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Binding of two desmin derivatives to the plasma membrane and the nuclear envelope of avian erythrocytes: Evidence for a conserved site-specificity in intermediate filament–membrane interactions

(lamin B/ankyrin/membrane skeleton/karyoskeleton)

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ABSTRACT **Using solution binding assays, we found that a 45-kDa fragment of desmin, lacking 67 residues from the N terminus, could specifically associate with avian erythrocyte nuclear envelopes but not with plasma membranes from the same cells. It was also observed that a 50-kDa desmin peptide, missing 27 C-terminal residues, retained the ability to bind to both membrane preparations. Displacement experiments with an excess of purified vimentin suggested that the two desmin derivatives were interacting with a previously identified vimentin receptor at the nuclear envelope, the protein lamin B [Georgatos, S. & Blobel, G. (1987) *J. Cell Biol.* 105, 117–127]. Additional analysis by affinity chromatography confirmed this conclusion. Employing an overlay assay, we demonstrated that the 50-kDa fragment, but not the 45-kDa desmin peptide, was capable of interacting with the plasma membrane polypeptide ankyrin (a known vimentin attachment site), as was intact vimentin. Conversely, the nuclear envelope protein lamin B was recognized by both fragments but not by a chymotryptic peptide composed solely of the helical rod domain of desmin. These data imply that the lamin B-binding site on desmin resides within the 21 residues following its helical rod domain, whereas the ankyrin-associating region is localized within its N-terminal head domain, exactly as in the case of vimentin.**

Earlier ultrastructural observations have revealed a close topological association of intermediate filaments (IF) with the nuclear envelope and the plasma membrane in several model systems (1–6). In line with these data, recent biochemical studies have suggested that at least vimentin IF may be anchored to the subplasmalemmal membrane-skeleton and the karyoskeleton via specific, proteinaceous linkers. Two types of such high-affinity vimentin "receptors" have been identified in avian and mammalian erythrocytes: the peripheral plasma membrane protein ankyrin, which also connects the spectrin–actin meshwork to the plasma membrane proper (7–10), and the polypeptide lamin B (11), a constituent of the fibrous nuclear lamina that lines the nucleoplasmic side of the inner nuclear membrane (12).

Vimentin, as well as all other IF subunits known so far, possesses a tripartite substructure. It consists of a central helical domain flanked by two nonhelical segments at the N- and C-terminal regions, the so-called "head" and "tail" domains (13). These peripheral domains exhibit distinctly different properties in terms of membrane binding: the N-terminal head domain of vimentin binds to ankyrin, while its C-terminal tail region binds to lamin B. Because the head domain seems also to be involved in filament formation (14, 15), ankyrin, by binding to it, could behave as a filament "capping" factor blocking IF elongation at the association sites. Lamin B, on the other hand, seems to promote filament nucleation by associating in a cooperative fashion with the tail domain. The central domain does not appear to be involved in any of these interactions. On the basis of the above information, a functional polarization during IF assembly *in vivo* has been postulated (11), in spite of the apparent structural apolarity of 10-nm filaments reconstituted *in vitro* from isolated subunits (16, 17).

To test the general validity of this hypothesis, we decided to examine the binding properties of a different IF subunit, desmin, taking advantage of the structural and functional similarities between desmin and vimentin (18, 19) and exploiting two desmin fragments that had been previously characterized (15). Here, we provide evidence that desmin and vimentin could share the same attachment sites. Since we also detect a significant affinity difference in the binding of desmin versus vimentin to avian erythrocyte lamin B, we predict the existence in muscle cells of a lamin B isotype that may be more finely tailored for connecting the desmin skeleton to the nucleus.

MATERIALS AND METHODS

Membranes and Probes. Nuclear envelopes and plasma membranes from turkey erythrocytes (depleted or not depleted of vimentin) were isolated as described (11), except that the envelopes were salt-washed with 1 M KCl/20 mM sodium phosphate/4 mM EDTA/1 mM dithiothreitol/0.2 mM phenylmethylsulfonyl fluoride, pH 8.0, for 15 min on ice, to enrich the preparation in nonhistone polypeptides.

Calf lens vimentin, turkey erythrocyte lamins, and rat liver lamins were purified as described (11). The two chicken desmin derivatives employed in this study (T-desmin and L-desmin, derived by digestion with thrombin and endoproteinase Lys-C, respectively) were isolated as specified (15), whereas the helical rod domain of desmin was obtained from one of the derivatives (the thrombic fragment) after brief treatment with chymotrypsin followed by ion-exchange chromatography (13).

The purified proteins were ^{125}I-labeled under mild conditions with Bolton–Hunter reagent (10).

Anti-ankyrin antibodies (prepared against human erythrocyte ankyrin) as well as anti-spectrin antibodies were a generous gift of V. Marchesi (Yale University); the characterization of the anti-lamin B antibodies has been reported (11, 20). Anti-lamin A and C antibodies were kindly provided by L. Gerace (Johns Hopkins University).

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Abbreviations: IF, intermediate filament(s); T-desmin, thrombic fragment of desmin; L-desmin, fragment of desmin obtained by digestion with the lysine-specific endoproteinase Lys-C.

Assays. Solution binding assays with iodinated proteins and isolated membrane fractions were performed as previously (11), except that the assay buffer contained 10 mM Tris·HCl (pH 7.3), 150 mM NaCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1 mg of bovine serum albumin per ml, unless stated otherwise. All data points in Fig. 1 are averages of at least four independent determinations. In Fig. 1 only the specific binding is shown; the nonspecific binding was measured using proteolyzed membranes and urea-extracted envelopes as in ref. 11 and amounted to about 20% of the total. The quantities of radiolabeled L- and T-desmin that were pelleted in the absence of membranes were about 2 orders of magnitude less than the specific binding. Affinity chromatography was conducted exactly as described (11), using a lamin B-agarose column. Overlay binding assays were executed as follows. Membrane preparations or protein fractions were solubilized in Laemmli sample buffer (21) that also contained 6 M urea, without boiling. Samples were electrophoretically fractionated in 10% polyacrylamide gels (separating gel, 12 cm), run at 9 mA. The proteins were electrophoretically transferred to nitrocellulose filters (50 V, 5 hr, 23°C) in a buffer containing 4 g of $NaDodSO_4$, 57.65 g of glycine, 12.1 g of Tris (base), and 800 ml of methanol per 4 liters. After transfer, the filters were washed with 50% 2-propanol in water (5–10 min, 100 ml per strip), rinsed extensively with distilled water, and washed for 1 hr with 10 mM Tris·HCl/150 mM NaCl/0.1% Tween 20, pH 7.3. To renature the proteins, the filters were then treated for a minimum of 18 hr with buffer A (15 mM Tris·HCl/150 mM NaCl/2 mM $MgCl_2$/1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride/0.1% Tween 20/0.1% gelatin, pH 7.3, at 23°C). At the end of this incubation, the filters were bathed for 1 hr in fresh buffer and then incubated with buffer A containing the appropriate tracers as specified in the figure legends (3 hr, 23°C). Finally, the filters were washed six times with ≈900 ml of buffer A over a 2-hr period. After drying, they were autoradiographed on Kodak XAR film for 3–15 hr at −70°C.

Other Procedures. Electrophoresis was according to Laemmli (21) and protein determinations were made according to Lowry *et al.* (22) and Fenner *et al.* (23).

RESULTS

Quantitative Aspects of Binding. Previous studies (15) had established that upon prolonged thrombic digestion, the IF protein desmin (M_r 53,000) could be converted into a smaller derivative (T-desmin) that lacks the ability to polymerize and contains only the helical middle domain and the C-terminal tail of the original molecule. Under isotonic salt conditions and at neutral pH this preparation is fairly homogeneous and consists of tetramers with subunit M_r 45,000. To assess the membrane-binding activity of this fragment, ^{125}I-labeled T-desmin was combined with plasma membranes or nuclear envelopes depleted of endogenous IF (electrophoretic profiles shown in Fig. 1*C*) and the binding was measured by a sedimentation assay (11).

When T-desmin was coincubated with turkey erythrocyte plasma membranes, no significant specific binding was detected (Fig. 1*B*, lower curve). However, when nuclear

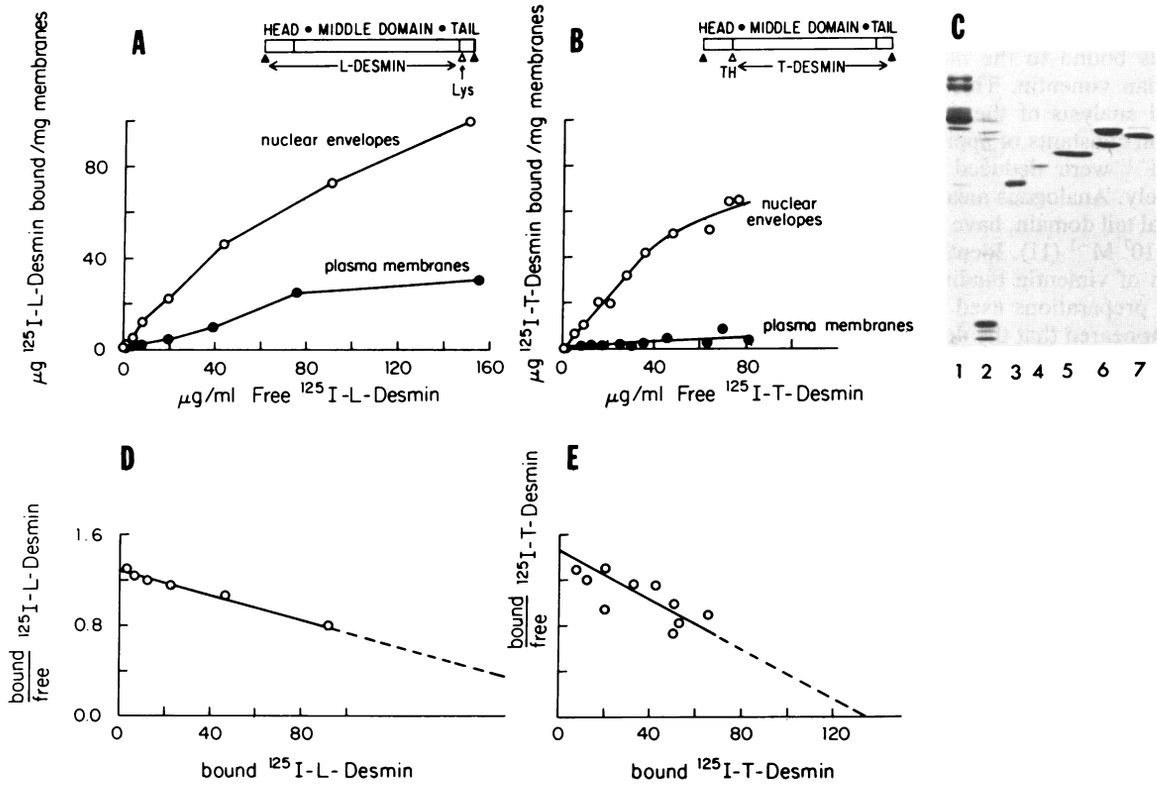

FIG. 1. Binding of chicken desmin fragments to plasma membranes and nuclear envelopes from turkey erythrocytes. (*A*) Binding of ^{125}I-labeled L-desmin to plasma membranes (●) or nuclear envelopes (○). The assay was executed as specified in *Materials and Methods* with iodinated desmin fragments at final specific activities of 2000–5500 cpm/μg, at 50 mM NaCl. (*B*) As in *A*, but with ^{125}I-labeled T-desmin (specific activity 4000 cpm/μg) at 150 mM NaCl. In both cases the nonspecific binding was subtracted. (*C*) Electrophoretic profiles of plasma membranes (lane 1), nuclear envelopes (lane 2), T-desmin (lane 3), L-desmin (lane 4), vimentin (lane 5), rat liver lamins A and C (lane 6), and rat liver lamin B (lane 7) used in the assays. Proteins were visualized by Coomassie blue staining. (*D* and *E*) Scatchard plots of the binding of L-desmin and T-desmin (respectively) to nuclear envelopes. The schemes in *A* and *B* represent the structural features of the two desmin derivatives. The site (Lys) where desmin is cleaved by the lysine-specific protease to produce L-desmin and the thrombin site (TH) along the desmin molecule where cleavage generates T-desmin are indicated. The major structural domains (head, middle, tail) of desmin are also indicated.

envelopes from the same cells were substituted for the plasma membranes, a significant quantity of the tracer was found to partition with the membrane pellets. Quantitative assessment of the binding revealed a concentration-dependent, saturable association (Fig. 1*B*, upper curve). In essence, T-desmin appeared to behave like some vimentin peptides that lack parts of or the entire head domain and thereby are incompetent to polymerize or associate with the ankyrin molecule (10, 14). On the other hand, T-desmin shared with a 6.6-kDa vimentin fragment (containing exclusively its tail domain) the ability to bind to nuclear envelopes in a saturable manner (11).

Because these results were consistent with the notion that desmin subunits, like vimentin subunits, may utilize opposite end-domains to interact with the nuclear envelope and the plasma membrane, we used another derivative of desmin in order to map the active sites along the desmin molecule more precisely. A proteolytic product was prepared by digesting isolated desmin with a lysine-specific protease, yielding L-desmin, which contains an intact N-terminal domain, the entire middle domain, and part of the tail domain, missing only 27 residues from the C-terminus. L-desmin is polymerization-competent and behaves like intact desmin under hypotonic salt conditions (50 mM salt). At physiological ionic strength it can form bundled filaments (15).

When binding was assessed using radiolabeled L-desmin (at 50 mM NaCl), a saturable association with the plasma membranes was observed (Fig. 1*A*, lower curve). The same probe bound also to nuclear envelopes in a concentration-dependent manner (Fig. 1*A*, upper curve).

Despite the qualitative similarities in the behavior of desmin and vimentin, there were some noteworthy differences in their binding to the nuclear envelope. Both desmin derivatives bound to the nuclear envelopes with a lower affinity than vimentin. This was particularly evident after Scatchard analysis of the data (Fig. 1*D* and *E*), whereby association constants of approximately $2 \times 10^6\ M^{-1}$ and $1.2 \times 10^6\ M^{-1}$ were deduced for L-desmin and T-desmin, respectively. Analogous measurements with vimentin, or its C-terminal tail domain, have yielded values ranging from 10^7 to $2.1 \times 10^7\ M^{-1}$ (11). Identical values were obtained upon repetition of vimentin binding assays with the salt-washed envelope preparations used in this study (data not shown). Thus, it appeared that the desmin–envelope association was substantially weaker than the vimentin–envelope interaction.

Another difference in the quantitative features of the binding concerned the issue of cooperativity: in previous experiments it was shown that the primary interaction of the vimentin tail with its nuclear binding site was characterized by a pronounced positive cooperativity (11). This was not observed in the case of desmin, as evidenced by the shape of the Scatchard plots (Fig. 1*D* and *E*).

Identification of Binding Sites. To investigate the nature of the desmin binding sites at the nuclear envelope, as well as their relationship to the vimentin receptor, various amounts of unlabeled vimentin were coincubated with the iodinated desmin fragments and nuclear envelopes, and the displacement of the tracers was quantitated. As seen in Fig. 2*A*, vimentin was capable of inhibiting most of the T-desmin binding (the same happened with L-desmin, although higher amounts of unlabeled vimentin were required to achieve equivalent displacement; data not shown). These data suggested that the desmin derivatives and vimentin were binding to the same receptor sites at the nuclear envelope. Nevertheless, when Dixon analysis (24) was attempted, to check whether the inhibition we had observed was of a purely competitive mode, it was realized that, apart from a direct vimentin/desmin competition for the same site, the two proteins must have reacted also with each other (Fig. 2*B*). This was not unexpected, since independent studies (25) had shown that desmin and vimentin could copolymerize *in vitro* into heteropolymeric forms. However, the uncertainty associated with these interpretations dictated additional analysis in order to demonstrate direct interactions.

A lamin B-agarose column was used to examine whether the desmin peptides, like vimentin, could bind directly to lamin B. Both T- and L-desmin were quantitatively retained by the column and subsequently eluted with 7 M urea (Fig. 2*C*).

To confirm these results we decided to develop an overlay assay for examining the direct associations of both desmin and vimentin with their putative membrane receptors. High-specific-activity ^{125}I-labeled T-desmin, L-desmin, vimentin, or the desmin middle domain (obtained after cleaving T-desmin with chymotrypsin) were prepared and used to probe blotted electrophorograms of plasma membranes and nuclear envelopes that contained some endogenous vimentin serving as an internal marker. As shown in Fig. 3 (lanes E), L-desmin, T-desmin, and intact exogenous vimentin bound to lamin B and to endogenous vimentin contained in the nuclear enve-

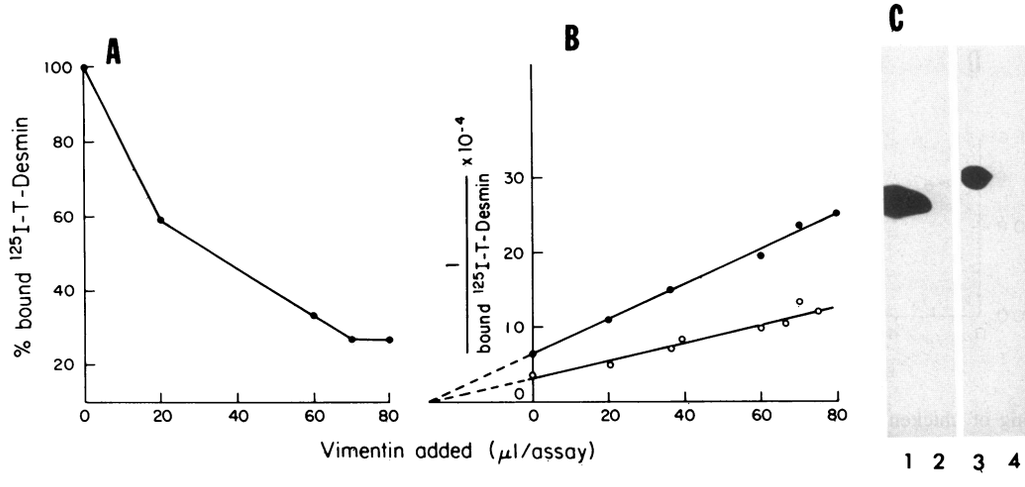

FIG. 2. (*A*) Displacement of ^{125}I-labeled T-desmin from nuclear envelopes by an excess of unlabeled vimentin. The assay mixture (100 μl) contained 0.2 μg of ^{125}I-labeled T-desmin (specific activity 80,000 cpm/μg) and 15 μg of nuclear envelopes. Vimentin was added as a 150-μg/ml solution. (*B*) Two assays similar to the one shown in *A*, with ^{125}I-labeled T-desmin at 1.5 μg/ml (●) or 4.0 μg/ml (○). Data are plotted according to Dixon (24). (*C*) Binding of the two desmin derivatives to isolated lamin B as detected by affinity chromatography. For details see ref. 11. Lanes: 1, bound ^{125}I-labeled T-desmin; 2, column flow-through (not bound); 3, bound ^{125}I-labeled L-desmin; 4, column flow-through.

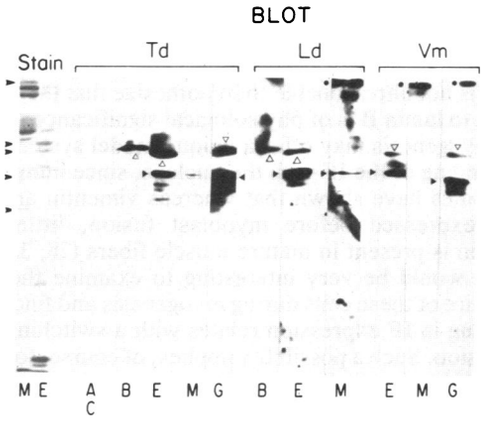

FIG. 3. Detection of desmin- and vimentin-binding polypeptides by an overlay assay. Isolated rat liver lamins A and C (lane A/C), lamin B (lanes B), nuclear envelopes (lanes E), plasma membranes (lanes M), or whole erythrocyte membrane "ghosts" (lanes G) were electrophoresed and blotted as described in *Materials and Methods*. The blots were then incubated with iodinated T-desmin (61,000 cpm/μg, group Td), iodinated L-desmin (48,000 cpm/μg, group Ld), or iodinated vimentin (89,000 cpm/μg, group Vm). The positions of lamin B (open arrowheads), vimentin (solid arrowheads), and ankyrin (solid circles) are indicated. The bands seen below vimentin represent some of its degradation products that are also detected by specific antibodies. On the left, Coomassie blue-stained gels of plasma membranes (M) and nuclear envelopes (E) are depicted. Solid arrowheads indicate (from top to bottom) the positions of erythrocyte ankyrin, lamin A, lamin B, vimentin, and actin.

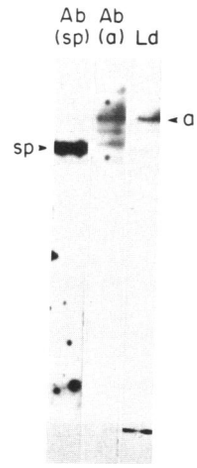

FIG. 4. Binding of anti-α-spectrin [lane Ab-(sp)], anti-ankyrin [lane Ab-(a)], or L-desmin (lane Ld) to plasma membrane preparations. The positions of α spectrin (sp) and ankyrin (a) are indicated by arrowheads. Immunoblotting was done as described (11), and the solid-phase binding assay with L-desmin was done as in *Materials and Methods*.

lope fractions. Lamin A, or other constitutive proteins of the nuclear envelopes, did not react with these tracers. Vimentin and L-desmin, but not T-desmin, bound to a band comigrating with turkey erythrocyte ankyrin in plasma membrane blots (Fig. 3, lanes M). L- and T-desmin were able to bind to isolated rat liver lamin B (Fig. 3, lanes B), as well as to isolated bovine lens vimentin (data not shown). Spectrin, actin, and proteins 4.1 and 3, all present in the plasma membrane fractions, did not associate with any of the probes (compare staining pattern and blot patterns in Fig. 3). We noticed also that the iodinated probes reacted with a minor band slightly heavier than lamin B (for example, lanes E) that did not correspond to lamin A, as judged by immunoblotting. The same minor component can be detected in certain fractions of purified rat liver lamin B (Fig. 1*C*, lane 7) and in immunoblots of rat nuclear envelopes (20). Therefore, this polypeptide may represent a minor isotype of lamin B that also possesses a vimentin-binding activity. To ensure that the detected reactivities were not due to the removal of other, perhaps more interesting, "receptors" during subcellular fractionation, we tested whole "ghost" preparations from which no proteins other than the soluble cytoplasmic ones had been removed. As seen in Fig. 3 (lanes G), intact vimentin was able to recognize ankyrin, lamin B, and endogenous vimentin, while T-desmin bound only to the latter two. No additional reactivities were detected in the ghost preparations.

To show that the desmin fragments were recognizing exactly the same proteins that constituted the vimentin receptors, we employed immunochemical approaches in combination with our overlay assays. When blots containing plasma membrane fractions were tested, a polyclonal anti-ankyrin antibody recognized the same band that reacted with L-desmin, while an antibody against the α subunit of erythroid spectrin crossreacted with a polypeptide migrating below it (Fig. 4). Thus we were unable to confirm, at least by this technique, a previous report of a direct desmin–spectrin association (26).

Finally, when urea-extracted proteins from nuclear envelopes (fractionated by ion-exchange chromatography and PAGE; Fig. 5, DEAE) were tested, using anti-lamin B antibodies (Anti-B), it was found that, like exogenous vimentin, L- and T-desmin recognized always a band that corresponded to the lamin B antigen (Fig. 5, Vm, Td, and Ld). Variable binding to endogenous vimentin (coeluted with lamin B under these conditions) was also detected, but no binding to lamin A was apparent. In sharp contrast, when the radiolabeled middle domain of desmin was applied to the same fractions there was no detectable binding (Fig. 5, Rd).

DISCUSSION

Applying several biochemical criteria, we have shown that the IF protein desmin, which is specifically expressed in myogenic cells, interacts with the same membrane receptors as vimentin, another IF subunit, expressed only in mesenchyma-derived tissues (27, 28). We deduced that desmin and vimentin (11) utilize analogous domains to associate with the same nuclear-envelope and plasma-membrane attachment sites because (*i*) elimination of the N-terminal head domains abolishes binding to the plasma membrane in both cases; (*ii*) the middle domains do not possess any binding potential; (*iii*) fragments containing the appropriate domains but missing other parts of the molecule retain their characteristic functional properties (that is, recognition of lamin B and ankyrin, respectively); (*iv*) purified vimentin displaces the desmin derivatives from the nuclear envelope; and (*v*) direct protein–protein associations of desmin and vimentin with the same membrane components can be demonstrated *in vitro*.

Using a solid-phase overlay assay, we obtained also some hints concerning the desmin–vimentin interactions: not only did desmin and vimentin seem able to bind to each other; in addition, they appeared to interact in a site-specific fashion. For example, after cleavage of vimentin with 2-nitro-5-thiocyanobenzoic acid (8), it was found that only its N-terminal peptide associated with T-desmin (S.G., unpublished data). Therefore, in the desmin–vimentin "hybrid," some type of site-specific interactions must be important. Furthermore, cleavage of the lamin B molecule at cysteine residues abolished vimentin and desmin binding to all of the lamin B fragments larger than 15 kDa, as previously described (11).

The conservation of such binding activities across species (birds, mammals) and across tissues (muscle, erythrocytes)

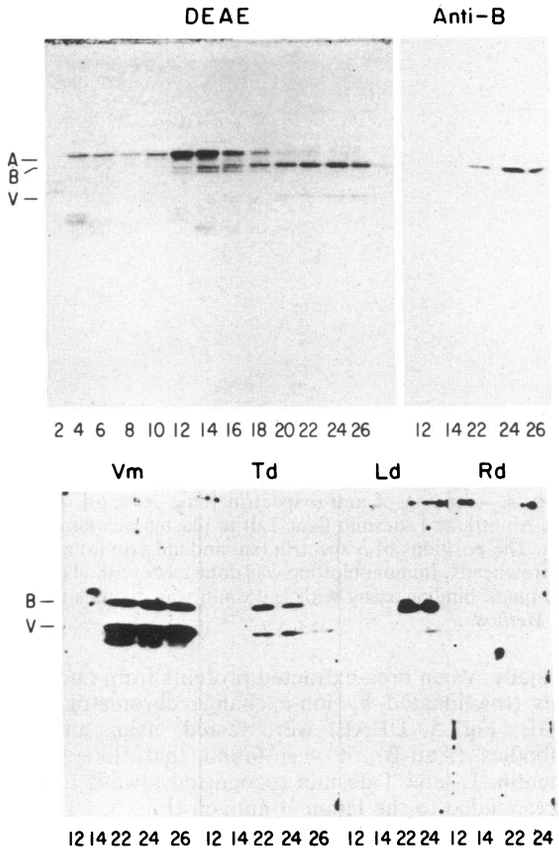

FIG. 5. Binding of desmin fragments and vimentin to fractionated polypeptides obtained from urea extracts of nuclear envelopes. Nuclear envelopes were prepared, extracted with 8 M urea, and fractionated by ion-exchange chromatography (11). Panel DEAE shows the electrophoretic profile of the Coomassie blue-stained polypeptides in the various fractions as indicated below the lanes. Panel Anti-B shows an autoradiogram of an immunoblot of the indicated fractions with a lamin B-specific antibody. Panels Vm, Td, Ld, and Rd represent autoradiograms of blots probed with labeled vimentin, T-desmin, L-desmin, and the desmin rod domain, respectively. Markers at left show positions of lamins A and B and of vimentin (V) contained in each column fraction. The bands comigrating with lamin B in fractions 12–16 (panel DEAE) are degradation products of lamin A, as indicated by immunoblotting (data not shown).

suggests that the functional polarity of the filament subunits must be of fundamental importance for the cellular physiology. At this point it is not clear that the functional polarity in IF–membrane interactions implies necessarily a structural polarity of the filaments *per se*. However, a polar arrangement of the filamentous matrix might be important for its function if IF were used as "tracks" for transporting ribonucleoprotein particles or proteins from the nucleus to the cell surface and vice versa. However, such a general role of this system would conflict with the observation that several cultured cell lines can grow normally without apparently expressing any of the known types of IF (29–31).

The different affinities that were detected in the associations of erythrocyte lamin B with the desmin and vimentin molecules may imply the occurrence of a distinct lamin isotype in muscle cells that is more specialized in recognizing desmin rather than vimentin. Morphological studies on striated muscle cells (32, 33) revealed a close spatial association of Z-disc 10-nm filaments with some focal areas on the nuclear surface where scalloping of the nuclear contour is observed together with a greater concentration of nuclear pores. On the other hand, cells that coexpress desmin and vimentin, as for example BHK-21 cells, possess perinuclear IF composed of both desmin and vimentin (19, 28). Therefore, it is not unreasonable to hypothesize that the binding of desmin to lamin B is of physiological significance. In fact, *in vitro* myogenesis may offer a unique model system to study the coupling of the IF with the nucleus, since immunochemical studies have shown that whereas vimentin and desmin are coexpressed before myoblast fusion, little (if any) vimentin is present in mature muscle fibers (28, 33). Therefore, it would be very interesting to examine the lamin B repertoire of these cells during myogenesis and find out if the switching in IF expression relates with a switching in lamin expression. Such a possibility applies, of course, to other cell types and in particular to neurons, where the expression of neurofilament components is developmentally regulated (18).

Note Added in Proof. We have recently synthesized a desmin peptide extending from the end of the rod domain (residue 415) to residue 444. This 29-mer, which contains approximately half the carboxyl-terminal "tail" of desmin, recognizes specifically lamin B but not lamins A and C.

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