Filensin: A New Vimentin-binding, Polymerization-competent, and Membrane-associated Protein of the Lens Fiber Cell

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Abstract. We have studied the molecular properties of a 100-kD protein, termed filensin, which we have isolated from porcine lens membranes. Filensin represents a membrane-associated element, resistent to salt and nonionic detergent treatment, and extractable only by alkali or high concentrations of urea. By indirect immunofluorescence and immunoelectron microscopy, this protein can be localized at the periphery of the lens fiber cells. Immunochemical analysis suggests that filensin originates from a larger 110-kD component which is abundantly expressed in lens but not in other tissues. Purified filensin polymerizes in a salt-dependent fashion and forms irregular fibrils (~10 nm in diameter) when reconstituted into buffers of physiological ionic strength and neutral pH. Radiolabeled filensin binds specifically to lens vimentin under isotonic conditions, as demonstrated by affinity chromatography and ligand-blotting assays. By the latter approach, filensin also reacts with a 47-kD peripheral membrane protein of the lens cells. Purified filensin binds to PI, a synthetic peptide modelled after a segment of the COOH-terminal domain of peripherin (a type III intermediate filament protein highly homologous to vimentin), but not to various other peptides including the NH_2-terminal headpiece of vimentin and derivatives of its middle (rod) domain. The filensin-PI binding is inhibited by purified lamin B, which is known to interact in vitro with PI (Djabali, K., M.-M. Portier, F. Gros, G. Blobel, and S. D. Georgatos. 1991. *Cell.* 64:109–121). Finally, limited proteolysis indicates that the filensin–vimentin interaction involves a 30-kD segment of the filensin molecule. Based on these observations, we postulate that the lens fiber cells express a polymerization-competent protein which is tightly associated with the plasma membrane and has the potential to serve as an anchorage site for vimentin intermediate filaments.

BECAUSE of its "extreme" specialization, the eye lens has been a useful experimental system for exploring the structure and function of intercellular junctions and for studying the programs of tissue-specific gene expression (for a comprehensive review see Bloemendal, 1982). One particular cell type in this tissue, the lens fiber cell (LFC),[1] also provides unique opportunities to study the interactions between the plasma membrane and the cytoskeleton.

LFCs originate from the lens epithelium, a population of ectoderm-derived cells that cover the surface of the lens (only the anterior aspect of it in the mature organ). During embryonic development, the posterior cells of the lens vesicle elongate to form the primary lens fibers, which gradually fill the cavity of the vesicle. Later, and throughout the adult life of an animal, epithelial cells located near the equator of the lens differentiate into secondary, tubular fibers which add up to the primary lens fibers. Accumulating LFCs finally form an "onion-like" structure which can be divided into a "cortex" (around the periphery of the lens, beneath the epithelium) and a "nucleus" (at the very center of the organ). The process of epithelial cell differentiation is paralleled by profound changes in the intracellular architecture of the LFCs. The cell nucleus disappears, RNA synthesis ceases, while the cells develop an extensive system of specialized junctional complexes. Remarkably, despite the slowing down of the cellular machinery, the LFCs remain functional for decades, maintaining a high degree of intracellular order and making efficient use of the available metabolic resources (Maisel et al., 1981).

The mature LFC can be considered analogous to the anucleate and terminally differentiated erythrocyte. In fact, most of the typical components of the erythrocyte membrane-skeleton (spectrin, actin, protein 4.1, ankyrin, band 3, etc.) also occur in the lens (Allen et al., 1987; Aster et al., 1984*a*, *b*, 1986). However, unlike the erythrocyte, the LFC also contains an extensive *trans*-cytoplasmic skeleton that comprises microfilaments, intermediate filaments (IFs), and microtubules (Benedetti et al., 1981; Ramaekers and Bloemendal, 1981). Because nucleus-associated structures have been implicated in the organization of cytoskeletal elements (Eckert et al., 1982; Georgatos and Blobel, 1987*a*,*b*; Goldman et al., 1985; Katsuma et al., 1987; Vikstrom et al., 1989; Wang, 1985; for a discussion see also Albers and Fuchs, 1987, 1989), one suspects that the LFC plasma membrane

1. *Abbreviations used in this paper*: BF, beaded filament; IF, intermediate filament; LFC, lens fiber cell.

may be uniquely equipped with some compensating factors that modulate the cytoskeleton when the nucleus disappears. The latter notion becomes particularly interesting in the light of recent studies that have claimed the existence of karyoskeletal protein analogues (such as lamin B) in specialized plasma membrane domains of epithelial and muscular cells (Cartaud et al., 1989, 1990), as well as the occurrence of a similar protein in the LFC membrane (Dunia et al., 1990).

To systematically examine the molecular interactions that are responsible for the arrangement of the cytoskeletal elements in the absence of a cell nucleus, we have studied the properties of a 100-kD polypeptide which appears to be strongly associated with the LFC membrane. We show here that this 100-kD protein possesses a polymer-forming ability and binds specifically to vimentin. We have named this protein filensin, because it forms filament-like aggregates and is apparently lens specific.

Materials and Methods

Cell Fractionation

Porcine eyes were obtained from a local slaughterhouse. The lenses were removed and washed three times with ice-cold TKM-buffer (Ramaekers et al., 1982; 50 mM Tris-HCl, pH 8.0, 25 mM KCl, 5 mM $MgCl_2$) containing 1 mM DTT, 1 mM PMSF, and 2 μg/ml leupeptin. After homogenization in a Waring blender at 4°C, the homogenate was spun first at 1,000 *g* for 10 min at 4°C to remove debris and contaminating nuclei and then or 30 min at 4°C at 12,000–38,000 *g* to pellet the lens membranes. After centrifugation, the membrane pellet was washed twice with cold TKM-buffer, resuspended in 20 mM Tris-HCl, pH 8.0, 0.9% NaCl, 1 mM PMSF (0°C) and spun in the same fashion. In some of the experiments, the membranes (in TKM buffer) were further purified by sucrose step-gradient centrifugation, as previously described (Ramaekers et al., 1982). To prepare the cytosolic and the "microsomal" fractions, a postmembrane (38,000 *g*, 30 min) supernatant was further centrifuged at 356,000 *g* for 30 min (k factor = 12) and the resulting pellet and supernatant fractions were collected separately. For investigating the partitioning of filensin upon chemical extraction of the lens membranes, aliquots of washed membranes were resuspended in 8 vol of one of the following buffers: (*a*) 0.9% NaCl (wt/vol), 25 mM Tris-HCl, pH 7.3, 2 mM $MgCl_2$, 1 mM PMSF, and 0.5% (vol/vol) Triton-X-100 (Triton buffer): (*b*) 3–6% NaCl (wt/vol), 25 mM Tris-HCl, pH 7.3, 2 mM $MgCl_2$, and 1 mM PMSF (high salt buffer): (*c*) 10 mM Tris-HCl, pH 7.3, 2 mM EDTA, and 1 mM PMSF (low salt buffer): (*d*) 7–8 M urea, 10 mM Tris-HCl, pH 7.3, and 1 mM PMSF (urea buffer); and (*e*) 0.1 N NaOH (alkali solution). After 10 min at room temperature, the samples were spun at 356,000 *g* for 35 min at 18°C. Portions of the supernatants (concentrated by acid precipitation) and the solubilized pellets were analyzed by SDS-PAGE.

Protein Chemical Procedures

Isolation of Filensin. A lens membrane pellet (see above) was extracted for 2 h at room temperature with 10 vol of 7 M urea in 10 mM Tris-HCl, pH 7.6, 2 mM EDTA, 1 mM DTT, and 0.5 mM PMSF. After centrifugation at 12,000 *g* for 20 min (18°C), the supernatant was collected and mixed with diaminoethyl cellulose (DE52; Whatman, Maidstone/Kent, UK) equilibrated in the above buffer. The resin was pelleted, washed with 25 vol of urea buffer, resuspended in a small amount of buffer, and loaded onto a column. Bound proteins were eluted with a linear gradient of 0–150 mM NaCl (in the same urea buffer). Fractions containing the 100-kD protein were identified by SDS-PAGE, pooled, and dialyzed against 7 M urea, 10 mM NaPi, pH 7.6, 1 mM DTT, and 0.5 mM PMSF. This material was loaded on a hydroxylapatite column (Bio-Rad Laboratories, CA). Vimentin was recovered in the flowthrough and subsequent wash with 15 ml of the urea/phosphate buffer. The 100-kD protein was eluted with a linear gradient of 10–60 mM Na-phosphate.

Isolation of IF Proteins. Lens vimentin was purified as specified above. Nuclear lamins were prepared from rat liver as described by Georgatos and Blobel (1987*b*). Vimentin NH_2-terminal domain fragments were a gift from P. Kouklis (European Molecular Biology Laboratory). The fusion protein termed "vimentin rod" that is shown in Fig. 8 contains vimentin sequences 245–366 and was prepared by P. Kouklis (Kouklis, 1990). The synthetic peptides used in this study were prepared in the biopolymer facility of The Rockefeller University (New York, NY) and correspond to residues 405–434, 432–461 of rat peripherin (PI and PII, respectively) and to residues 1–32 of human lamins A/C (LI).

Radiolabeling. Labeling of purified vimentin and filensin with the ^{125}I-Bolton-Hunter reagent was performed as previously described by Georgatos et al. (1985).

Immunological and Immunochemical Procedures

To generate polyclonal antibodies, column fractions containing the 100-kD protein were electrophoresed on preparative 10% SDS-polyacrylamide gels. The 100-kD bands were cut out, homogenized in PBS, and injected with complete Freund's adjuvant (Sigma Chemical Co., St. Louis, MO) in the thigh lymph nodes and subcutaneously in rabbits. After boosting in weeks 3 and 5, blood was collected in week 7 after immunization. Affinity-purified antibodies were produced by adsorption of the antiserum against the 110- or 100-kD proteins immobilized on nitrocellulose after SDS-PAGE and electrotransfer. The antibodies were eluted with 200 mM glycine-HCl, 500 mM NaCl, pH 2.3, and rapidly neutralized with Na_2HPO_4.

Light and Electron Microscopy

Porcine eye lenses were embedded in Tissue-Tek (Miles Laboratories Inc., Elkhart, IN) and manually sectioned in a cryostat. The sections were fixed in 3.5% formaldehyde solution in PBS. After quenching with 50 mM NH_4Cl and extraction with 0.15% Triton X-100, the sections were incubated with anti-filensin antibodies, diluted 1:100. As a secondary antibody, FITC-coupled goat-antirabbit antibody (Cappel, Organon Teknika, West Chester, USA) was used. The sections were examined in a modular confocal microscope (MCM, developed and constructed by E. H. K. Stelzer et al. at the EMBL, Heidelberg, Germany). Immunoelectron microscopy was performed on ultrathin frozen sections of porcine lenses, fixed overnight in 8% paraformaldehyde in 250 mM Hepes, pH 7.4, and processed as specified by Griffiths et al. (1984). The sections were decorated with antifilensin antibodies diluted 1:50 and protein A coupled to 9-nm gold. EM of isolated lens plasma membranes was performed on ultrathin-sectioned, epon-embedded material according to Bloemendal et al. (1972). The assembly of the 100-kD protein into filaments was investigated by dialyzing samples of purified filensin from a 7 M urea solution against one of the following buffers: (*a*) 170 mM KCl, 20 mM Tris, 0.1 mM EGTA, 1 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF, pH 7.4; (*b*) 130 mM KCl, 25 mM MES, 1 mM $CaCl_2$, 1 mM DTT, 0.5 mM PMSF, pH 6.5; (*c*) 2 mM Tris-HCl, 1 mM DTT, 0.5 mM PMSF, pH 7.4. Dialysis was performed overnight at 4°C and subsequently for 3 h at 37°C. Negative staining was performed on carbon-coated copper grids, using 1.5% uranyl acetate or 0.75% uranyl formate. Filensin filaments were decorated on the grid with antifilensin antibodies and protein A coupled to 5-nm gold. For rotary shadowing the dialyzed protein solution was mixed with glycerol (at a final concentration of 30%) and sprayed onto freshly cleaved mica. The samples were further processed according to Fowler and Aebi (1983).

Assays

Ligand blotting assays were based on previously reported protocols (Djabali et al., 1991; Georgatos et al., 1987) and were executed as follows: 1 mm-thick 10% SDS-polyacrylamide gels were made using Bio-Rad (Bio-Rad Laboratories) ultrapure electrophoresis reagents. Gels were left to "age" overnight at 4°C. Proteins were resolved in this system using a constant current of 25 mAmps and a cooling device. Electroblotting involved transfer onto nitrocellulose membranes (35 V, 2 h, room temperature) in a buffer containing 57.65 g glycine, 12.1 g Tris (base), 4.0 g SDS, and 800 ml 100% methanol per 4 liters of solution. Blots were washed three times with "washing buffer" (0.9% (wt/vol) NaCl, 20 mM Tris-HCl, pH 7.3, 0.1% Tween-20) and incubated at either 4°C, or at room temperature with "gelatin buffer" (0.9% [wt/vol] NaCl, 20 mM Tris-HCl, pH 7.3, 1 mM $MgCl_2$, 1 mM DTT, 0.1% Tween-20, 0.2% [wt/vol] boiled fish gelatin, and 0.2 mM PMSF). Probing of the blots was executed at various temperatures in the gelatin buffer that contained ^{125}I-filensin or ^{125}I-vimentin. The blots were washed four to five times with 25 ml of "gelatin buffer" over a period of 60 min before air drying and further processing for autoradiography. Dot-blots were done as specified previously (Djabali et al., 1991). Affinity chromatography was performed as described by Georgatos and Blobel (1987*b*).

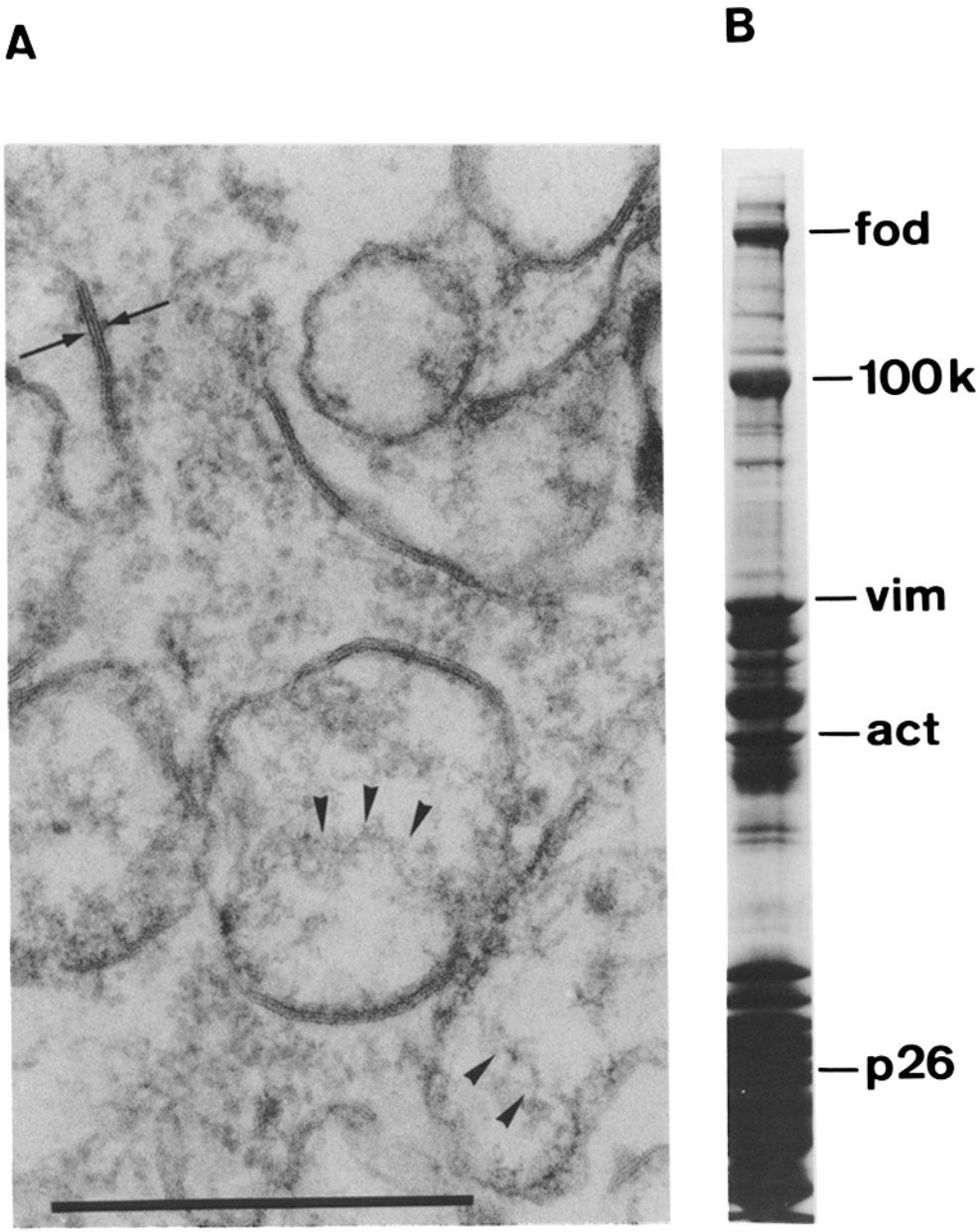


Figure 1. Characterization of membrane–cytoskeleton complexes from porcine lens. (*A*) An electron microscopic profile of an ultrathin section of epon-embedded lens membranes prepared as described under Materials and Methods. The arrowheads point to "fuzzy" fibrillar material which is attached to the membranes. Penta-layer structures, representing junctional complexes between lens fiber cells, are often found in these preparations (*arrows*). (*B*) SDS-PAGE profile of a sample similar to the one shown in (*A*). Several of the major bands have been previously characterized as fodrin (*fod*) (Aster et al., 1984*b*), vimentin (*vim*), actin (*act*), and as the major intrinsic protein of the lens junctional complexes, p26 (for a review see Bloemendal, 1981). The position of filensin (*100k*) is indicated. Bar, 0.5 μm.

Results

Previous studies have shown that membrane–cytoskeleton complexes can be isolated from mammalian lenses using a rapid fractionation procedure (Ramaekers et al., 1982). We employed a slightly modified version of this method to prepare such fractions from porcine lens (EM profile shown in Fig. 1 *A*) and used them as a source of factors that may be involved in the coupling of the cytoskeleton to the plasma membrane of the lens cells.

The SDS-PAGE profile of the membrane–cytoskeleton complexes, shown in Fig. 1 *B*, reveals several major bands that have already been characterized in previous studies (see legend to Fig. 1 *B*). Taking into account that some of these components also occur in other (nucleated) cell types, we focused our attention on a characteristic lens protein with an apparent M_r of 100 kD, which we termed filensin. To proceed, we prepared two different polyclonal antibodies against column-purified filensin (see below) and studied its properties by biochemical and immunochemical means.

Filensin Constitutes a Peripheral Membrane Protein of the LFC

To study the properties of the membrane-associated filensin, we extracted lens membranes under various conditions and examined the partitioning of the 100-kD polypeptide. Fig. 2 *A* shows that Triton-X-100, high salt, or low salt/EDTA extractions do not affect filensin, whereas 7–8 M urea extraction completely solubilizes this protein. Extraction with alkali (0.1 N NaOH) also removes all of the filensin from the plasma membranes (data not shown). By immunoblotting, the antifilensin antibodies decorate, in addition to the 100-kD band, a 54-kD band. Minor reactivities are seen at 110, 68, 51, and 40 kD (Fig. 2 *B*). The lower molecular mass bands (especially the 54/51 kD doublet) represent reproduci-

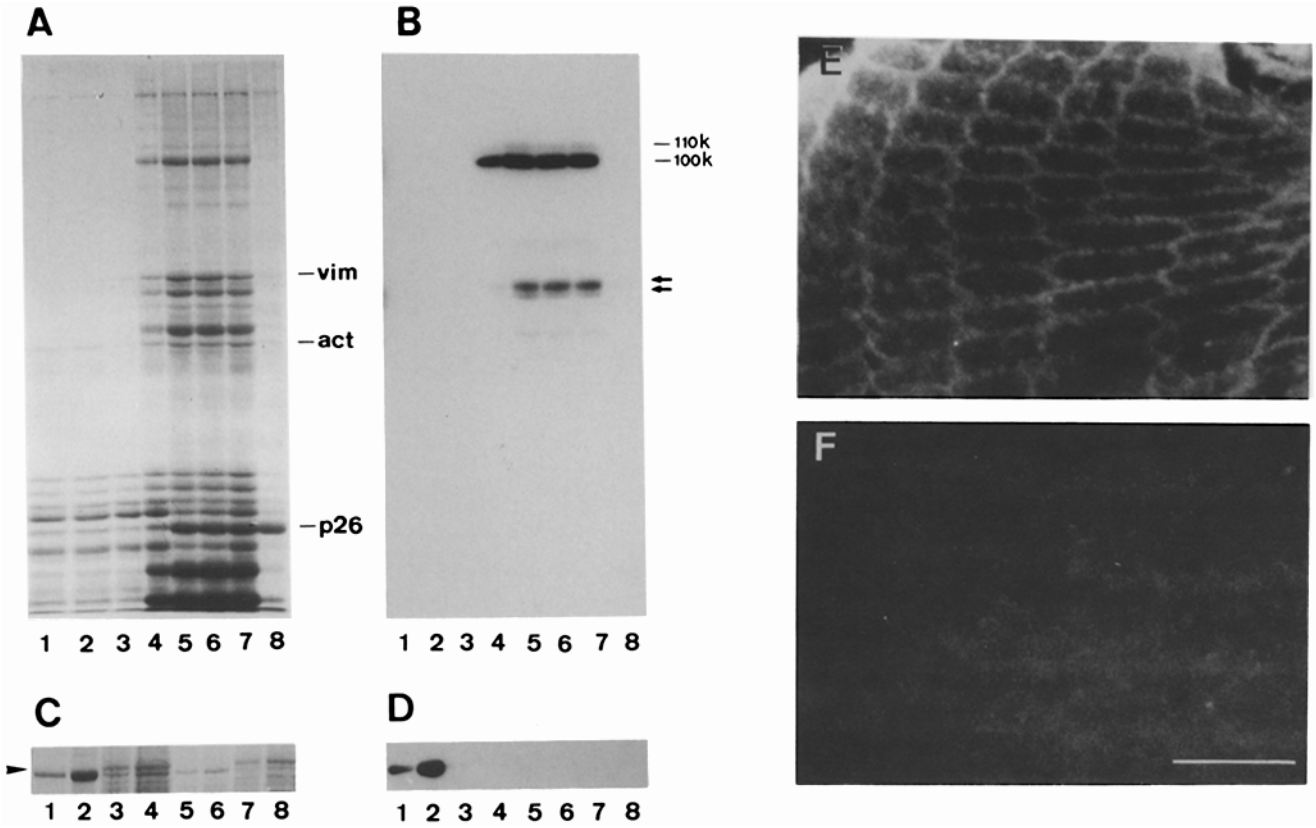
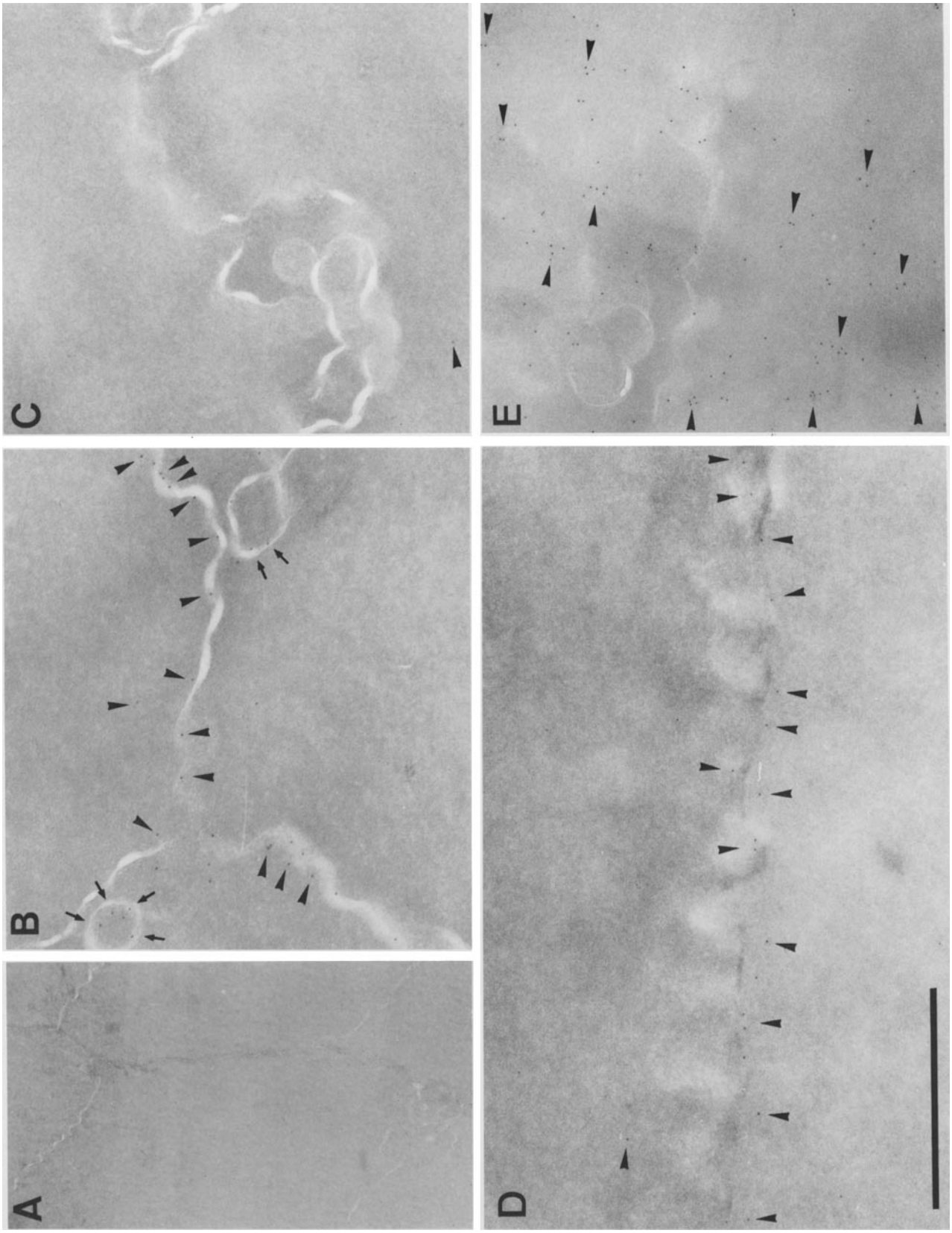


Figure 2. Filensin is a peripheral membrane protein of the lens fiber cells. *A* and *B* show a Coomassie blue–stained gel and a corresponding immunoblot of lens membranes, extracted with various solvents and fractionated by centrifugation into a supernatant (extract) and a pellet (membranous) fraction (as specified in Materials and Methods): extraction with Triton X-100 (supernatant: lane *1*, pellet: lane *5*); extraction with high-salt buffer (sup.: lane *2*, pellet: lane *6*); extraction with low-salt buffer (sup.: lane *3*, pellet: lane *7*); extraction with urea buffer (sup.: lane *4*, pellet: lane *8*). The immunoblot (*B*), probed with a polyclonal antibody (diluted 1:600) against the 100-kD protein, shows that filensin in solubilized by urea extraction (lane *4*), but not by high/low salt, or detergent treatment. Double arrow indicates the 51/54-kD degradation products of filensin. (*C* and *D*) Subcellular fractionation of pig lens (for details see Materials and Methods). After homogenization, lens material was subjected to differential centrifugation to generate a crude membrane pellet (lanes *1* and *2*), and a postmembrane supernatant (lanes *3* and *4*). The postmembrane supernatant was further centrifuged, yielding a "microsomal" pellet (lanes *5* and *6*) and a cytosolic supernatant (lanes *7* and *8*). A Coomassie blue–stained gel (*A*) and an immunoblot (*B*) of these fractions are shown (only the area of ~100 kD is depicted here; for more detail see Fig. 3). Even-numbered lanes contain double the amount of protein of the odd-numbered lanes. The immunoblot (*D*), probed with the antiserum against filensin (diluted 1:400), shows a strong reactivity at 100 kD in the lens membrane pellet (lanes *1* and *2*, *arrowhead*), whereas the other fractions lack any significant reactivity. Negative staining of each individual fraction shows the presence of membranes in the first pellet only (A. Merdes and S. D. Georgatos, unpublished observations). (*E* and *F*) Cryostat sections of a pig lens decorated by the polyclonal antifilensin antibodies. (*E*) is a confocal immunofluorescence micrograph of a lens specimen in cross-section, decorated by immune serum and showing immunostaining around the hexahedral outline of the LFCs. (*F*) is a similar micrograph showing the absence of immunostaining with the preimmune serum. Bar, 10 μm.

bly obtained degradation products of filensin and increase upon storage or prolonged handling of the preparation, whereas the 110-kD species appears to constitute the original form of the filensin molecule (see below).

To examine the intracellular distribution of filensin, we continued with subcellular fractionation experiments. When a lens homogenate is fractionated into a crude plasma membrane fraction and a postmembrane supernatant fraction, the

Figure 3. Filensin can be localized at the lens fiber cell membrane by immunoelectron microscopy. (*A*) An overview of an ultrathin frozen cross-section of lens fiber cells is shown. (*B*) At higher magnification, a cross-section of the same specimen, probed with antifilensin antibodies and gold labeled protein A, shows the almost exclusive location of filensin along the inner aspect of the plasma membrane (*arrowheads*). "Ball-and-socket structures" at the contact sites between neighboring cells are also heavily decorated (*arrows*). The borders between neighboring cell membranes are artifactually broken in some regions during sample preparations. (*C*) A cross-section similar to the one shown in *B* probed with the preimmune serum. (*D*) A longitudinal section of lens fiber cells, probed with the antifilensin antibodies, as in *B*. In this orientation, filensin can be detected almost exclusively in the neighborhood of the plasma membrane (*arrowheads*). (*E*) A longitudinal section of lens fiber cells probed with a polyclonal antibody against vimentin and decorated with protein A-gold. Vimentin is localized throughout the cytoplasm of the LFCs (*arrowheads*). Bar, 1 μm.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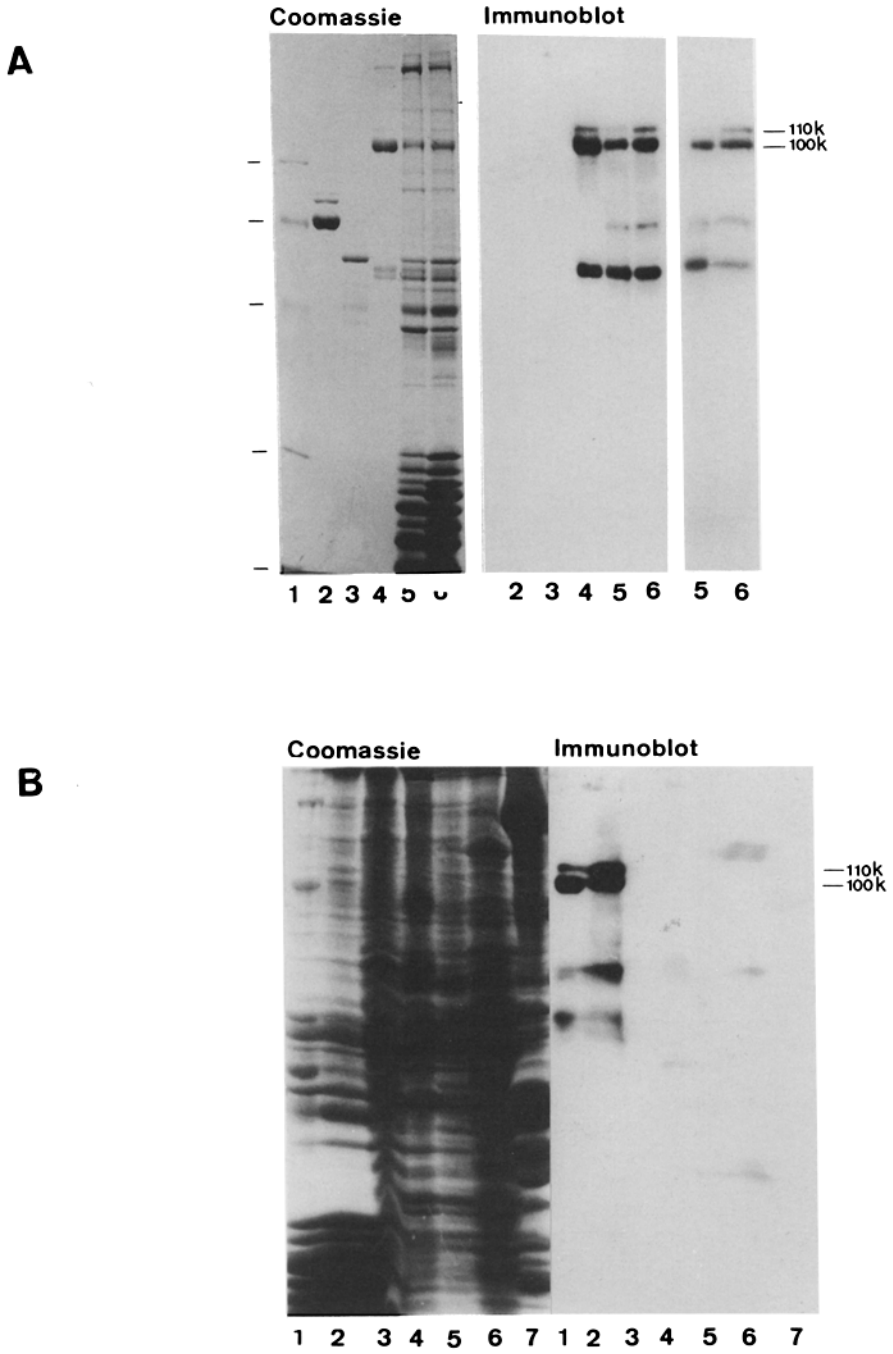

Figure 4. Filensin originates from a 100-kD protein specifically expressed in lens. (*A*) Fractions of purified lamin B from rat liver (lane *2*), pig lens vimentin (lane *3*), partially purified filensin (lane *4*), a urea extract of lens membranes (lane *5*), and washed lens membranes (lane *6*) were separated on a 10% SDS-polyacrylamide gel and electrotransferred onto nitrocellulose filters. The nitrocellulose strips were probed with the affinity-purified antifilensin antibodies after absorbing the immune serum against either the 100-kD band (*left strip*), or the 100-kDa band (*right strip*, lanes *5* and *6* only). Both antibodies show identical reactivities with the 110- and 100-kD bands, as well as with degradation products, mainly at 54 and 51 kD. (lane *1*) Molecular mass markers of 97.4, 66.2, 45.0, 31.0, and 21.5 kD. (*B*) A membrane preparation from pig lens (lane *1*) as well as SDS-boiled samples from whole pig lens (lane *2*), pig brain (lane *3*), pig spleen (lane *4*), pig kidney (lane *5*), pig liver (lane *6*), and pig diaphragm muscle (*7*) were separated by SDS-PAGE, transferred to nitrocellulose filters, and probed with the antifilensin antibodies (diluted 1:500). Filensin is exclusively detected in the lens samples. Lens material immediately boiled in SDS shows a higher ratio of 110/100 kD polypeptides (lane *2*), compared to the washed lens membranes (lane *1*).

main 100-kD cross-reactive component is almost exclusively detected in the membrane pellets (Fig. 2 *D*, lanes *1* and *2*). Interestingly, examination of Coomassie blue–staining gels of such fractions reveals two other bands, with apparent M_r values of ~100 kD, which occur in the postmembrane supernatant (Fig. 2 *C*, lanes *3* and *4*). One of these proteins appears to be exclusively cytosolic, whereas the other one can be pelleted by further centrifugation of the post-membrane supernatant (with sedimentation forces that will clear particles >24S). Since none of these two components cross-react to any significant extend (i.e., 3-d exposure of the autoradiograms) with the antifilensin antibodies (Fig. 2 *D*, lanes *5–8*), we conclude that they represent species distinct from the 100-kD filensin antigen.

To localize the filensin molecules in he LFC, we performed indirect immunofluorescence, decorating cryostat sections of porcine lens with the antifilensin antibodies. By confocal microscopy, the filensin antigen appears to be concentrated at the periphery of the hexahedral LFC (Fig. 2 *E*). This decoration is specific because preimmune sera do not show such a staining pattern (Fig. 2 *F*). Indirect immunofluorescence on longitudinal sections of pig lens also shows that filensin is scarce in the lens epithelium (data not shown).

To resolve the intracellular distribution of filensin in more detail, we also performed immunoelectron microscopy on ultrathin frozen sections. To prevent any artifactual randomization of the antigen position during sample processing, we avoided detergent extraction of the sections and exposure to

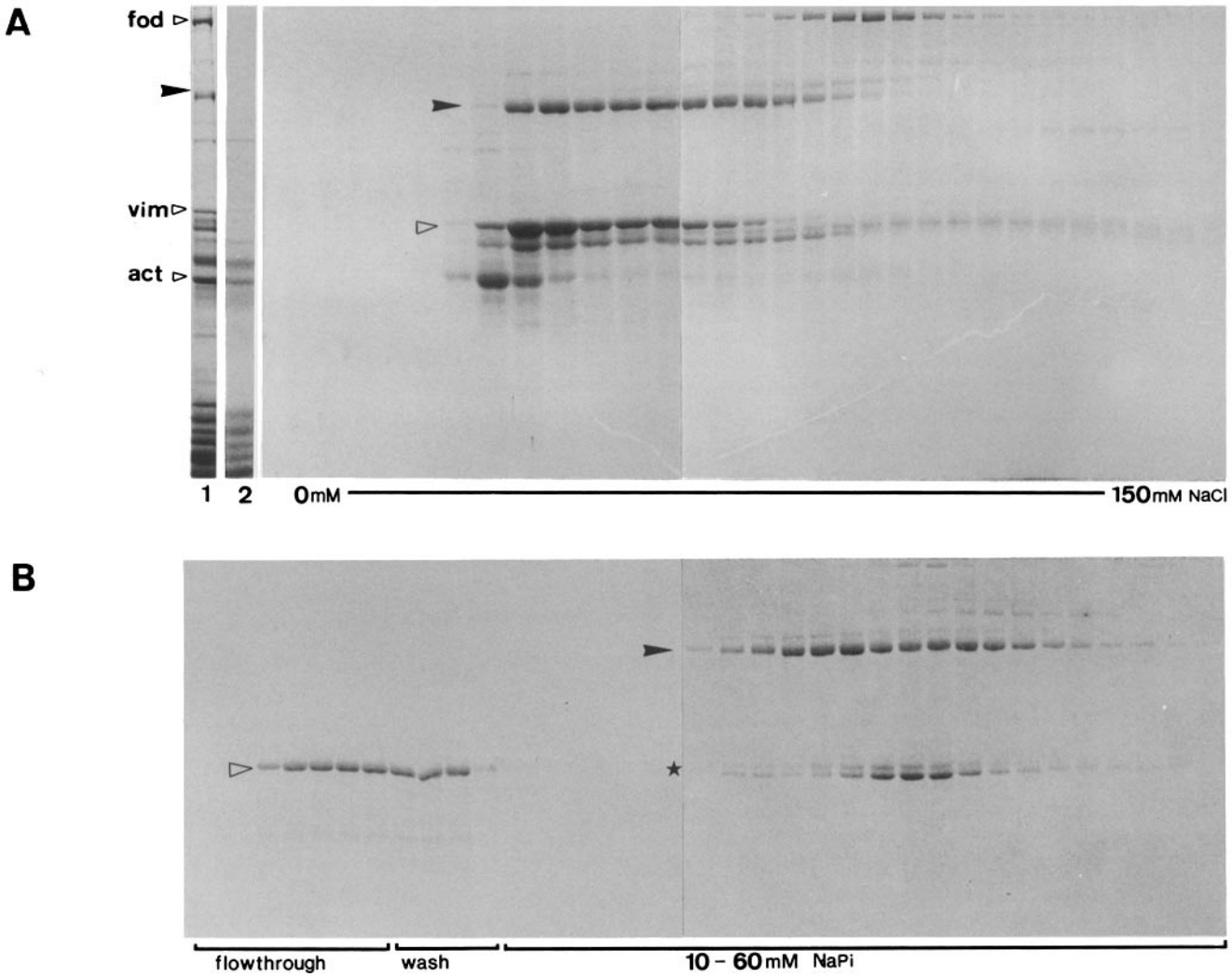

Figure 5. Purification of filensin from pig lens membranes. (*A*) Urea extract from lens membranes (lane *1*) was loaded onto a DE 52 column. The nonbound material was collected (lane *2*), and the resin was first washed with low-salt buffer and then eluted with a linear salt gradient (for details see Materials and Methods). Filensin is eluted at ~40 mM NaCl (*closed arrowhead*) together with vimentin (*open arrowhead*). In lane 1, the electrophoretic mobilities of the following proteins are indicated: fodrin (*fod*), filensin (*closed arrowhead*), vimentin (*vim*), and actin (*act*). (*B*) Fractions from the DE 52 column containing filensin were further chromatographed on a hydroxylapatite column. Vimentin (*open arrowhead*) is recovered in the flowthrough and wash fractions. Filensin (*closed arrowhead*) and its degradation products (*double band* at 54/51 kD, indicated by an *asterisk*) are eluted by a NaPi gradient. For assembly and binding studies, purified filensin of the first half of the gradient (showing no substantial contamination) was used.

antibodies before fixation (see Discussion), although filensin is not extracted by Triton-X100 (Fig. 2 *A*). As depicted in Fig. 3 (*B* and *D*), in paraformaldehyde-fixed sections of whole porcine lens, the antifilensin antibodies decorate very heavily the LFC membrane, whereas no decoration is detected in the cytoplasmic region (at any specimen orientation). No "clustering" or "segmental" distribution of the antigen can be seen in the decorated sections. A similar sample probed with preimmune serum shows the complete absence of immunostaining (Fig. 3 *C*). To confirm the exclusively peripheral localization of filensin and assess the accessibility of cytoplasmic antigens to antibody probes under our sample preparation conditions, we also performed immunoelectron microscopical surveys with antivimentin antibodies. These experiments reveal a heavy cytoplasmic decoration of the lens sections, although the vimentin filaments are not directly visualized (Fig. 3 *E*). Thus, the specific membrane localization of the filensin antigen is not due to "masking" of potential cytoplasmic sites by other structures. Taken together with the biochemical data, our morphological observations strongly support that filensin constitutes a typical peripheral membrane protein of the LFC.

Filensin is Derived from a 110-kD Polypeptide Which Is Specifically Expressed in Lens

To investigate more closely the relationship between filensin and the 110-kD band that cross-reacts with the antifilensin antibodies, we affinity purified the antibodies against the 110- or the 100-kD bands. Results shown in Fig. 4 *A* demonstrate that the antibodies eluted from the 110- or the 100-kD polypeptides give indistinguishable immunostaining patterns (cross-reaction with the main 100-kD band and the characteristic ladder of degradation products), strongly suggesting

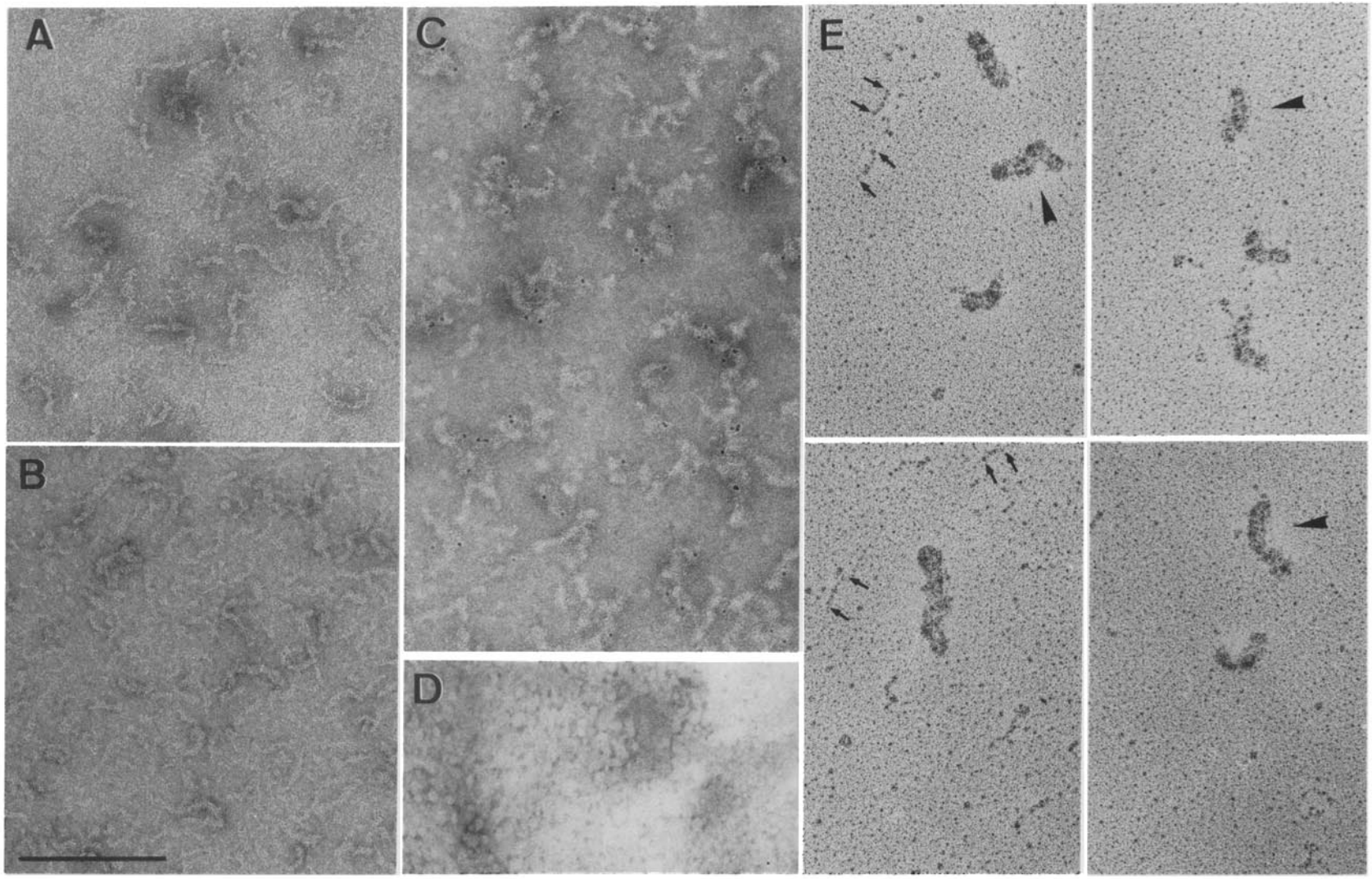

Figure 6. Filensin assembles into short 10-nm filaments under in vitro conditions. (*A*) Negatively stained filaments of porcine filensin, assembled by dialyzing a preparation (~200 μg/ml of purified protein) against isotonic buffer at neutral pH (for details see Materials and Methods). (*B*) A 100-kD protein from bovine lens (very homologous to filensin by protein sequencing), purified according to the same protocol as described for the porcine protein, reconstituted (at ~400 μg/ml) and visualized as in *A*. Note that filensin from different sources yields similar 10 nm fibrils. (*C*) Purified porcine filensin filaments assembled as in *A* and labeled with antifilensin antibodies and protein A, coupled to 5-nm gold. (*D*) Porcine filensin reconstituted under low ionic strength conditions and visualized as in *A*. Notice the absence of filamentous material. (*E*) Porcine filensin filaments visualized by rotary shadowing (*arrowheads*). Note that thinner thread-like structures (*arrows*) are also detected by this technique. These may represent filensin assembly intermediates. Bar, 0.2 μm.

that the filensin molecule is a fragment of the 110-kD protein. Consistently, immunoprecipitation of newly made proteins from in vitro translated lens RNA yields only one high molecular weight band (F. Gounari and S. D. Georgatos, unpublished observation). This latter polypeptide appears to constitute the original form of the 100-kD protein which is proteolytically cleaved during membrane isolation and sample processing.

To substantiate this interpretation, and to examine the occurrence of filensin in other tissues, we prepared tissue specimens under conditions known to minimize proteolysis (immediate boiling in SDS in the presence of protease inhibitors). As it could be seen in Fig. 4 *B*, the relative ratio between the 110- and the 100-kD bands is significantly increased, the 54-kD immunoreactive band is less pronounced, and the 68-kD band is more apparent, if the sample is processed in the above manner. Given that the 100-kD band appears even in samples immediately boiled in SDS, it is probable that the proteolysis of the 110-kD protein occurs in vivo and it is only facilitated by the in vitro manipulations. Microsequencing experiments show that the 100-kD polypeptide has a blocked NH_2-terminus. Therefore, the cleavage of the 110-kD protein to the 100-kD species should involve COOH-terminal "trimming" of the molecule.

The antifilensin antibodies do not decorate any component similar to filensin in overloaded blots containing material from five different porcine tissues (Fig. 4 *B*). A similar result is obtained by indirect immunofluorescence screening of cryostat sections of various porcine organs (not shown). On the basis of these data, and although a low degree of filensin expression in some other tissue can not be formally excluded, we conclude that filensin is a lens-specific protein.

Filensin Assembles Into Filaments

To isolate filensin, we used as a starting material 7 M urea extracts of washed lens membranes. The urea-solubilized proteins were further fractionated by ion-exchange chromatography in DEAE-cellulose, as depicted in Fig. 5 *A*. We noticed that filensin behaves as an acidic protein, being quantitatively retained by the DEAE-cellulose column and coeluting, together with a number of other proteins, at ~40 mM NaCl (Fig. 5 *A*, *arrowhead*). Subsequent chromatographic separation in hydroxylapatite columns, purifies filensin to near homogeneity (Fig. 5 *B*), the most persistent contaminant being a minor doublet migrating slightly faster than lens vimentin (consult also Fig. 4 *A*, lanes *3* and *4*). Because we could show that these minor bands are in fact degradation products of the main 100 kD polypeptide and species distinct from vimentin (see Fig. 4 *A*, lanes *3* and *4*), we did not proceed in further purification. We emphasize here that the material used for assembly and binding studies was free of non-filensin proteins (containing only traces of the 51/54-kD bands) and was routinely checked by immunoblotting to rule out even trace contamination with vimentin. However, the influence of the 51/54-kD filensin degradation products on the assembly and the binding properties of filensin is presently unknown.

Sedimentation experiments with purified filensin suggested that it may self-associate and form higher order structures (data not shown). To investigate the aggregation properties of filensin in a systematic fashion, purified material from porcine or bovine lens was dialyzed from a 7 M urea solution into different electrolyte buffers. The dialyzed preparations were then processed for negative staining and rotary shadowing. Negative staining shows that the filensin molecules incubated at isotonic salt and neutral pH form short fibrils that have a "rough" surface (Fig. 6, *A* and *B*). The diameter of these aggregates is ~10 nm and their average length (at the concentrations tested) is ~80 nm. Anti-filensin antibodies specifically decorate these structures, documenting that they consist of filensin and do not arise from a minor contaminant present in the preparation (Fig. 6 *C*). Consistently, antivimentin antibodies do not decorate the filensin fibrils (data not shown). The filensin polymers seem to closely resemble the short 10-nm fibrils formed by purified neurofilament-M protein under similar in vitro conditions (Troncoso et al., 1989). However, no such structures can be detected at low ionic strength (Fig. 6 *D*). At slightly acidic pH and in the presence of Ca^{++}, the filensin fibrils become "contracted" (not shown), indicating a subtle dependence of the assembly process on $[Ca^{++}]$ and $[H^+]$. Rotary shadowing on samples dialyzed against isotonic salt at neutral pH confirms the existence of fibrils in the filensin preparations, and also reveals less abundant thread-like forms with a much smaller diameter that may correspond to filensin assembly intermediates (Fig. 6 *E*). From these data we conclude that filensin is a polymerization-competent protein and can aggregate in a salt-dependent fashion.

Filensin Behaves as a Vimentin-binding Protein

To examine the potential interactions between filensin and other cytoskeletal elements, we radiolabeled purified filensin and probed the entire complement of the urea-soluble and urea-insoluble proteins of the LFC membrane using a previously established ligand-blotting method (Georgatos et al., 1987). Fig. 7 *A* shows that ^{125}I-filensin reacts strongly with two other proteins: lens vimentin and a urea-soluble 47-kD polypeptide (distinct from actin) of unknown identity. Binding of ^{125}I-filensin to endogenous filensin is (under such conditions) very weak. This is probably due to the low protein concentrations that are used in such ligand-blotting assays (~1 μg/ml versus 200 μg/ml that are used for assembly experiments), or to partial inactivation of filensin by the Bolton-Hunter reagent. The first explanation is favoured by data we have obtained in affinity chromatography experiments (M. Brunkener and S. D. Georgatos, unpublished observations). The reactions we have detected by ligand blotting appear to be fairly specific because binding of ^{125}I-filensin to vimentin and to the 47-kD protein is significantly reduced by an excess of nonlabeled filensin (Fig. 7 *B*). The specificity of the detected interactions is further supported by the fact that other prominent bands on the same blots (as for example the p26 component present in the urea-insoluble fraction and several very abundant crystallins) do not react with the radioactive tracer.

In a different version of the same experiment, we also probed purified filensin, in parallel with rat liver lamins A/B and C, with ^{125}I-vimentin. Fig. 7 *C* shows that under physiological (i.e., isotonic) conditions. ^{125}I-vimentin binds avidly to filensin. Strong binding of ^{125}I-vimentin to lamin B (positive control), but only background labeling of lamins A and C (negative control), is also detected, as it has been previ-

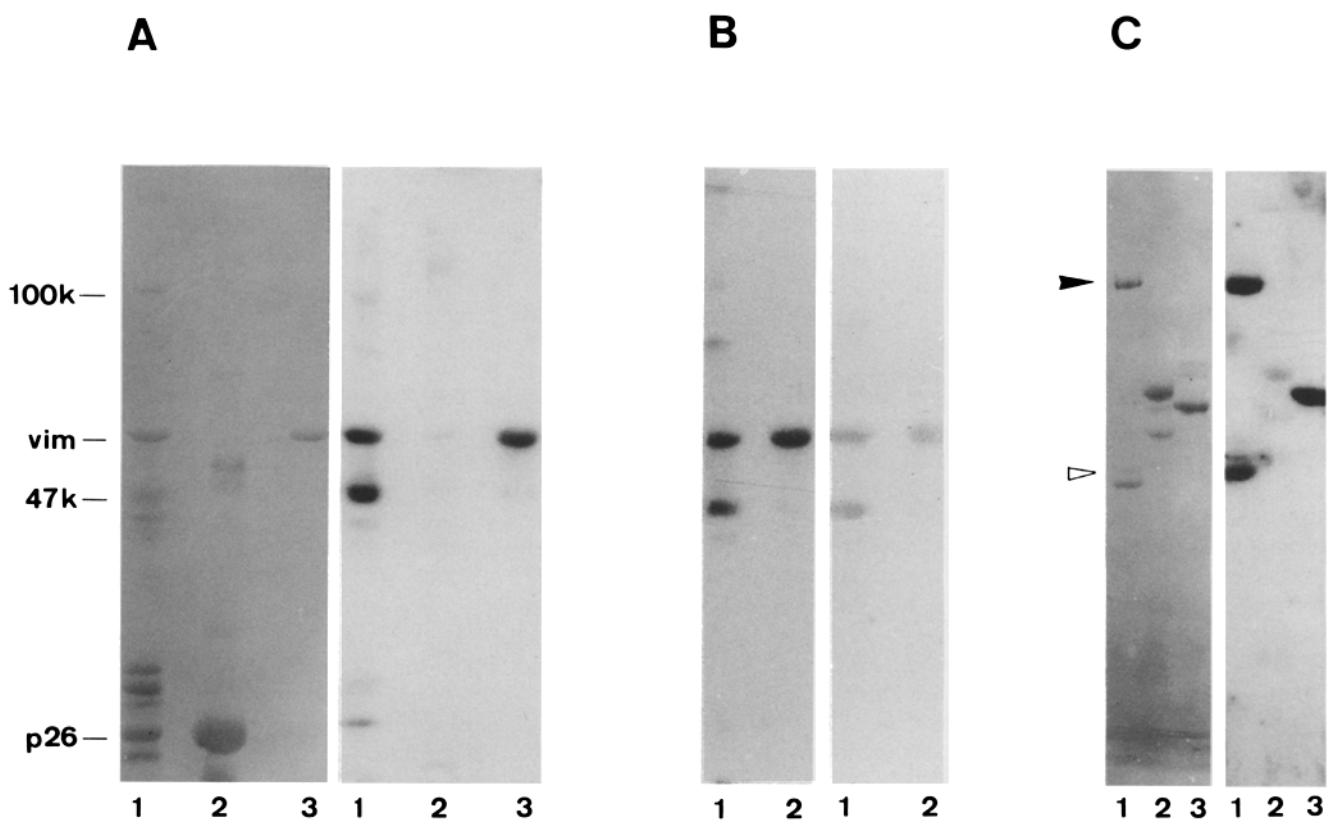

Figure 7. Binding of filensin to lens vimentin can be detected by a ligand-blotting assay. (*A*) Binding of ^{125}I-filensin to lens proteins. Polypeptides of the urea extract of lens membranes (lanes *1*), the urea insoluble fraction of lens membranes (lanes *2*, consult also Fig. 2) and purified lens vimentin (lanes *3*) were resolved by SDS-PAGE, transferred to nitrocellulose and incubated with ^{125}I-filensin (163,000 cpm/ml; 1 μg/ml) for 15 h at 4°C, as specified under Materials and Methods. The blot on the left was mock-incubated and then stained with Coomassie blue (0.1% Coomassie brilliant blue for 2–5 min at room temperature), whereas the replica blot on the right was incubated with radioactive filensin and then autoradiographed. Note the reaction with vimentin (*vim*) in lanes *1* and *3*, the 47-kD protein (*47k*) in lane *1*, and the lack of a reaction with the major intrinsic protein of the lens membranes (*p26*) in lane 2. (*B*) Competition between labeled and unlabeled filensin for binding to vimentin and the 47-kD protein. Replica blots of a lens membrane urea extract (lanes *1*) and purified lens vimentin (lanes *2*) were assayed as in A either with ^{125}I-filensin alone (*left*), or with a mixture of ^{125}I-filensin and unlabeled filensin (~1:10; *right*). (*C*) Binding of ^{125}I-vimentin to filensin. A fraction of partially purified filensin (lanes *1*), purified rat liver lamins A/C (lanes *2*), and purified lamin B (lanes *3*) were run on 10% SDS-polyacrylamide gels, transferred to nitrocellulose filters, and probed with ^{125}I-vimentin (659,000 cpm/ml; 1 μg/ml) for 2 h at room temperature, as specified in Materials and Methods. The blot on the left was mock-incubated and then stained with Coomassie blue, whereas the replica blot on the right was incubated with radiolabeled vimentin and then autoradiographed. Note the reaction of ^{125}I-vimentin with filensin (*closed arrowhead*) and its 54/51 kD degradation products (*open arrowhead*), with lamin B (positive control) and the background reaction with lamins A/C (negative control).

ously reported (Djabali et al., 1991; Georgatos et al., 1987). This experiment confirms that the binding of filensin to vimentin that we have described in Fig. 7 *A* is not due to a trace contamination of the filensin preparation by vimentin. The filensin–vimentin interaction appears to be of a nonelectrostatic nature because binding is abolished when the assay is performed under low-salt conditions (not shown).

Finally, to confirm these results by another method, we combined a trace quantity of ^{125}I-filensin with an excess of an unrelated protein (BSA) and applied this mixture to a vimentin-agarose affinity column. Fig. 8 (A and B) shows that, under these conditions, more than 70% of the ^{125}I-filensin is retained in the vimentin matrix, whereas the bulk of the BSA is recovered in the flowthrough.

Binding of Vimentin to Filensin is Site Specific

To further characterize the filensin–vimentin interaction, we tested various other IF proteins, synthetic peptides and fragments of vimentin, using a dot-blot method that involves incubation of the substrates with unlabeled filensin and anti-filensin antibodies (see also Djabali et al., 1991). We found that filensin binds specifically to a synthetic peptide (PI) representing the proximal half of the COOH-terminal domain of rat peripherin (a protein similar to vimentin, desmin, and gliol fibrillary acidic protein; see Djabali et al., 1991). Filensin does not bind significantly to other synthetic peptides, lamin B, or fragments of the middle (rod) and the NH_2-terminal domains of vimentin (Fig. 9 *A*, lanes *1* and *2*). The PI-filensin interaction can be competed off by soluble lamin B (Fig. 9 *A*, lane *3*), which is known to associate with PI in vitro (Djabali et al., 1991). These data suggest that filensin binds to the COOH-terminal region of type III IF proteins, near or at the lamin B–binding site.

To examine the site specificity of the filensin–vimentin association, we first performed limited proteolysis on ^{125}I-

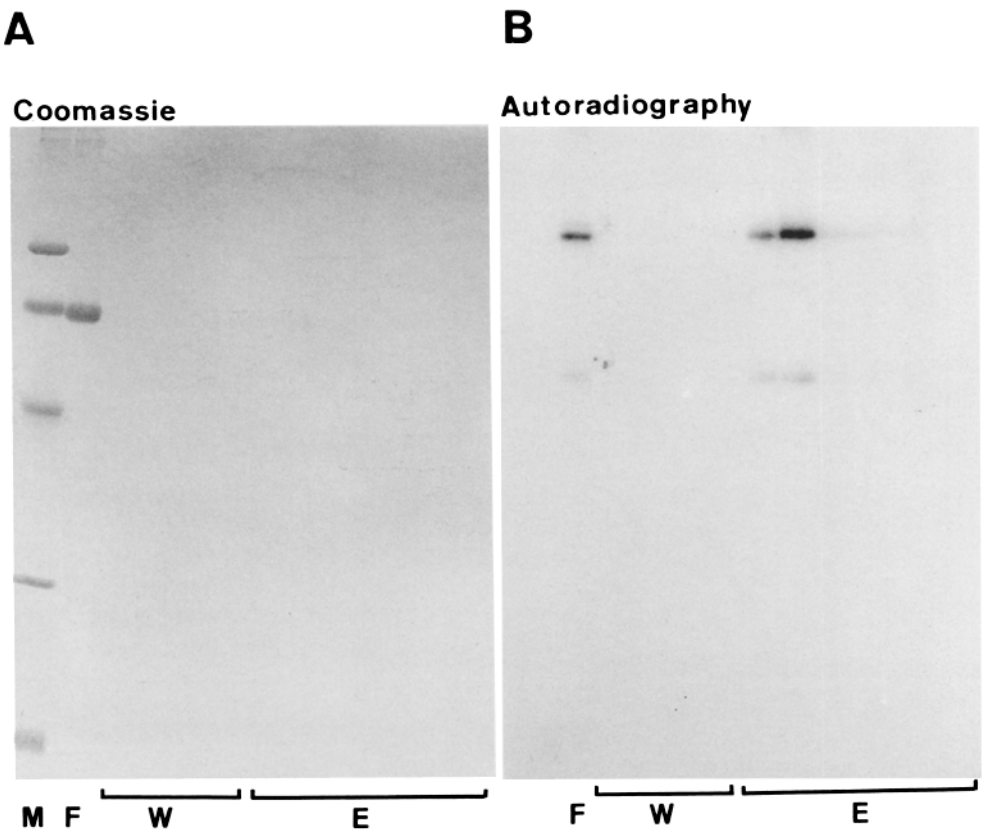

Figure 8. Binding of ^{125}I-filensin to vimentin can be detected by affinity chromatography. A vimentin-affigel-15 column (~0.2 mg of vimentin coupled /ml of resin) was constructed exactly as specified by Georgatos and Blobel (1987). 2 ml of this resin were incubated with a mixture of ^{125}I-filensin (853,000 cpm/ml; ~20 μg/ml) and BSA (500 μg/ml) reconstituted into 0.9% NaCl (wt/vol), 20 mM Tris-HCl, pH 7.3, and 0.1% Tween-20. After a 2-h incubation, the slurry was packaged into a disposable Bio-Rad column (Bio-Rad Laboratories, Richmond, CA). The flowthrough was collected and the column was washed with a total of 20 ml (10 column volumes) of the above buffer. Bound protein was eluted with 1% SDS, 7 M urea, 150 mM Tris-HCl, pH 7.4, 2 mM EDTA, 1 mM DTT, and 0.5 mM PMSF. All fractions were counted in a gamma counter to calculate the degree of binding, and then 30 μl of each fraction were analyzed by SDS-PAGE and autoradiography. *A* and *B* denote the Coomassie blue-stained gel and its corresponding autoradiogram. Lane M indicates molecular weight markers (as specified in Fig. 2 *A*), lane F corresponds to the flowthrough, lanes labeled W represent the wash, and lanes labeled E correspond to material eluted from the column. Note that the bulk of unlabeled BSA is recovered in the flowthrough, whereas most of the ^{125}I-filensin (70% of the counts) is found in the eluted material.

filensin. Data depicted in Fig. 9 *B* (left part) show that brief tryptic digestion of filensin (and its 54/51-kD degradation products) yields two groups of fragments with approximate M_r values of 50 and 30 kD, respectively. Proteolysis with lower concentrations of trypsin (Fig. 9 *B*, *right part*) yields, in addition to the 50- and 30-kD peptides, a 43-kD set of fragments. The latter appear to be cleavage intermediates that are finally degraded into the 30-kD species. Judging from the profiles of the digestion time course, our provisional interpretation is that the 50- and 43-kD peptides represent fragments with minimal or no overlap (50 kD +43 kD = 93 kD ~100 kD), originating from different parts of the filensin molecule. The fact that filensin is cleaved into groups of similar-sized fragments rather than into distinct peptide species indicates the existence of multiple and closely-spaced protease-sensitive sites that flank a protease-resistent domain.

Taking advantage of the previous observations, a 10-min tryptic digest of ^{125}I-filensin (profile shown in Fig. 9 *B*, lane *5* on the right hand side group of digests) was applied on a vimentin-affinity column and the ability of the various filensin peptides to bind to the matrix was assessed. As it could be seen in Fig. 9 *C*, vimentin binds selectively the two (presumably related) fragments with M_r values of 43 and 30 kD, whereas the 50-kD peptide is quantitatively recovered in the nonbound fraction. From these experiments, we conclude that the vimentin-binding site of filensin can be assigned to a 30-kD subfragment of this molecule.

Discussion

Is Filensin Related to Other Cytoskeletal Proteins?

We have identified a 100-kD protein and characterized it as a component of the ·LFC membrane-skeleton by immunochemical, morphological, and biochemical means. Furthermore, we have shown that this protein, filensin, is derived from a protease-sensitive 110-kD component that is abundantly expressed in lens but not in other tissues. At this point we do not know whether the proteolysis of the 110-kD species is an in vivo or an in vitro process. Due to the fact that the 110-kD protein is rapidly converted into the 100-kD form, we cannot comment here on the whole spectrum of properties that intact filensin may possess. However, since the partially proteolyzed filensin remains strongly associated with the membranes, apparently retaining some of its binding properties, it is worth discussing the molecular features of the 100-kD polypeptide.

Filensin forms short 10-nm fibrils which, at a first glance, bear a similarity to the structures assembled from isolated neurofilament M subunits (Troncoso et al., 1989). Keeping in mind that these fibrils may acquire different characteristics if assembled from the uncleaved 110-kD form, we could, nevertheless, review here some other reports also suggesting a relationship between filensin and other cytoskeletal proteins. A recent communication (Remington, S. G. 1990. *J.*

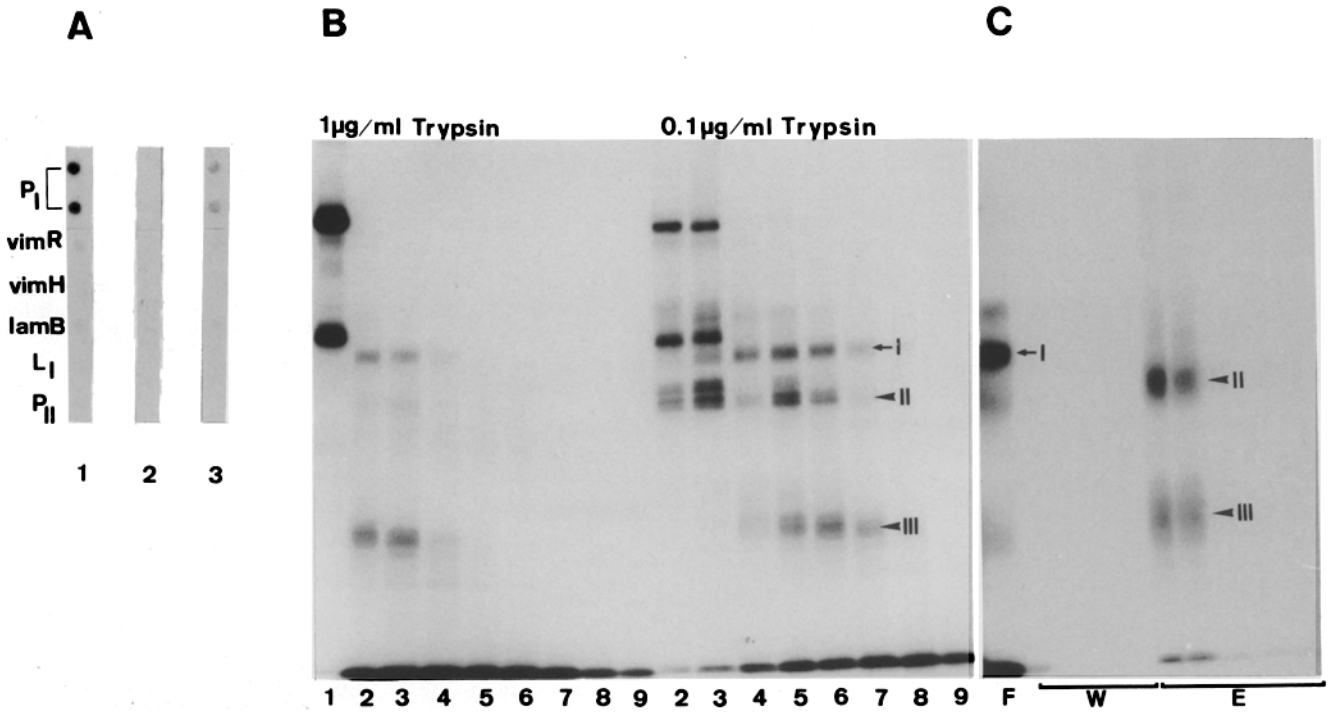

Figure 9. Binding of filensin to vimentin is site-specific. (*A*) Binding of unlabeled filensin to synthetic peptides (for designations see Materials and Methods), vimentin fragments, and lamin B using a dot-blot assay. Roughly equivalent molar amounts (1.3 nmol) of the synthetic peptides PI (*PI*), PII (*PII*), LI (*LI*), and the proteins lamin B (*lamB*), a fusion protein containing a fragment of the rod domain of vimentin (residues 245–366; "vimR"), and vimentin NH_2-terminal domain (*vimH*) were immobilized on nitrocellulose filters, as previously described (Djabali et al., 1991). After appropriate blocking, the strips were incubated with ~45 μg/ml purified filensin (track *1*), buffer alone (track *2*), or a mixture of 45 μg/ml filensin and ~50 μg/ml of purified rat liver lamin B in "gelatin buffer" (track *3*). After a 3-h incubation at room temperature, the strips were washed with the same buffer and then probed with antifilensin antibodies (diluted 1:500). Note the specific reaction of filensin with the PI peptide and the inhibition of binding in the presence of lamin B. (*B*) Limited proteolysis of ^{125}I-filensin. 12 μg of ^{125}I-filensin diluted in 80 μl of TBS were combined with 20 μl of 1 μg/ml or 0.1 μg/ml of TCPK-trypsin and incubated for 0 min (lanes *1*), 1 min (lanes *2*), 2 min (lanes *3*), 5 min (lanes *4*), 10 min (lanes *5*), 15 min (lanes *6*), 30 min (lanes *7*), 60 min (lanes *8*), and 120 min (lanes *9*) at 0°C. The reactions were stopped by adding trypsin inhibitor (10-fold excess over trypsin) and PMSF (to 1.3 mM). Portions of each digest were analyzed by SDS-PAGE on a 12% gel which was then dried and autoradiographed. Note the gradual degradation of the labeled filensin into species with molecular masses of 50 (*I*), 43 (*II*), and 30 kD (*III*). The second strong band in lane 1 represents the endogenous degradation product of filensin (51/54 kD) which increases upon storage of the preparation. (*C*) Binding of ^{125}I-filensin peptides to vimentin as detected by affinity chromatography. A 10-min tryptic digest of ^{125}I-filensin was prepared, reconstituted with an excess of BSA, and chromatographed over a vimentin-agarose column exactly as described in Fig. 8. An autoradiograph of the flowthrough (lane *F*), the wash (*W*), and the eluted (*E*) fractions is shown. Note that peptide I remains in the non-bound fraction, whereas peptides II and III bind to the vimentin column.

Cell Biol. 111:44a) has described the occurrence of a lens-specific 110-kD protein that possesses a sequence motif highly conserved among all IF subunits (in the coil 1a and coil 2b region; see Conway and Parry, 1988). Consistently, limited proteolysis of filensin generates a protease-resistent fragment with a size (50 kD) that roughly corresponds to the middle helical domains of cytoplasmic IF proteins and nuclear lamins. Whether or not this peptide conforms to an alpha-helix remains to be seen after isolation and CD analysis. Finally, the in vitro binding of vimentin to the filensin molecule (the physiological significance of which remains to be further examined) and the site specificity of this interaction suggest a "functional" relatedness between filensin and nuclear lamin B. Filensin does not colocalize with vimentin filaments in the cytoplasm of LFCs and is located at the plasma membrane, assembling into separate short fibrils by itself. Also, filensin does not appear to copolymerize with vimentin in vitro (A. Merdes and S. D. Georgatos, unpublished observations). Therefore, it is more likely that the 100-kD protein constitutes a hypothetical "attachment-site" for IFs, than an accessory component which coassembles with vimentin subunits. It will be interesting to examine whether a "lamin B-like" antigen that has recently been localized by immunoelectron microscopy in bovine lens membranes (Dunia et al., 1990) corresponds to filensin.

Having cited the favorable evidence for a relationship between filensin and IF proteins, we ought to stress, nevertheless, that a site-specific antibody that recognizes lamins A/B/C, as well as an antibody recognizing an epitope conserved among IF proteins (intermediate filament antibody epitope; Pruss et al., 1981), do not react with filensin in immunoblots. However, two anti-idiotypic antibodies recognizing lamin B react with filensin under certain conditions. To meaningfully answer these questions we are now trying to clone the cDNA coding for filensin and determine its primary structure.

Is Filensin a Component of the "Beaded Filaments"?

Polypeptides with molecular masses similar to that of filensin (95 and 115 kD) have previously been identified as constituents of the "beaded filament" (BF), a structure found exclusively in LFC (FitzGerald, 1990; FitzGerald and Gottlieb, 1989; Ireland and Maisel, 1984; Maisel and Perry, 1972). These proteins seem to colocalize and copurify with a 49-kD component that may correspond to the 47-kD filensin-binding protein that we have identified. Since filensin binding to vimentin appears to be as strong as the binding of filensin to the 47-kD protein, this raises two exciting possibilities: (*a*) that the core of the BF is built according to the principles that govern IF formation from filensin and 47-kD protein subunits; and (*b*) that the BFs associate with IFs via an interaction between a hypothetical "end-domain" of filensin and the COOH-terminal domain of vimentin subunits. The second possibility is not favored by ultrastructural data showing a complete segregation of BFs away from IFs in situ (e.g., FitzGerald, 1990).

However, any correlation between filensin and the 95/115-kD BF proteins should be made with caution because the 100-kD region of lens electrophoretograms is considerably complex and comprises several different polypeptide chains (only one of which crossreacts with the antifilensin antibodies), as indicated in the Results. It should also be taken into account that whereas BFs are clearly cytoplasmic structures, the antifilensin antibodies recognize antigens exclusively localized on the LFC periphery and not in the cytoplasmic region (at all specimen orientations).

How is Filensin Targeted and Stabilized onto the Plasma Membrane?

All pieces of evidence that we have obtained converge to the idea that filensin is strongly associated with the plasmalemma of the LFC. However, preliminary experiments suggest that the 100-kD polypeptide does not directly bind to urea-stripped membranes or to lens integral membrane proteins fractionated by SDS-PAGE and immobilized on nitrocellulose. Although the possibility of a low-affinity interaction with the lipid bilayer cannot be excluded at present, it is interesting that filensin binds specifically to another 47-kD peripheral protein of the lens membrane. The latter may therefore represent a factor responsible for the targetting and the subsequent stabilization of filensin filaments to the plasmalemma. We are currently examining this possibility, employing in vitro reconstitution assays and solution binding studies.

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