

Lamin B Constitutes an Intermediate Filament Attachment Site at the Nuclear Envelope

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Abstract. We found that urea extraction of turkey erythrocyte nuclear envelopes abolished their ability to bind exogenous ^{125}I -vimentin, while, at the same time, it removed the nuclear lamins from the membranes. After purification of the lamins from such urea extracts, a specific binding between isolated vimentin and lamin B, or a lamin A+B hetero-oligomer, was detected by affinity chromatography. Similar analysis revealed that the 6.6-kD vimentin tail piece was involved in this interaction. By other approaches (quantitative immunoprecipitation, rate zonal sedimentation, turbidometric assays) a substoichiometric lamin B-vi-

mentin binding was determined under in vitro conditions. It was also observed that anti-lamin B antibodies but not other sera (anti-lamin A, anti-ankyrin, preimmune) were able to block 70% of the binding of ^{125}I -vimentin to native, vimentin-depleted, nuclear envelopes. These data, which were confirmed by using rat liver nuclear lamins, indicate that intermediate filaments may be anchored directly to the nuclear lamina, providing a continuous network connecting the plasma membrane skeleton with the karyoskeleton of eukaryotic cells.

ALTHOUGH an association between 10-nm filaments and the nuclear envelope has been proposed several times during the past on the basis of morphological observations (9, 14), evidence for such an interaction on the molecular level was obtained only recently. We have demonstrated in a preceding article (7) that vimentin subunits can bind under in vitro conditions to some proteinaceous receptor site displayed along the nuclear periphery. In this interaction vimentin uses its 6.6-kD carboxy-terminal tail domain to associate with the nuclear membrane and then polymerizes in situ, forming extensive filament arrays surrounding the nuclear envelope.

Knowing that the vimentin receptor at the nuclear surface is of a proteinaceous nature and exploiting the in vitro binding assay established previously, we tried to identify such a filament attachment site. Pivotal criteria in this survey have been the following: (a) removal of the putative receptor from the envelopes by chemical extraction should in principle decrease the binding of ^{125}I -vimentin to the nuclear envelopes, (b) blockage of the same site by antibodies should affect the binding, and (c) the receptor protein should be able to bind vimentin in vitro and with the same site specificity as the previously described nuclear attachment site (7).

Materials and Methods

Extractions of Nuclear Membranes

Equal amounts of turkey RBC nuclear envelopes (287 μg of protein) were washed once with 10 vol of 10 mM NaPO_4 , 0.5 mM phenylmethylsulfonyl fluoride (PMSF) pH 7.6 at 4°C, pelleted, and resuspended in 0.5 ml of either

(a) 20 mM NaPO_4 , 140 mM NaCl, 1 mM MgCl_2 , 0.1 mM PMSF, pH 7.6 (PBS+), (b) 1 mM Na_2CO_3 , 1 mM EDTA, 2 mM β -mercaptoethanol (β -me), 0.1 mM PMSF, pH 9.6, (c) 25 mM Tris-HCl, 140 mM NaCl, 1 mM EDTA, 2 mM β -me, 0.1 mM PMSF, 1% Tween-20, pH 7.6, or (d) 10 mM NaPO_4 , 1 mM EDTA, 2 mM β -me, 0.1 mM PMSF, 1.5–6 M urea, pH 7.6. The samples were incubated for 30 min at 0°C and then centrifuged at 35,000 g for 35 min. The pellets were resuspended by vortexing (10 s) in ice-cold PBS+ (1 ml) and recentrifuged. This step was repeated twice more. After a brief dialysis (3 h, 4°C) against PBS+ and subsequent centrifugation (15,000 g, 24 min), the pellets were resuspended in 120 μl of PBS+. Aliquots were analyzed by SDS-PAGE or used for binding assays.

Protein Purification and Radiolabeling

Calf lens vimentin was isolated as previously described (7). For purification of turkey erythrocyte lamins A and B, two methods were used.

Method A. 2 ml of packed nuclear envelopes (12 mg of total protein), derived from 40 ml of whole turkey blood, were resuspended in 9 vol of double-distilled water, incubated for 15 min on ice, and spun at 35,000 g for 20 min. The pellets were resuspended in 9 vol of 10 mM NaPO_4 , 1 mM EDTA, 2 mM β -me, 0.1 mM PMSF, and 6 M urea pH 7.9 (buffer Ch), vortexed, and extracted on ice for 30 min. The sample was then centrifuged in a Beckman TL-100 rotor (model TL-100; Beckman Instruments, Inc., Palo Alto, CA) (128,000 g, for 20 min at 4°C), and the supernatant was collected and dialyzed against the extraction buffer (buffer Ch) for 18 h at 4°C. The dialyzed extract was incubated with 2 ml of DEAE-cellulose, previously equilibrated in buffer Ch, and the mixture was turned end-over-end for 45 min at 4°C. The resin was pelleted, washed with a total of 250 ml of buffer Ch, and packed into a column. The column was washed with 20 ml of buffer Ch and eluted with 100 ml of a 0–400 mM NaCl gradient at a flow rate of 0.21 ml/min at 4°C. Lamin-containing fractions, after identification by SDS-PAGE, were pooled and dialyzed against 18 liters of 100 mM NaPO_4 , 1 mM EDTA, 0.2 mM PMSF, pH 8.1 at 4°C.

Method B. This method was similar to method A except that chromatography was conducted at room temperature in buffer Ch containing 8 M urea and that column elution was done with a 36 ml gradient of 0–150 mM NaCl. Rat liver lamins were isolated essentially as described in method B from

nuclear envelopes prepared as described by Aaronson and Blobel (1). To remove traces of lamins A+C that contaminated lamin B, appropriate column fractions were pooled, dialyzed against low-salt buffer (Ch), and rechromatographed using a 0–80 mM NaCl gradient. Radiolabeling of the purified proteins with the Bolton–Hunter–¹²⁵I reagent was done as reported previously for bovine lens vimentin (7). Siliconized glassware was used throughout the purification procedure. For storage of the purified material the lamins were dialyzed against 2× PBS, 5 mM β-me, 0.2 mM PMSF pH 8.0 and kept at 0°C or –20°C.

Binding Assays

Binding with ¹²⁵I-vimentin and nuclear envelopes were done exactly as described by Georgatos and Blobel (7).

Immunoprecipitation

For quantitative immunoprecipitation, 60 ng of purified lamin B or 90 ng of a complex of lamin A and B (isolated by method A) were pre-incubated with increasing quantities of ¹²⁵I-vimentin (5,270 cpm/μg) in PBS, 1.5 mg/ml BSA (final volume 100 μl) for 60 min at room temperature. Then, 2 μl of anti-lamin B antiserum (diluted into 10 μl of PBS, 1 mM PMSF and 1% Triton X-100) was added. After another 60 min incubation, 40 μl of a 50% suspension of protein A–Sepharose 4B in PBS, 1 mM PMSF and 1% Triton X-100 was added. After a 15-min incubation the samples were loaded onto a 0.5-ml cushion of 1 M sucrose in the above buffer and centrifuged at 15,000 g for 10 min. The pellets were resuspended in PBS, 0.1 mM PMSF, 0.1% Triton X-100, and 1.5 mg/ml BSA pH 7.6 and recentrifuged in the same fashion. This step was repeated three more times. Finally, the radioactivity of the pellets and of the combined supernatant was determined.

Rate Zonal Sedimentation

A complex of turkey RBC lamins A and B, isolated by method A, ¹²⁵I-labeled (0.75 μg, 1.2 × 10⁶ cpm/μg), was incubated with or without 6 μg of purified lens vimentin in PBS, 1 mM MgCl₂, 5 mM β-me, 0.1 mM PMSF, pH 7.6 in a final volume of 462 μl for 1 h at room temperature. The samples were loaded onto 13 ml of a sucrose gradient (5–20%) poured into ultraclear plastic tubes precoated with 0.1% gelatin (to prevent nonspecific adsorption). The gradients were centrifuged at 120,000 g in a Beckman Ti 40 rotor (4°C) for 22 h. 600-μl fractions were collected and portions (400 μl) were mixed with 20 μg of DNase I (carrier) and precipitated at 0°C with 10% TCA. The radioactivity in each fraction was determined by counting in a gamma counter. The material in the pellet was dissolved in 600 μl of Laemmli lysis buffer (2% SDS) and counted directly.

Turbidometry

Purified lens vimentin (150 μg/ml) in 2 mM NaPO₄, 2 mM β-me, 0.1 mM PMSF, pH 7.6 was induced to polymerize in the presence or in the absence of 1.5 μg/ml of lamin B or a complex of lamin A and B by the addition of salt (NaCl to isotonic). Polymerization was monitored by recording the development of turbidity at 350 nm (22°C) using virgin polyethylene microcuvettes.

Affinity Chromatography

Purified lamin A+B complex from turkey RBC (~200 μg) or lens vimentin (~1 mg) were dialyzed extensively against 100 mM HEPES, 1 mM EDTA, 0.1 mM PMSF and 6 M urea pH 7.5. The proteins were mixed with 5 ml of derivatized agarose beads (Affigel-15), prewashed with 2-propanol, distilled water, and the above buffer, in this order. The mixtures were turned end-over-end for 1 h at room temperature and for 3 h at 4°C. After this incubation the resin was allowed to settle and the supernatants were removed. The Affigel was washed three times with 40 ml of 1 M Tris-HCl pH 8.0, 1 mM EDTA and turned end-over-end for 18 h at 4°C in the same buffer to block all remaining coupling sites. Finally, the gel was washed with (a) 10 mM NaPO₄, 1 mM EDTA, 5 mM β-me, 0.1 mM PMSF, 7 M urea pH 7.6 (40 ml), (b) 10 mM NaPO₄, 140 mM NaCl, 1 mM MgCl₂, 0.2 mM PMSF, 5 mM β-me pH 7.6 (600 ml), and (c) 10 mM NaPO₄, 1 mM EDTA, 5 mM β-me, 0.1 mM PMSF, 0.2% NaN₃, and kept in this buffer at 0°C.

Before each assay, the gel was packed into a 2.5-ml column, washed with at least 50 vol of 10 mM NaPO₄, 140 mM NaCl, 1 mM MgCl₂, 5 mM β-me, 0.1 mM PMSF pH 7.6, and equilibrated for 1 h at room temperature.

Other Procedures

One-dimensional gel electrophoresis was according to Laemmli (13). Urea-SDS gels were made according to Sorti et al. (17). Chymotryptic cleavage of vimentin was performed as previously described (6). Immunoblotting was done as described by Granger and Lazarides (10). Immune precipitation of purified ¹²⁵I-lamin B and vimentin with anti-vimentin antibodies was executed as described in Quantitative Immunoprecipitation with 2 μg of vimentin and 0.5 μg of ¹²⁵I-lamin B (500,000 cpm/μg).

Results

Urea Extraction Abolishes Vimentin Binding and Removes the Lamins from the Nuclear Envelope

To investigate whether the vimentin binding site at the nuclear envelope is represented by a peripheral or an integral membrane protein, isolated nuclear envelopes were extracted with various solvents and the ability of the resultant residual structures to bind ¹²⁵I-vimentin was assessed. Data presented in Table I indicated that the only treatment that could substantially lower the binding was urea extraction, while treatment with non-ionic detergents, or sodium carbonate at high pH, did not affect the association (the nominal increase in the % binding of Na₂CO₃-extracted membranes is due to the removal of histone proteins and some lamin A and the resulting enrichment in other authentic membrane polypeptides on a mg/mg scale).

Examination of urea-extracted envelopes by SDS-PAGE revealed that the lamins were completely removed, whereas a 62-kD polypeptide and a low-molecular weight protein of an approximate size of 18 kD were not extracted (Fig. 1, compare lanes *e*, *p*, and *s*). Therefore, we inferred from these experiments that some peripheral membrane proteins, and perhaps the lamins, may play a role in connecting intermediate filaments (IF)¹ to the nuclear envelope.

Screening with Antibodies

Searching for putative vimentin receptors at the nuclear envelope we looked for antibodies that might affect the binding of ¹²⁵I-vimentin to the membranes using the assay system established previously (7). From the antibodies analyzed, we found that only an autoimmune anti-lamin B serum from a systemic lupus erythematosus patient (16, see also Fig. 6 A) was able to block ~70% of the binding, while

Table I. Vimentin Binding Capacities of Extracted Nuclear Envelopes

Membranes	¹²⁵ I-Vimentin	¹²⁵ I-Vimentin bound	Binding %
	μg/ml	μg/mg	
Nonextracted	3.6	7.85 ± 0.54	100
Urea-extracted*	3.6	2.22 ± 0.30	28
Nonextracted	6.5	20.94 ± 0.08	100
Urea-extracted‡	6.5	6.51 ± 0.80	31
Na ₂ CO ₃ -extracted	6.5	40.1 ± 0.00	191
Tween-extracted	6.5	21.8 ± 0.56	104

All assays were executed as described by Georgatos and Blobel (7).

* 4 M urea.

‡ 1.5 M urea.

1. Abbreviation used in this paper: IF, intermediate filament(s).

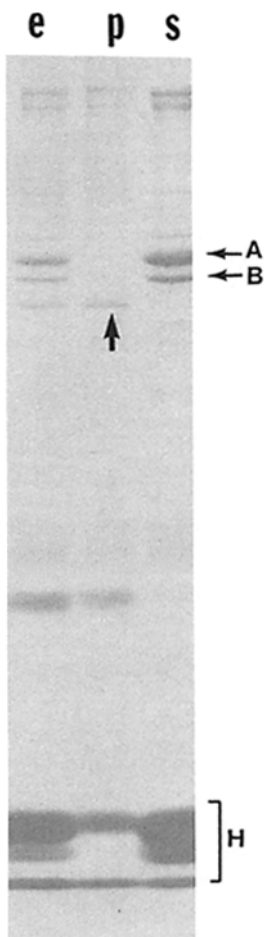


Figure 1. SDS-PAGE profiles of urea-extracted nuclear envelopes. The nuclear envelopes were extracted with 6 M urea, as specified in Materials and Methods. e, non-extracted membranes; p, membrane pellet after extraction; s, extracted proteins (from three times as much material as the pellet). 1.5–4 M urea extraction gave similar profiles (data not shown). Arrow indicates a nuclear envelope polypeptide (with an M_r similar to that of lamin C) that was not removed by urea (note the higher load of lane s). Arrowheads point to lamins A and B, histones are indicated by H. A 10% acrylamide gel is shown stained with Coomassie Blue.

anti-lamin A antibodies, anti-ankyrin antibodies, and non-immune sera had no effect (Fig. 2). This finding provided a first indication that a vimentin-lamin B interaction might be responsible for the linking of IF to the nuclear envelope.

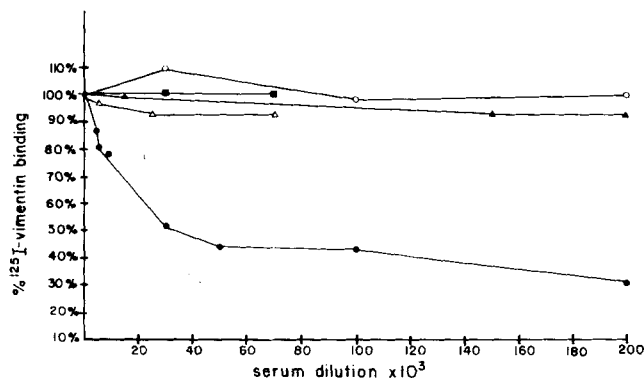


Figure 2. Effect of various antibodies on the binding of ^{125}I -vimentin to vimentin-depleted nuclear envelopes. The membranes were preincubated at the indicated dilutions of antibodies at 4°C for 45 min in PBS-1 mM PMSF, pH 7.6. All samples then received $6.2 \mu\text{g}$ of ^{125}I -vimentin (219,000 cpm/ μg) and were processed as described (7). % binding was expressed in reference to controls receiving no antibodies. (●) Anti-lamin B (human); (○) normal serum (human); (▲) anti-lamin A and C (hamster); (△) anti-ankyrin (rabbit); (■) normal serum (rabbit).

Vimentin-Lamin Interactions In Vitro

Purification of the Nuclear Lamins. To identify more precisely vimentin-binding proteins in our nuclear membrane system, a 6 M urea extract of turkey RBC nuclear envelopes was fractionated by ion-exchange chromatography in the presence of 6 M urea (see Materials and Methods). About 50% of lamin A was found, under these conditions, in the fraction of polypeptides that did not bind to the ion exchanger DEAE-cellulose (not shown). However, after elution of such columns by a salt gradient, fractions containing both lamins A and B in an apparent 1:1 stoichiometry were obtained (Fig. 3 A). The co-purification of lamin A and B in 6 M urea was unexpected as the two lamin forms exhibit a significant pI difference, with lamin B being much more acidic (12; and our unpublished results). This suggested that 6 M urea caused fragmentation of the nuclear lamina into lamin A homo-oligomers and lamin A+B hetero-oligomers (note that there is about twice as much lamin A than B in these cells). The complex of the lamins could be dissociated when chromatography was carried out in the presence of 8 M urea. Under these conditions the two lamins were eluted as distinct peaks, with the less acidic lamin A eluting first and the more acidic lamin B eluting after it (Fig. 3 B). A similar method was used to purify rat liver lamins. Lamins A and C remained under these conditions associated, while a complete separation between them and lamin B was achieved. The purified lamins were radiolabeled by the Bolton-Hunter reagent- ^{125}I . Autoradiographic profiles of ^{125}I -lamins analyzed by SDS-PAGE are depicted in Fig. 3 C. The high degree of purity of this material (as indicated by the overexposed autoradiogram) is apparent.

Rate Zonal Sedimentation Assays. When a small amount of turkey RBC lamins A+B (renatured after removal of urea and radiolabeled with ^{125}I) was sedimented through sucrose gradients in neutral electrolyte solutions, $\sim 60\%$ of it remained at the top of the gradient, while the rest was pelleted (Fig. 4). Thus, at least a portion of the purified material existed in a soluble form under these conditions. The rest of the (pelletable) lamins were most probably in a polymeric state. The relative amounts of soluble versus polymeric lamins could be altered by manipulating the total lamin concentrations as monitored either by direct sedimentation assays or by nondenaturing gel electrophoresis (to be reported elsewhere).

Upon prior co-incubation with vimentin, the lamin peak migrated significantly faster and coincident with the vimentin peak (Fig. 4). Thus, vimentin homo-oligomers and lamin hetero-oligomers appeared to form a complex in vitro. In addition, as shown in Fig. 4 (*inset*), a substantial increase in the amount of pelletable ^{125}I -lamins was detected when these proteins were co-incubated with vimentin. Since all pelleted vimentin is thought to consist of filaments, this experiment suggested that lamins also bind to filamentous vimentin.

Affinity Chromatography. To confirm these results, purified turkey RBC lamin A+B complex or isolated lens vimentin were coupled to agarose (Affigel-15) and binding was assessed. When a mixture of ^{125}I -vimentin and BSA was passed through the lamin column, we observed that almost all the radioactive material bound to the matrix and was eluted by 7 M urea. Conversely, all of the BSA was recovered in the nonbound fraction and the subsequent column wash

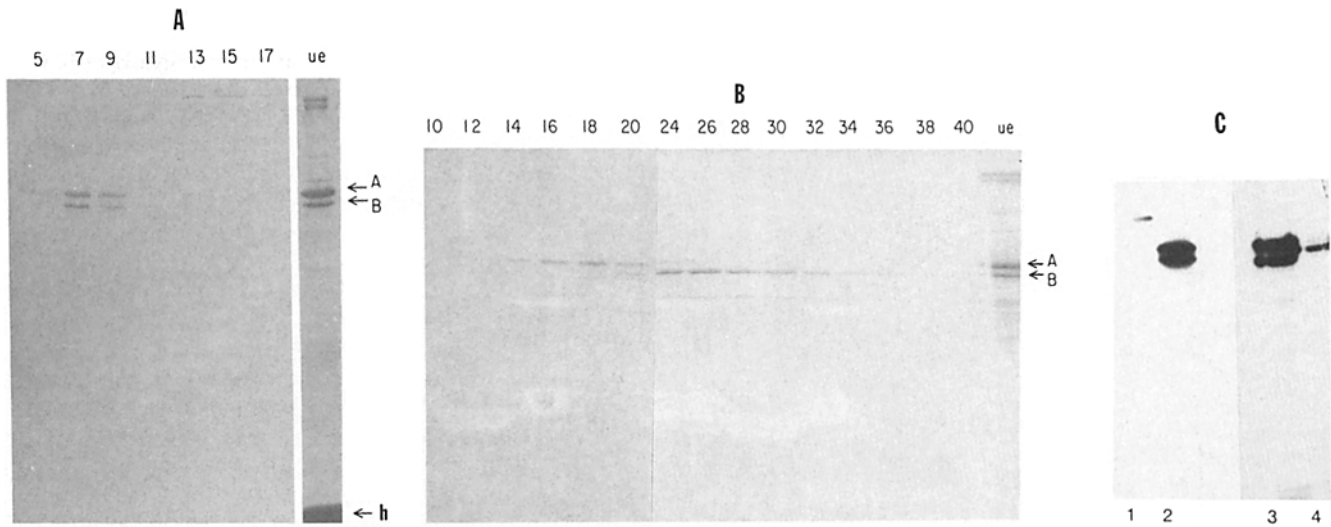


Figure 3. Purification of turkey RBC lamins from urea-extracts of nuclear envelopes. (A) Column elution profile according to method A (see Materials and Methods). The fraction numbers are indicated. ue, urea extract applied to DEAE-cellulose. A and B indicate lamins A and B; h, histones (10% acrylamide gel). (B) Column elution profile according to method B (check Materials and Methods). Note the separation of lamins A and B. The fraction numbers are indicated. (C) Purified lamins radiolabeled by the ^{125}I -Bolton-Hunter reagent. Lane 1, a marker 100-kD protein purified from lens. Lane 2, turkey RBC lamins A and B. Lane 3, rat liver lamins A+C. Lane 4, rat liver lamin B (purified according to method B). Autoradiograms are shown.

(Fig. 5 A). When a chymotryptic digest of ^{125}I -vimentin was passed through the same column it was noticed that only the 6.6-kD tail piece was retained (Fig. 5 A, inset). Thus, the lamin A+B complex seemed to specifically recognize the same segment of vimentin that associates with the previously described nuclear receptor (7).

In contrast, when turkey RBC ^{125}I -lamin B or ^{125}I -lamin A were passed through the vimentin affinity column we noticed that vimentin was able to retain selectively lamin B but not lamin A (Fig. 5 B). Moreover, when turkey RBC ^{125}I -lamin B was briefly treated with α -chymotrypsin and then tested, there was a significant increase in the radioactivity associated with the nonbound fraction and a corresponding decrease in the amounts of the tracer eluted by 7 M urea. BSA

in all cases was recovered in the nonbound fraction. Likewise, treatment of lamin B with thrombin (for 18 h at room temperature) completely abolished the binding (data not shown). The same results were obtained after testing rat liver lamin B (Fig. 5 C). Furthermore, when rat liver lamin B was cleaved at its Cys residues by 2-nitro-5-thiocyanobenzoic acid (NTCB) we found that the vimentin column did not retain any of the cleaved products (Fig. 5 C), suggesting that the region of lamin B that binds to vimentin should contain at least one Cys residue.

Immunochemical Approaches. Using antibodies that specifically recognize rat liver lamin B (16) and turkey erythrocyte lamin B (Fig. 6 A), we attempted to quantitate the binding of ^{125}I -vimentin to the lamin A+B complex, or to

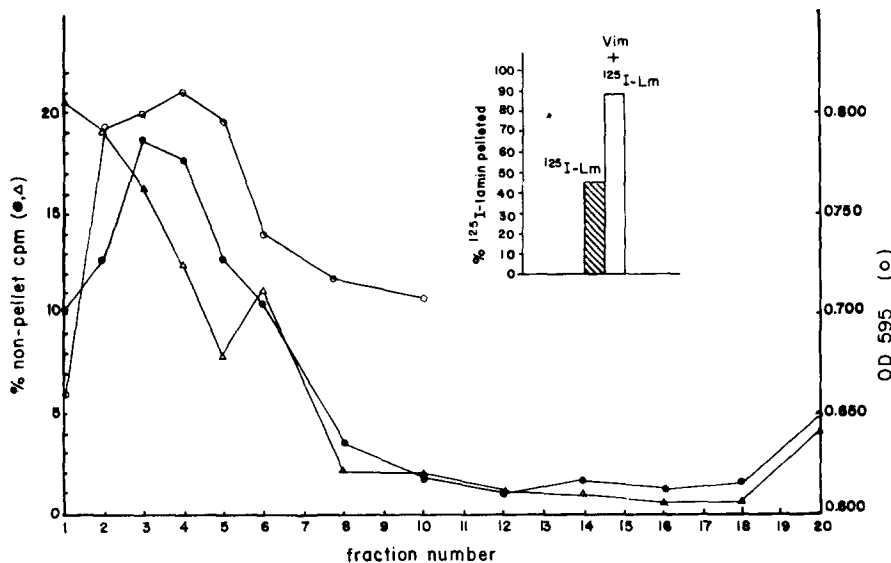
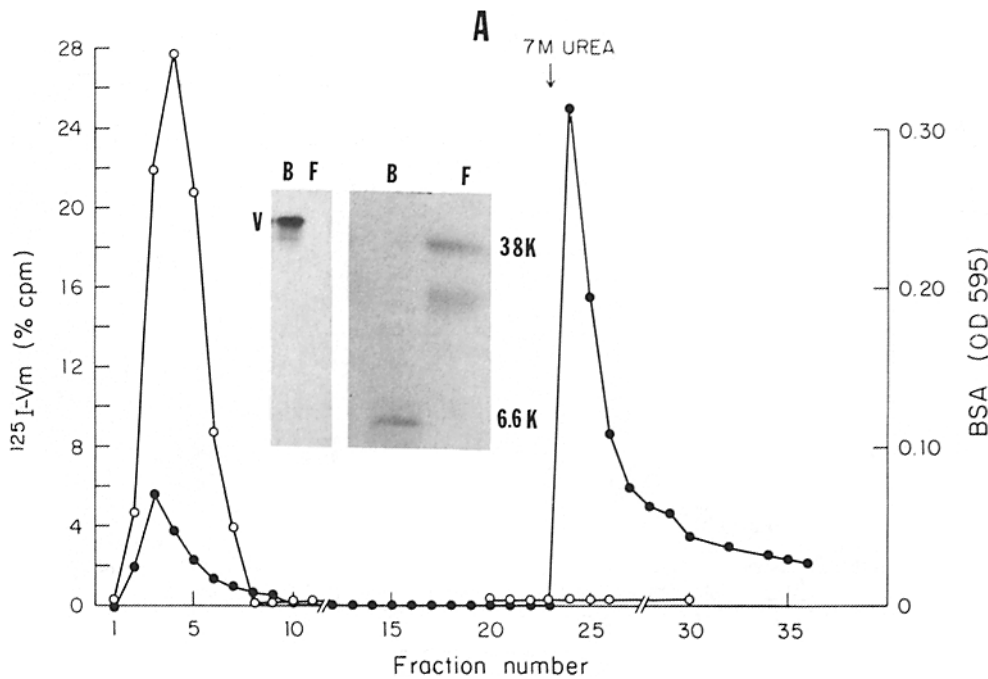
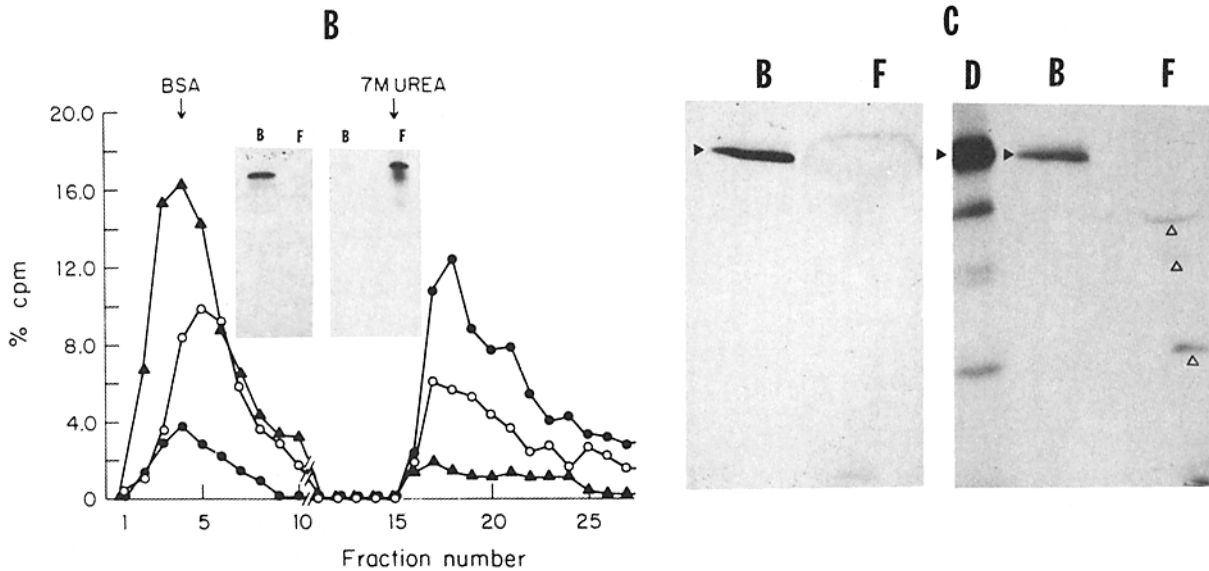


Figure 4. Binding of ^{125}I -lamin A and B complex to purified vimentin as detected by rate zonal sedimentation (see Materials and Methods). (Δ) ^{125}I -Lamins without added vimentin. (\bullet) ^{125}I -Lamins plus vimentin. (\circ) Vimentin alone (assayed by the Biuret reaction and spectrophotometry at 595 nm). (Inset) Percentage ^{125}I -lamin complex pelleted in the absence (hatched bar) and presence (open bar) of vimentin.



activity or assayed for protein by the Biuret reagent. (○) BSA (as detected by protein determination); (●) ¹²⁵I-Vimentin. (Inset) SDS-PAGE and autoradiographic analysis of fractions eluted from the lamin-affinity column. (Left) ¹²⁵I-Vimentin. (Right) ¹²⁵I-Vimentin digest. B, bound fraction; F, flowthrough; V, vimentin. The positions of the 6.6-kD tail piece and the 38-kD middle domain are indicated. (B) Three experiments similar to the one shown in Fig. 5 A with a vimentin-agarose column. A chymotryptic digest of ¹²⁵I-lamin B (10 μg/ml, 10⁶ cpm/μg), intact ¹²⁵I-lamin B, and intact ¹²⁵I-lamin A (10 μg/ml, 200,000 cpm/μg) were passed sequentially through the same column. After each assay the column was washed with 50 ml of PBS-PMSF allowed to equilibrate for 5 h and then re-used. Recoveries of radioactivity ranged from 85 to 95%. (▲) ¹²⁵I-Lamin A; (●) ¹²⁵I-lamin B; (○) a 30-s chymotryptic digest of ¹²⁵I-lamin B (enzyme/protein ratio 1:200, incubation at room temperature). (Inset) SDS-PAGE and autoradiographic analysis of fractions eluted from the vimentin-affinity column. (Left) ¹²⁵I-Lamin B. (Right) ¹²⁵I-lamin A. B, bound material; F, flowthrough. (C) SDS-PAGE and autoradiographic analysis of fractions recovered from the same vimentin column after applying ¹²⁵I-lamin B prepared from rat liver. (Left) ¹²⁵I-lamin B. (Right) ¹²⁵I-Lamin B NTCB-digest. D, the whole digest; B, bound fraction; F, flowthrough.



isolated lamin B, by immunoprecipitation. To avoid polymerization during the assay, the purified proteins were co-incubated at low concentrations and the resulting complexes were precipitated by anti-lamin B antibodies and protein A-bearing Sepharose beads. Under these circumstances, al-

though the nonspecific adsorption of ¹²⁵I-vimentin to the beads was relatively high, the specific binding of lamins A+B to vimentin generated a saturable isotherm with an apparent plateau corresponding to a stoichiometry of 2.4 vimentin tetramers per lamin A+B hybrid (i.e., a monomer

Figure 5. Vimentin-lamin interactions as detected by affinity chromatography. (A) Agarose-linked turkey RBC lamin A and B complex (0.5 ml of packed resin; see Materials and Methods) was poured into a column. ¹²⁵I-Vimentin (50 μg/ml 150,000 cpm/μg) or a chymotryptic digest of ¹²⁵I-vimentin was mixed with an equal volume (200 μl) of 0.1 mg/ml BSA in PBS, 1 mM MgCl₂, 5 mM β-me, and 0.1 mM PMSF pH 7.6 and applied to the column. After collection of the nonbound fraction the column was washed with 10 ml of the above buffer, and then the bound protein was recovered by elution with 10 mM NaPO₄, 1 mM EDTA, 5 mM β-me, 0.1 mM PMSF, and 7 M urea pH 7.6. 0.25-ml fractions were collected and portions (70 μl) were counted for radioac-

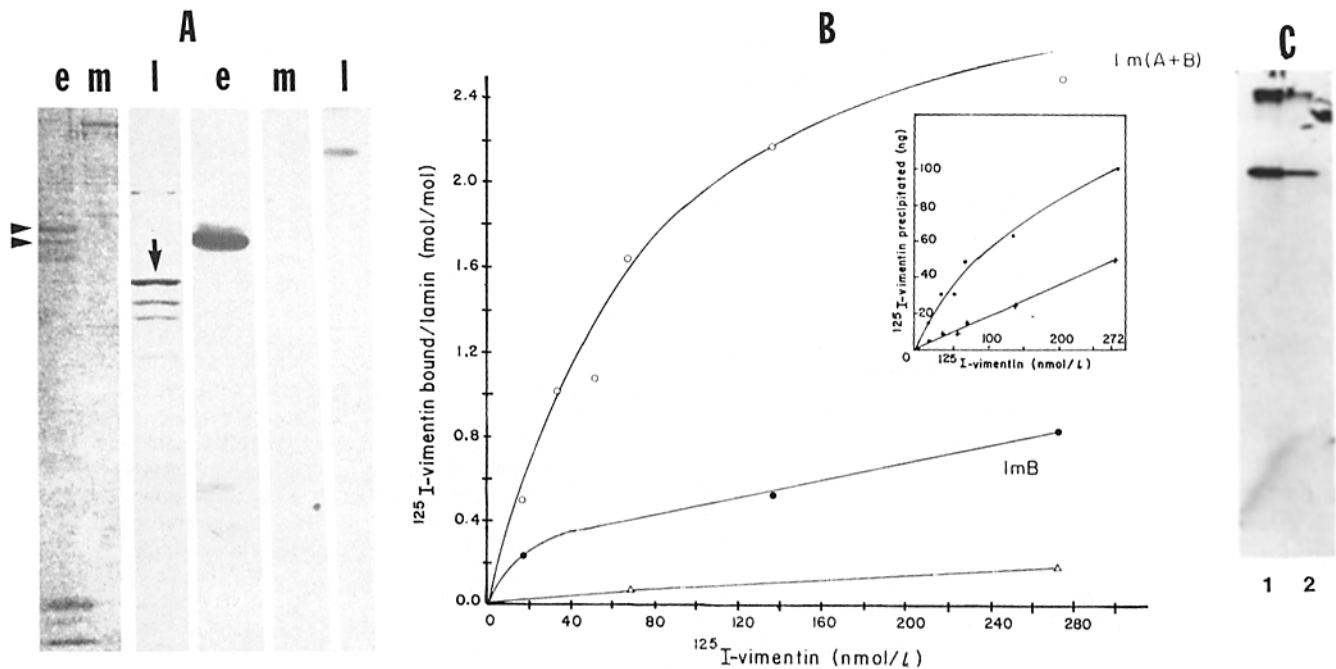


Figure 6. (A) Immunoblotting with an anti-lamin B antibody. (Left) Protein-stained gel slices of turkey RBC plasma membranes (m), nuclear membranes (e), and bovine lens cytoskeleton urea-extracts (l). (Right) Corresponding autoradiogram of replicas probed with antibodies and ^{125}I -protein A. Note the lack of cross-reaction with vimentin (arrow). A 10% acrylamide gel is shown. (B) Binding of ^{125}I -vimentin to the complex of lamins A and B, or lamin B, as detected by quantitative immunoprecipitation with anti-lamin B serum (for details check Materials and Methods). (○) ^{125}I -Vimentin plus purified lamins A and B. (●) ^{125}I -Vimentin plus lamin B (specific binding). (Δ) ^{125}I -Vimentin plus lamin A. (Inset) Nonspecific binding control. (●) Total ^{125}I -vimentin precipitated; (+) ^{125}I -vimentin precipitated in the absence of lamins. Because of the uncertainty associated with the aggregation state of lamins 1 mole equivalent signifies here one tetramer of vimentin and one hybrid monomer of lamin A and B. (C) Co-precipitation of purified ^{125}I -lamin B with vimentin and anti-vimentin antibodies. The assay was executed as described in Materials and Methods. An autoradiogram is shown. Lane 1, ^{125}I -lamin B, plus vimentin, plus antibodies. Lane 2, ^{125}I -lamin B, without vimentin, plus antibodies (12% acrylamide gels).

with a M_r equal to the mean of lamin A and B molecular masses). In the case of isolated lamin B there was no indication for saturation at the concentrations tested but the specific binding was substantial (Fig. 6 B). Isolated turkey RBC lamin A bound minimal amounts of ^{125}I -vimentin, indistinguishable from the ones obtained for controls containing no lamin. From the binding data, an association constant (K_a) of $2 \times 10^7 \text{ M}^{-1}$ could be estimated for the vimentin-lamin complex. This value agrees with the apparent K_a calculated from the binding of intact vimentin to nuclear envelopes ($2.1 \times 10^7 \text{ M}^{-1}$) or from the binding of the vimentin fragment to the same membranes ($1 \times 10^7 \text{ M}^{-1}$), as shown previously (7).

Likewise, in the presence of purified vimentin, specific anti-vimentin antibodies could immunoprecipitate purified ^{125}I -lamin B, while in the absence of vimentin significantly smaller quantities of the tracer were found in the immunoprecipitate (Fig. 6 C).

Turbidometric Assays. To gain insight into the possible effects of the lamins on vimentin polymerization, the method of Zackroff and Goldman (19) was used to monitor these interactions. We observed that inclusion of substoichiometric amounts of turkey RBC lamin A+B complex in reaction mixtures containing pure vimentin resulted in a fourfold increase of the plateau turbidity (developed after the addition of salt) with respect to controls, which contained only vimentin. The inclusion of isolated B (at the same approximate concentrations) augmented the turbidity even more (seven-

fold increase, see Fig. 7). Since the magnitude of the plateau turbidity is independent of the polymer length and proportional to the weight concentration of the polymer formed, these results indicated that lamins A+B, or lamin B, facilitated the assembly of IF (thermodynamically). In addition, since the polymerization rate in the presence of lamins was greater than in their absence, the same data suggested that the lamins behaved as an IF nucleator in kinetic terms. The decrease in the plateau turbidity detected after the first 30 min of assembly (Fig. 7) indicated a kinetic overshooting: initially, a few nucleation sites were used to assemble a large polymer mass because the elongation reaction was probably faster than the nucleation process. Upon equilibration, however, the weight concentrations dictated by the relative affinities were established, resulting in a polymer redistribution as all nucleation sites were occupied now.

Quantitative Assessment of Lamin-Vimentin Interactions in the Context of the Nuclear Envelope. After characterizing the nuclear lamins, and in particular lamin B, as a possible vimentin anchorage site at the nuclear envelope, additional means of analysis were sought to understand better the stoichiometries involved. Operating under the assumption that the primary binding of ^{125}I -vimentin to the nuclear envelopes (at low concentrations of the ligand) can be described by a saturable isotherm (because of the pseudo-plateau seen in Fig. 2 A of the preceding article, the results obtained by immunoprecipitating ^{125}I -vimentin and soluble

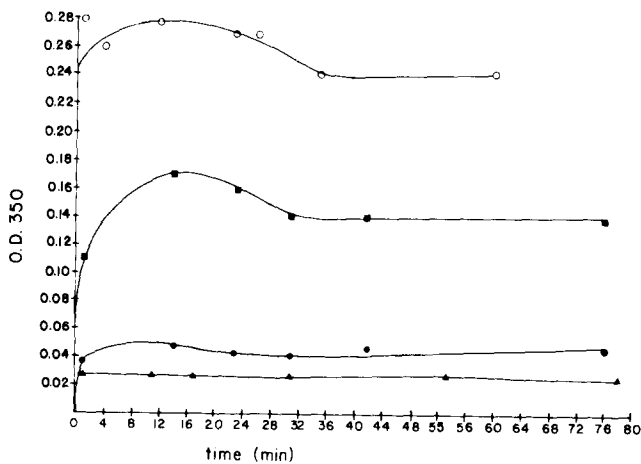


Figure 7. Effect of purified lamins on vimentin polymerization in vitro. The assay was carried out essentially as described by Zackroff and Goldman (19) with a few modifications (see Materials and Methods). (●) Vimentin alone; (■) vimentin plus lamins A and B; (○) vimentin plus lamin B; (▲) lamins A and B alone. Transmission with the cuvettes used at 350 nm was 55% of the maximum (at 800 nm as specified by the manufacturer). Assays with isolated lamin B gave similar values as the lamin A+B control (not shown).

lamins A and B, Fig. 5 B, and the saturable binding of the vimentin tail to nuclear envelopes, Fig. 8 B of the previous report), the question of the ligand and receptor valency was investigated.

A modified form of Scatchard analysis (11) was used and a computer program was constructed to calculate the relative affinity and the corresponding valency parameters. To apply this method, preliminary runs of the program were done to select the best parameters that could describe the above situation. Considering the empirically determined dissociation constant (from the values of the relative equilibrium vimentin concentration needed to reach 50% of the pseudo-plateau and the saturable binding of the vimentin fragment to nuclear envelope), which was in the order of 2.1×10^{-7} M, the following rationale was adopted: in an aggregate size of four (i.e., a tetrameric soluble state) vimentin could have a valency (f) of one (the whole unit comprising the binding entity), or two (each of the two parallel strands of the dimer binds), or four (each individual strand binds separately). To select a meaningful value, different f values were introduced into the program and the relative affinity constants were calculated. By these means the best fit to the experimentally obtained dissociation constant was derived for an $f = 1$ (3.4×10^{-7} M). Plotting the data for $f = 1$ permitted the estimation of $q \times m_A$, i.e., of the product of receptor valency (q) and the total concentration of receptor protein in the assay mixture (m_A).

By extracting the Coomassie Blue stain from electrophorograms of nuclear envelopes (3), a lamin B content of $7.7 \mu\text{g/ml}$ of assay mixture was estimated. From the plot presented in Fig. 8, a $q \times m_A$ amounting to $141.2 \mu\text{g/ml}$ was calculated. Thus, a value of q of ~ 18 can be derived, pointing to a stoichiometry of vimentin to lamin B equal to 18:1. This value is close to that obtained from solution studies that indicated that 2.4 vimentin tetramers associated with 1/2 lamin B molecule (in the lamin A/lamin B hybrid) or that 2×2.4

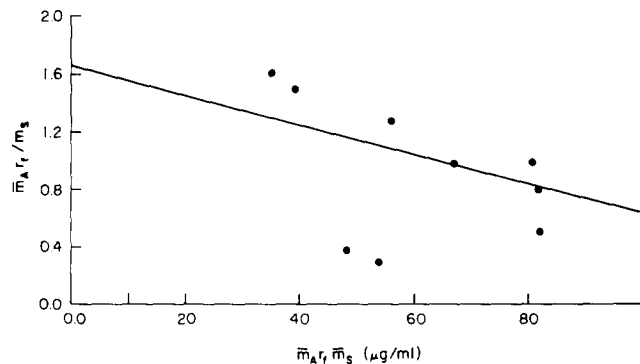


Figure 8. Quantitative assessment of lamin-vimentin interactions in the context of the nuclear envelope. The binding data presented before (Fig. 2 A in reference 7) were analyzed taking into account that lamin B constitutes the nuclear binding site for vimentin. The lamin B content of the assayed envelopes was estimated by pyridine extraction of Coomassie Blue-stained gels (1). The results were plotted according to the Hogg-Winzor equations (11) $r_f/(m_s)^{1/f} = qK_A - fK_A r_f (m_s)^{(f-1)/f}$ where $r_f = [(m_s)^{1/f} - (m_s)^{1/f}] / m_A$. m_A is total concentration of lamin B, m_s is equilibrium concentrations of non-bound ^{125}I -vimentin, \bar{m}_s is total concentration of ^{125}I -vimentin, K_A is equilibrium constant, f is vimentin's valency ($f = 1$), and q is lamin B valency.

$\times 4 = 19.2$ vimentin molecules bind to each lamin B moiety (see Fig. 6 B).

Discussion

Molecular Aspects

Using conventional in vitro methods we have attempted to characterize a vimentin attachment site occurring at the nuclear envelope of avian erythrocytes. We have arrived at the conclusion that lamin B, a previously identified component of the fibrous nuclear lamina (8), serves also as a vimentin receptor, since all necessary conditions for such a role have been met: removal of lamin B from the nuclear envelope by urea extraction or blockage with anti-lamin B antibodies reduced the binding of vimentin to these membranes. Furthermore, a specific vimentin-lamin B association could be detected by rate zonal sedimentation, affinity chromatography, and immunoprecipitation, using both ^{125}I -vimentin or ^{125}I -lamins, from avian or mammalian sources. It should be noted, however, that lamin B bound more avidly to vimentin when lamin A was also present (Fig. 6 B). Thus, lamin A, albeit not directly involved in this interaction, may play a role in maintaining a favorable conformation of lamin B in which the latter recognizes better the vimentin molecule. The lamin complex was capable of associating with the same vimentin tail piece fragment which binds to whole nuclear envelopes (Fig. 5 A, inset). The same site specificity was found by assessing the binding of a thrombic vimentin fragment to a rat liver lamin B column. This peptide lacks the first 60 amino terminal residues, that is the entire head domain, and it binds to lamin B (Georgatos, S., unpublished results).

The examination of such molecular interactions required the purification of the nuclear lamins for which no data were available concerning their solution behavior. We found, much to our surprise, that avian nuclear lamina-derived lam-

ins A and B, after depolymerization in 6 M urea, disassembled only partially. Moreover, upon removal of urea these proteins retained biological activity. Not only were they capable of recognizing the carboxy-terminal tail of vimentin (this article) but they also seemed competent to polymerize in a concentration-dependent fashion and to bind back to urea-extracted envelopes (to be reported elsewhere).

The low solubility of the lamins, combined with the well-known insolubility of IF, necessitated binding assays under conditions of low protein concentrations to avoid self-association reactions that would obscure the stoichiometries involved. Unfortunately, these restrictions did not allow a detailed assessment of the cooperativity parameters in lamin-vimentin interactions by immunoprecipitation. This is mainly due to the dilution factor, introduced by the multiple washings applied to reduce the nonspecific binding, during immunoprecipitation, which could affect small binding differences. Nevertheless, some other aspects of these interactions could be indirectly approached by examining the effect of the lamins on the polymerization of IF. Pertinent to this issue is the fact that substoichiometric amounts of purified lamins A+B, or lamin B, could augment the turbidity of vimentin solutions under polymerization-favoring conditions.

Despite the presented *in vitro* evidence, which favors a physiologically meaningful interpretation of vimentin-lamin associations, one should be cautious in evaluating the data. Recent analyses (4, 15) have revealed an extensive sequence and secondary structure homology between lamins A, C, and IF subunits. Therefore, the possibility of a stochastic copolymerization of homologous subunits *in vitro* should be considered.

Arguing against the latter interpretation, vimentin associates with the less homologous partner of the lamin A and B complex (lamin B) using its nonconserved, tail domain (4, 18). In addition, a defined (substoichiometric) molar ratio between vimentin and lamin B can be deduced both in the context of the nuclear envelope and in solution, under conditions not favoring filament formation. If our calculations are correct, each lamin B unit will "concentrate" four to five vimentin tetramers, exactly the number of protofilaments usually seen at IF ends. This elementary nucleus will then be the substrate onto which more subunits may be added.

Topological Considerations

So far, the only convincing evidence that exists indicates that both the lamins and the lamina are confined to the inner face of the inner nuclear membrane (8). It is therefore logical to hypothesize that IF subunits, to establish connections with the nuclear lamins, must somehow gain access to the nuclear interior. For cytoplasmic proteins the only conceivable way to achieve such an access is by passing through the nuclear pores. In this regard, it is useful to consider here the data concerning the binding of vimentin to detergent-extracted nuclear envelopes (Table I). Since the lipid bilayer barrier was removed by these treatments, one anticipates higher amounts of the radioactive probe to associate with the extracted envelopes. However, no such increase in the binding was observed. Therefore, vimentin subunits seem to associate with already exposed foci along the lamina and not diffusely and homogeneously with each individual lamina subunit. In other words, the nuclear lamina constitutes an

homogeneous matrix as far as the anchorage of IF is concerned. Taking into account the apparent hetero-polymeric structure of the lamina (12), it would not be unreasonable to assume that lamin B (complexed with lamin A) may occur preferentially at certain focal regions flanking the inner annuli of the nuclear pores.

The notion that IF may associate with the nuclear envelope at distinct foci, coinciding with the nuclear pores, is not novel. In fact, previous morphological studies (2, 9, 14) have suggested a co-localization of IF with the nuclear pores. The diameter of the pores (60–90 nm) (5) could easily accommodate a passage of IF subunits, or even filaments, through this canal. We are now concentrating efforts in our laboratory to examine in detail this interesting scenario using conventional and immune electron microscopy.

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References

1. Aaronson, R. P., and G. Blobel. 1974. Isolation of nuclear pore complexes in association with a lamina. *Proc. Natl. Acad. Sci. USA.* 72:1007–1012.
2. Franke, W. W. 1971. Relationship of nuclear membranes with filaments and microtubules. *Protoplasma.* 73:263–292.
3. Fenner, C., R. R. Traut, D. T. Mason, and J. Wilkman-Coffelt. 1975. Quantification of Coomassie-blue stained proteins in polyacrylamide gels based on analysis of eluted dyes. *Anal. Biochem.* 63:595–602.
4. Fisher, D. Z., N. Chaudhary, and G. Blobel. 1986. cDNA sequencing of nuclear lamins A and C reveals primary and secondary structural homology to intermediate filament proteins. *Proc. Natl. Acad. Sci. USA.* 83:6450–6454.
5. Franke, W. W., U. Scheer, G. Krohne, and E. D. Jarasch. 1981. The nuclear envelope and the architecture of the nuclear periphery. *J. Cell Biol.* 91(3, Pt 2):395–505.
6. Geisler, N., E. Kaufmann, and K. Weber. 1982. Protein chemical characterization of three structurally distinct domains along the protofilament unit of desmin 10 nm filaments. *Cell.* 30:277–286.
7. Georgatos, S. D., and G. Blobel. 1987. Two distinct attachment sites for vimentin along the plasma membrane and the nuclear envelope in avian erythrocytes. *J. Cell Biol.* 105:105–115.
8. Gerace, L., A. Blum, and G. Blobel. 1978. Immunochemical localization of the major polypeptides of the nuclear pore complex lamina fraction. Interphase and mitotic distribution. *J. Cell Biol.* 79:546–566.
9. Goldman, R., A. Goldman, K. Green, J. Jones, N. Lieska, and H.-Y. Yang. 1985. Intermediate filaments: possible function as cytoskeletal connecting links between the nucleus and the cell surface. *Ann. NY Acad. Sci.* 455:1–17.
10. Granger, B. L., and E. Lazarides. 1984. Membrane skeletal protein 4.1 of avian erythrocytes is composed of multiple variants that exhibit tissue-specific expression. *Cell.* 37:595–607.
11. Hogg, P. J., and D. J. Winsor. 1985. Effects of ligand multivalency in binding studies: a general counterpart of the Scatchard analysis. *Biochem. Biophys. Acta.* 843:159–163.
12. Krohne, G., E. Debus, M. Osborn, K. Weber, and W. W. Franke. 1984. A monoclonal antibody against nuclear lamina proteins reveals cell-type-specificity in *Xenopus laevis*. *Exp. Cell Res.* 150:47–59.
13. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the heads of bacteriophage T4. *Nature (Lond.).* 227:680–685.
14. Lehto, V. P., I. Virtanen, and P. Kurki. 1978. Intermediate filaments anchor the nuclei in nuclear monolayers of cultured human fibroblasts. *Nature (Lond.).* 272:175–177.
15. McKeon, F., M. Kirschner, and D. Caput. 1986. Homologies in both primary and secondary structure between nuclear envelope and intermediate filament proteins. *Nature (Lond.).* 319:463–468.

16. Reeves, W. H., N. Chaudhary, A. Salerno, and G. Blobel. 1987. Lamin B autoantibodies in sera of certain patients with systemic lupus erythematosus. *J. Exp. Med.* 165:750-762.
17. Sorti, R. V., D. M. Coen, and A. Rich. 1975. Tissue-specific forms of actin in the developing chick. *Cell.* 8:521-527.
18. Steinert, P. M., W. M. Idler, and R. D. Goldman. 1980. Intermediate filaments of BHK-21 cells and bovine epidermal keratinocytes have similar ultrastructures and subunit domain structure. *Proc. Natl. Acad. Sci. USA.* 77:4534-4538.
19. Zackroff, R. V., and R. D. Goldman. 1979. *In vitro* assembly of intermediate filaments from BHK-21 cells. *Proc. Natl. Acad. Sci. USA.* 76:6226-6230.