studies, we have found p58, by using immunofluorescence microscopy, in a variety of avian cell types. We have also detected a cross-reactive protein in rat hepatocyte nuclear envelopes with a molecular mass of 52 kDa. Furthermore, the presence of a specific integral membrane protein receptor for lamin B would explain why lamin B, unlike lamins A and C, remains membrane bound during mitosis when the nuclear envelope is vesiculated and the lamina depolymerized (12). This also suggests that the observed systemic hyperphosphorylation of the lamins in mitosis leading to their depolymerization (12) does not detach lamin B from its receptor.

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