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Membrane-binding properties of filensin, a cytoskeletal protein of the lens fiber cells

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Summary

Filensin is a 100/110 kDa membrane-associated protein found in lens fiber cells. Previous studies have shown that this protein polymerizes in vitro and binds strongly to vimentin and to another 47 kDa lens membrane protein. Using cosedimentation assays, flotation assays and immunoelectron microscopy, we have examined the properties of purified filensin and measured its binding to lens membranes. Filensin behaves as a urea-extractable, hydrophilic protein which does not partition with Triton X-114 and is not affected by 1 M hydroxylamine at alkaline pH, an agent known to release fatty-acylated proteins from the membrane. Immunoblotting of urea-extracted lens membranes with two different affinity-purified antibodies reveals that, unlike intact filensin, a COOH-terminal filensin degradation product (51 kDa) remains tightly associated with the membranes. Purified filensin binds directly to urea-stripped lens membranes, but not to protein-free vesicles reconstituted from total lens lipids. The binding of filensin is not significantly influenced by the purified 47 kDa protein. Interestingly, the filensin-binding capacity of urea-extracted membranes is increased at least two-fold after trypsin treatment, which removes entirely the 51 kDa peptide from the membranes and presumably unmasks additional filensin-acceptor sites. Consistent with this, filensin binds to trypsinized and non-trypsinized membranes with similar affinities (2×10^{-7} and 4×10^{-7} M, respectively). Treatment of the membranes with thrombin, which also eliminates the 51 kDa peptide, does not increase their binding capacity, apparently because filensin-acceptor sites are also destroyed during proteolysis. Finally, heat-treatment of the trypsinized membranes, or digestion of urea-stripped membranes with α-chymotrypsin and V8 protease, affect filensin binding to a variable degree. Based on these data, we conclude that filensin and its COOH-terminal proteolytic product have the potential to directly associate with intrinsic elements of the lens cell membrane.

Key words: binding assays, filensin, flotation, proteolysis.

Introduction

The eye lens is a multilayered structure which can be topographically divided into four distinct parts: (a) the capsule, a collageneous shell which surrounds the organ, (b) the anterior surface, which consists of a cuboidal epithelium, (c) the cortex, a region immediately beneath the epithelium, which comprises the so-called lens fiber cells (LFCs), and (d) the nucleus, a central area which contains tightly packed LFCs.

The LFCs originate from the epithelium and during the entire life of the animal continue to add to the upper layers of the cortex. The LFCs which are adjacent to the epithelium are still nucleated, whereas the cells found in deeper layers of the lens cortex are anucleate (the cell nucleus disintegrates during the final stages of LFC differentiation). Despite the lack of an RNA-synthesizing machinery, the LFCs maintain a well-developed cytoskeletal network (actin microfilaments, vimentin intermediate filaments, IFs) and numerous specialized intercellular junctions (for reviews see Bloemendal, 1981; Maisel, 1985).

The LFC cytoskeleton appears to be ultimately associated with the plasma membrane (Ramaekers et al., 1982). However, the components mediating this coupling remain unknown. Previous studies have demonstrated that, at least in some aspects, the LFC membrane resembles the mammalian erythrocyte membrane and possesses a spectrin-actin-protein 4.1 membrane-skeleton (Allen et al., 1987; Aster et al., 1984a,b; Aster et al., 1986; Granger and Lazarides, 1984). Nevertheless, due to its specialized architecture, the LFC plasma membrane also contains some unique components (e.g. Kistler and Bullivant, 1989). To characterize such lens-specific proteins, which might be involved in the anchoring of the cytoskeleton to the plasma membrane, we have recently studied the properties of a lens-specific 100/110 kDa polypeptide, which we have identified as a component of the LFC membrane-associated cytoskeleton by biochemical methods and by immunoelec-

tron microscopy (Merdes et al., 1991). This protein, termed filensin, forms characteristic 10 nm fibrils under in vitro conditions and binds strongly to two other proteins: the IF subunit vimentin and a 47 kDa peripheral protein of the LFC membrane. It is still unclear whether filensin relates to a 95-115 kDa protein which has been characterized as a constituent of a lens-specific structure, the so called "beaded filament" (FitzGerald and Gottlieb, 1989; Ireland and Maisel, 1984; Maisel and Perry, 1972).

In principle, there are three ways in which filensin may associate with the plasma membrane: (a) it may interact with another peripheral membrane protein (e.g., the 47 kDa polypeptide), (b) it may directly associate with an integral membrane protein, or, (c) it may insert into the lipid bilayer via a covalently-bound fatty acid tail. To explore these possibilities, we have developed assays to measure the binding of filensin to isolated lens membranes and to examine its solubility properties. The results presented below support the idea that filensin is able to associate independently of the 47 kDa component with intrinsic protein(s) of the lens cell membrane.

Materials and methods

Preparation of lens and erythrocyte membranes

Porcine and bovine lens membranes were isolated as previously described (Merdes et al., 1991). To remove peripheral proteins, the washed membranes were resuspended in 8 M urea, 10 mM Tris-HCl pH 8.0, 4 mM EDTA, 1 mM DTT and 1 mM PMSF (urea buffer), sonicated, and centrifuged at 218,000 ***g*** for 60 min at 18°C. The pellets were resuspended in urea buffer and recentrifuged at 356,000 ***g*** for 30 min at 18°C. This step was repeated twice. The urea-stripped membranes were kept (as a pellet) at −70°C until needed. Inside-out-vesicles (IOVs) were prepared from rabbit erythrocytes as specified previously (Bennett, 1983; Georgatos and Marchesi, 1985). Urea-extraction of these membranes was done as described above.

Extractions, protease digestions and other treatments of the membranes

Equal portions of a concentrated suspension of lens membranes were extracted in one of the following buffers: (a) 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM DTT, (low Tris buffer); (b) 2, 4, 5, or 8 M urea in low Tris buffer; (c) 1% Triton X-114 in 0.9% (w/v) NaCl and 20 mM Tris-HCl pH 7.4; (d) 1 M Tris-HCl pH 9.0; (e) 1 M hydroxylamine, pH 9.0. All buffers contained 1 mM PMSF. Samples resuspended in buffers (a) and (b) were incubated at room temperature for 45 min and spun at 356,000 ***g*** for 40 min at 18°C. Purified filensin and lens membranes reconstituted in buffer (c) were incubated on ice for 15 min and centrifuged at 10,000 ***g*** for 15 min at 4°C. The supernatant was warmed up to 37°C and then respun at 37°C to separate the water phase from the detergent phase. Membranes resuspended in buffers (d) and (e) were incubated at room temperature for 45 min and centrifuged at 10,000 ***g*** for 30 min at 4°C. Pellets and portions of the supernatants (concentrated by TCA precipitation) were solubilized in 4× electrophoresis sample buffer (250 mM Tris-HCl pH 6.8, 9.2% SDS, 40% glycerol, 0.2% (w/v) bromophenol blue, 100 mM DTT), and analyzed by SDS-PAGE. Urea-stripped lens membranes were washed twice with PBS and resuspended in PBS, before the addition of trypsin (ratio of membranes to protease~20:1 w/w). After a 30 min or an overnight incubation at room temperature, proteolysis was stopped by adding a 10-fold excess of trypsin inhibitor and 1 mM PMSF. After pelleting, the membranes were washed three times in assay buffer (see below) at 4°C, and resuspended in the same media. Treatment with other proteases was carried out in the same way, except that a protease inhibitor cocktail was used to stop proteolysis (34 mM DFP (diisopropyl fluorophosphate), 1 mM PMSF, 4 μg/ml leupeptin, 300 μg/ml trypsin inhibitor, 300 μg/ml trypsin/chymotrypsin inhibitor, and 2 μg/ml apronitin). In comparative experiments involving trypsin and other proteases, the digestion with trypsin was also terminated by the same anti-protease cocktail and the membranes washed with assay buffer. Heat-treatment was done by incubating membranes for 10 min at 95°C. NEM (*N*-ethylmaleimide) treatment was done by incubating the membranes with 2 mM NEM for 30 min at room temperature. The reaction was stopped by the addition of 4 mM DTT.

Reconstitution of vesicles from total lens lipids

The procedure of Folch was followed (Folch et al., 1957). Briefly, lenses were homogenized in a 2:1 (v/v) chloroform-methanol mixture, and the final volume was adjusted to 20-fold the weight of the tissue. The homogenate was filtered through a fat-free filter and 0.2 vol. of 0.05% (w/v) $CaCl_2$ in water were added to the filtrate. After mixing, the two phases were allowed to separate. The upper phase was removed and the interphase was washed three times with 3:48:47, chloroform-methanol-water. The lower phase was evaporated to almost dryness, resuspended in 10 mM Tris-acetate, pH 7.6 to a concentration of 10 mg/ml, and sonicated.

Isolation of proteins and radiolabelling

Filensin was purified according to Merdes et al. (1991). The 47 kDa protein was isolated from the same urea extract of lens membranes as filensin. After applying the urea extract on diaminoethyl cellulose (DE52; Whatman, Maidstone/Kent, UK), 47 kDa containing fractions were pooled and dialyzed against 8 M urea, 10 mM Tris-HCl, pH 8.0, 4 mM EDTA, 1 mM DTT, and 1 mM PMSF. This material was chromatographed on DE53. The 47 kDa protein was eluted with a linear gradient of 0 to 50 mM NaCl (in urea buffer). After pooling the fractions containing the 47 kDa protein and dialyzing the pool against the same urea buffer, the pool was rechromatographed on DE53 and eluted with a linear gradient of 0 to 25 mM NaCl (in urea buffer). Purified filensin was labelled with the ^{125}I-Bolton-Hunter reagent as described by Georgatos et al. (1985).

Immunological and immunochemical methods

Affinity-purified polyclonal anti-filensin antibodies were prepared and characterized as previously described (Merdes et al., 1991). The anti-peptide antibody anti-FL 2, was produced after injection of KLH-conjugated FL-2 into rabbits. FL-2, which is a synthetic peptide with the sequence AYEKVEVMESIEKFSTESI, has been modelled after authentic filensin COOH-terminal sequences obtained by direct sequencing of homogeneous bovine filensin after cleavage with trypsin. Its position along the filensin molecule has been mapped by comparison to a bovine filensin cDNA clone (Gounari, F., Merdes, A., Quinlan, R., Hess, J., FitzGerald, P., Ouzounis, C. and Georgatos, S. D., manuscript in preparation). Immunoblotting was performed as specified (Georgatos et al., 1987).

Assays

Sedimentation and flotation assays were carried out as follows. Purified filensin and 47 kDa protein were dialyzed, in the presence of 1% cold fish skin gelatin, against 0.9% (w/v) NaCl, 20 mM Tris-HCl pH 7.4, 2 mM $MgCl_2$ and 1 mM PMSF (assay buffer) at 4°C. When ^{125}I-filensin was used, the radiolabelled and the non-labelled protein were mixed in different ratios and dia-

lyzed against the assay buffer. Before performing the sedimentaton assay, the filensin stock solution was clarified by ultracentrifugation. Filensin was coincubated with 30-75 μg of membranes, (extensively washed and resuspended in the assay buffer), in a volume of 90-160 μl for 90 min at room temperature. At the end of the incubation, the membranes were sedimented by centrifugation in a Beckman TLA 100 rotor at 25,000 rpm for 10 min at 4°C. The pelleted material and a corresponding amount of the supernatant were solubilized in 4× electrophoresis sample buffer and analyzed by SDS-PAGE and immunoblotting (or, in the case of ^{125}I-filensin, by SDS-PAGE, autoradiography, and/or γ-counting). In the flotation assays, 95% sucrose in assay buffer was added to each reaction mixture after the incubation to give a final sucrose concentration of 75%. The mixture was transferred to the bottom of gelatin-coated SW 50 tubes (Beckman) to which 1 ml of 50% sucrose and 2 ml of 30% sucrose (in assay buffer) were added. The tubes were filled up with 20% sucrose solution and centrifuged at 245,000 ***g*** for 15 hours at 18°C. The gradients were fractionated with a Buchler HBI Auto Densi Flow-IIC apparatus into 250 μl fractions. The fractions were diluted in 4× electrophoresis sample buffer and analyzed by SDS-PAGE and immunoblotting.

Whole mount immunoelectron microscopy

Samples of floated membranes or lipid vesicles taken from the sucrose gradient fractions were first dialyzed at 4°C against assay buffer (to remove the sucrose) and then applied to carbon-coated EM grids for 3 min at room temperature. The samples were fixed with 3.5% formaldehyde in PBS for 10 min at room temperature, washed with PBS-100 mM glycine, and "blocked" for 20 min with 0.5% cold fish skin gelatin in assay buffer (gelatin buffer). Anti-FL 2 affinity-purified antibodies in gelatin buffer were then added for 45 min and, following 3 × 5 min wash with gelatin buffer, the grids were incubated with Protein A-gold (diluted in gelatin buffer). After a 30 min incubation the samples were washed again, rapidly rinsed over a droplet of distilled water, and stained with 1.5% uranyl acetate for 2 min. After air-drying, the specimens were visualized in a Philips 301 electron microscope operated at 80 kV.

Other methods

Polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli (1970). Protein determinations were done using a BioRad protein determination kit.

Results

Extraction of lens membranes and solubility properties of filensin

In previous studies we have found that filensin is removed from the lens membranes by high concentrations of urea or alkali (pH > 11), but not with Triton X-100 (Merdes et al., 1991). To examine the solubility properties of filensin in a more systematic manner, we performed additional chemical extractions on isolated lens membranes.

When the membranes are treated with a graded series (2-8 M) of urea solutions and then centrifuged, filensin gradually appears in the supernatant fraction. Solubilization starts at 2 M urea (Fig. 1a, lanes 2 and 2′), and is almost complete after 5-8 M urea extraction (Fig. 1a, lanes 4 and 4′, 5 and 5′). A trace amount of filensin which remains in the urea-extracted membranes (Fig. 1a, lane 5) can be entirely removed by further washes with 8 M urea solutions (see below). The selectivity of this extraction method is demonstrated by the fact that a known integral component of the lens cell membrane, the protein p26, is not removed by urea treatment (Fig. 1a, lanes 1-5, arrow), whereas all of the known peripheral proteins (e.g., fodrin, actin etc.) are extracted. When the membranes are treated with 1 M hydroxylamine at pH 9.0, a condition known to cleave thioester and oxyester-linked fatty acids from proteins (Berger and Schmidt, 1984; Magee et al., 1987; for a review see James and Olson, 1990), the bulk of filensin is still found in the membrane pellet (Fig. 1b, lanes 2 and 2′). Exactly the same is observed in a control extraction when hydroxylamine is substituted by 1 M Tris-HCl pH 9.0, to account for the effects of high ionic strength and alkaline pH (Fig. 1b, lanes 1 and 1′). Finally, extraction of the membranes with 1% Triton X-114 does not release detectable amounts of filensin (Fig. 1c, lanes 1 and 1′), but it does release some p26 which, upon phase separation at 37°C, partitions with the detergent phase (Fig. 1c, lane 1′, arrow). To directly examine the partitioning of filensin in the Triton X-114/water system, purified filensin was reconstituted in a buffer containing 1% Triton X-114 and the solution was warmed up to induce phase separation and spun. As it can be seen in Fig. 1c, lanes 2 and 2′, purified filensin partitions exclusively with the aqueous phase (the trace amount of filensin seen in the detergent phase represents contamination from material adhering to the test tube and is also detected when the experiment is repeated in the absence of detergent; M. B. and S. D. G., unpublished observations). These results support previous findings (Merdes et al., 1991) and indicate that filensin is tightly associated with the lens cell membrane. Its partitioning with the aqueous phase in the Triton X-114/water mixture and its resistance to hydroxylamine at high pH further suggest that filensin is not associated with the membrane lipids via a covalently attached fatty acid moiety (see below and in Discussion).

A 51 kDa, COOH-terminal fragment of filensin resists extraction and remains bound to lens cell membranes

To investigate the binding properties of purified filensin, we decided to use as a substrate membranes depleted from endogenous filensin by urea extraction. Although no residual filensin could be detected in the urea-stripped membranes (SDS-PAGE profile shown in Fig. 3a, lane N), when we probed blots of this material with affinity-purified antifilensin antibodies, we noticed a cross-reacting protein with an approximate molecular mass of 51 kDa (Fig. 2a, lane 2). This protein, which comigrates with one of the known degradation products of filensin, had escaped detection in previous immunoblotting experiments with urea-extracted membranes (Merdes et al., 1991), presumably because it is present in relatively low amounts.

To confirm that the 51 kDa protein represents a degradation product of filensin, we also probed blots of urea-extracted membranes with an affinity-purified antibody (anti-FL 2) developed against a synthetic peptide which corresponds to a COOH-terminal sequence of authentic filensin (the sequence of bovine lens filensin is to be reported elsewhere; F. Gounari, A. Merdes, R. Quinlan, J. Hess, P. FitzGerald, C. Ouzounis and S. D. Georgatos, manuscript in preparation). As it can be seen in Fig. 2b, lane 2, the

anti-FL 2 antibody reacts strongly with the 51 kDa protein, supporting the idea that the latter is indeed a COOH-terminal degradation product of filensin. The filensin peptide remains tightly bound to the membranes under a variety of conditions, including alkali and urea-high salt treatment of the membranes (M. B. and S. D. G., unpublished observa-

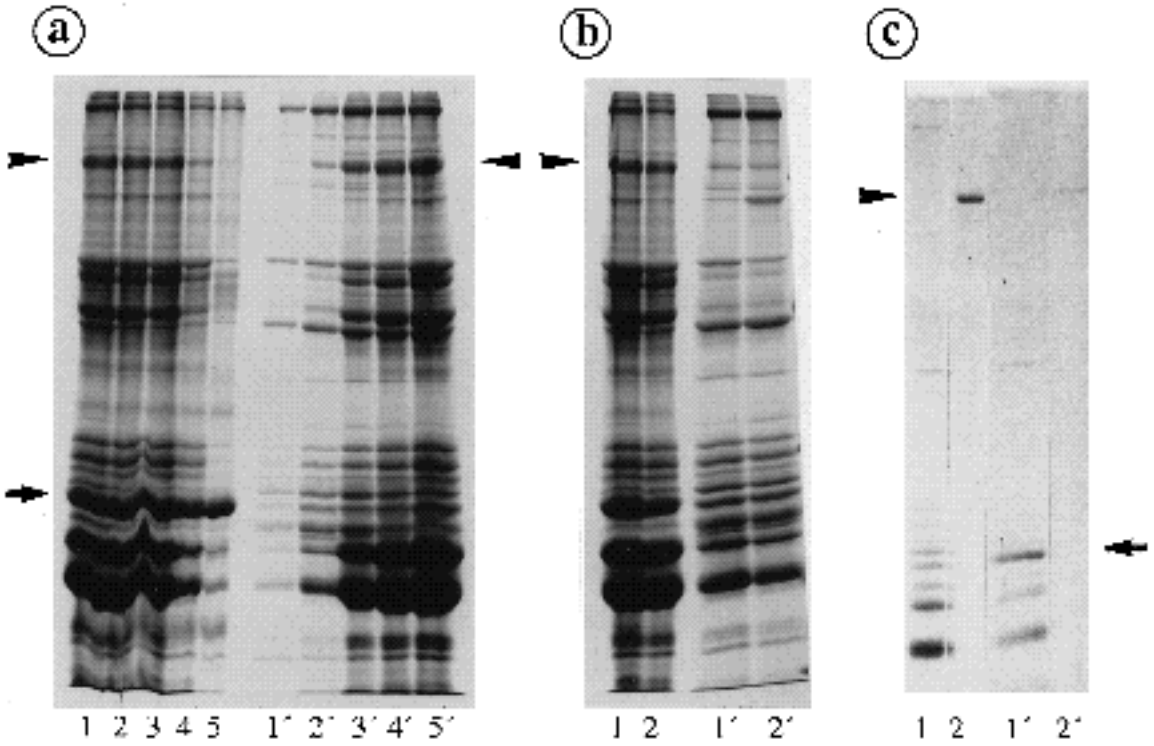

Fig. 1. Partitioning and solubility properties of filensin. Isolated lens membranes were extracted with various agents as specified in Materials and methods. The samples were separated into a pellet and a supernatant fraction by centrifugation and analyzed by SDS-PAGE. a: Extraction with low Tris buffer (pellet lane 1; supernatant lane 1′), with 2 M urea in low Tris buffer (pellet lane 2; supernatant lane 2′), with 4 M urea in low Tris buffer (pellet lane 3; supernatant lane 3′), with 5 M urea in low Tris buffer (pellet lane 4; supernatant lane 4′), and with 8 M urea in low Tris buffer (pellet lane 5; supernatant lane 5′). b: Extraction with 1 M Tris-HCl, pH 9.0 (pellet lane 1; supernatant lane 1′) and 1 M hydroxylamine, pH 9.0 (pellet lane 2; supernatant lane 2′). c: Extraction with Triton X-114. Lens membranes, or purified filensin, were reconstituted in 0.9% (w/v) NaCl, 20 mM Tris-HCl pH 7.4, and 1% Triton X-114 at 0°C. The samples were centrifuged at 4°C, and the supernatants (Triton X-114 extracts) were warmed up at 37°C to induce separation of the aqueous and the detergent phase. After another centrifugation, the two phases were collected separately and the material analyzed by SDS-PAGE (for details see Materials and methods). Lane 1 shows the water phase fraction after extraction of lens membranes; lane 2 shows the water phase fraction after reconstitution of purified filensin in 1% Triton X-114; lane 1′ is the the detergent phase fraction after extraction of lens membranes; lane 2′ represents the detergent phase fraction after reconstitution of purified filensin in 1% Triton X-114. Arrowheads indicate the position of filensin, whereas arrows mark the position of the integral membrane protein p26.

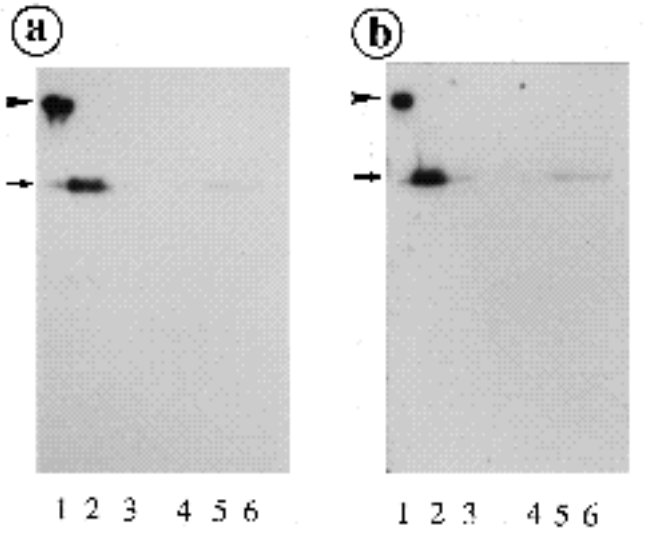

Fig. 2. Examination of urea-extracted and protease-treated lens membranes by immunoblotting. Lens membranes were extracted with 8 M urea, washed, and then treated with buffer (lanes 2), trypsin (lanes 3), α-chymotrypsin (lanes 4), thrombin (lanes 5), and V8 protease (lanes 6), as specified in Materials and methods. Samples were analyzed by SDS-PAGE, transferred to nitrocellulose filters and probed with antibodies. a: Immunoblot with polyclonal affinity-purified antifilensin antibodies (1 μg/ml) b: Immunoblot with affinity-purified anti-FL 2 antibodies (1 μg/ml, for explanations see text). Arrows mark the 51 kDa filensin degradation product which remains bound to the membranes and is removed by protease digestion. Arrowheads indicate intact filensin (lanes 1) included here as a control.

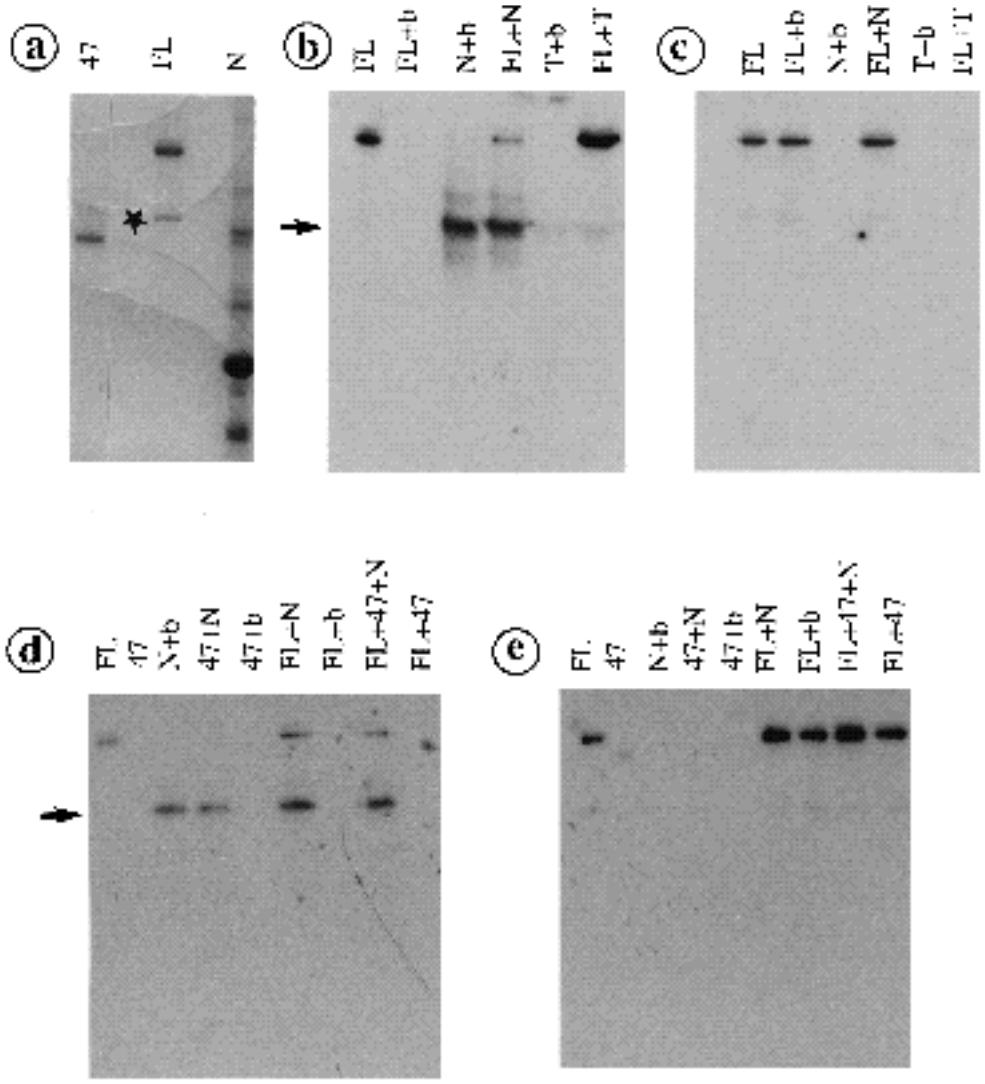

Fig. 3. Binding of purified filensin to urea-stripped membrane preparations, as detected by a cosedimentation assay. a: SDS-PAGE profile of purified 47 kDa protein (47), purified filensin (FL) and urea-stripped lens membranes (N). The proteins were stained with Coomassie brilliant blue. Asterisk indicates the position of a filensin degradation product. b: Binding of filensin to lens membranes. Eighteen μg of purified filensin were incubated with assay buffer (FL + b), 65 μg of urea-stripped membranes (FL + N), or the equivalent amount of trypsin digested/urea-stripped membranes (FL + T). Altertnatively, 65 μg of urea-stripped membranes (N + b), or the equivalent amount of urea-stripped/trypsin-digested membranes (T + b) were incubated with assay buffer alone. All samples were processed as described in Materials and methods and 20% of each pellet was analyzed by SDS-PAGE followed by immunoblotting with polyclonal antifilensin antibodies (diluted 1:400). FL is a sample of purified filensin included in the electrophoresis as a reference marker. Arrow indicates the 51 kDa filensin degradation product which remains bound to urea-stripped membranes. c: An immunoblot of the supernatants corresponding to the pellet samples shown in b. Each lane contains 20% of the original fraction. d: Binding of filensin to urea-stripped membranes in the presence and absence of the 47 kDa filensin-binding protein, as detected by the cosedimentation assay. Purified filensin was incubated, as indicated above, with urea-stripped membranes (FL + N), assay buffer (FL + b), urea-stripped membranes and 10 μg of purified 47kDa protein (FL + 47 + N), or only 10 μg of purified 47 kDa protein (FL + 47). Alternatively, the urea-stripped membranes were incubated with assay buffer (N + b), or with 10 μg of purified 47 kDa protein (47 + N). Finally, 10 μg of purified 47 kDa protein were incubated with assay buffer alone (47 + b). The samples were processed as explained in Materials and methods and 20% of each pellet were analyzed by SDS-PAGE followed by immunoblotting with polyclonal antifilensin antibodies. (FL) and (47) are samples of purified filensin and the 47 kDa protein, respectively, included in the electrophoresis as reference markers. Arrow indicates the 51 kDa filensin degradation product. e: An immunoblot of the supernatant fractions corresponding to the pellet samples shown in "d". Each lane contains 20% of the original fraction.

tions); however, because it can be completely removed (or destroyed) by digestion of the membranes with trypsin, thrombin, chymotrypsin, or V8 protease (Fig. 2a and b, lanes 3, 4, 5, 6), it is highly unlikely that this material is entrapped inside sealed membrane vesicles.

Purified filensin binds to trypsinized and non-trypsinized urea-stripped membranes

To proceed, purified porcine filensin (SDS-PAGE profile shown in Fig. 3a, lane FL) was incubated either with urea-stripped membranes (Fig. 3a, lane N), or with urea-stripped membranes which had been digested with trypsin (profile shown in Fig. 5A, lane T). After pelleting the membranes, binding was detected by resolving the sedimented material electrophoretically and performing immunoblotting with specific antifilensin antibodies. The results of these experiments are shown in Fig. 3b and c and can be summarized as follows. First, filensin does not sediment on its own under the conditions employed in the assay (lanes FL+b). Second, the urea-stripped membranes which have been digested with trypsin seem to bind substantially more filensin than the non-trypsinized membranes (compare lane FL+N with lane FL+T).

Because filensin has been found to react with another 47

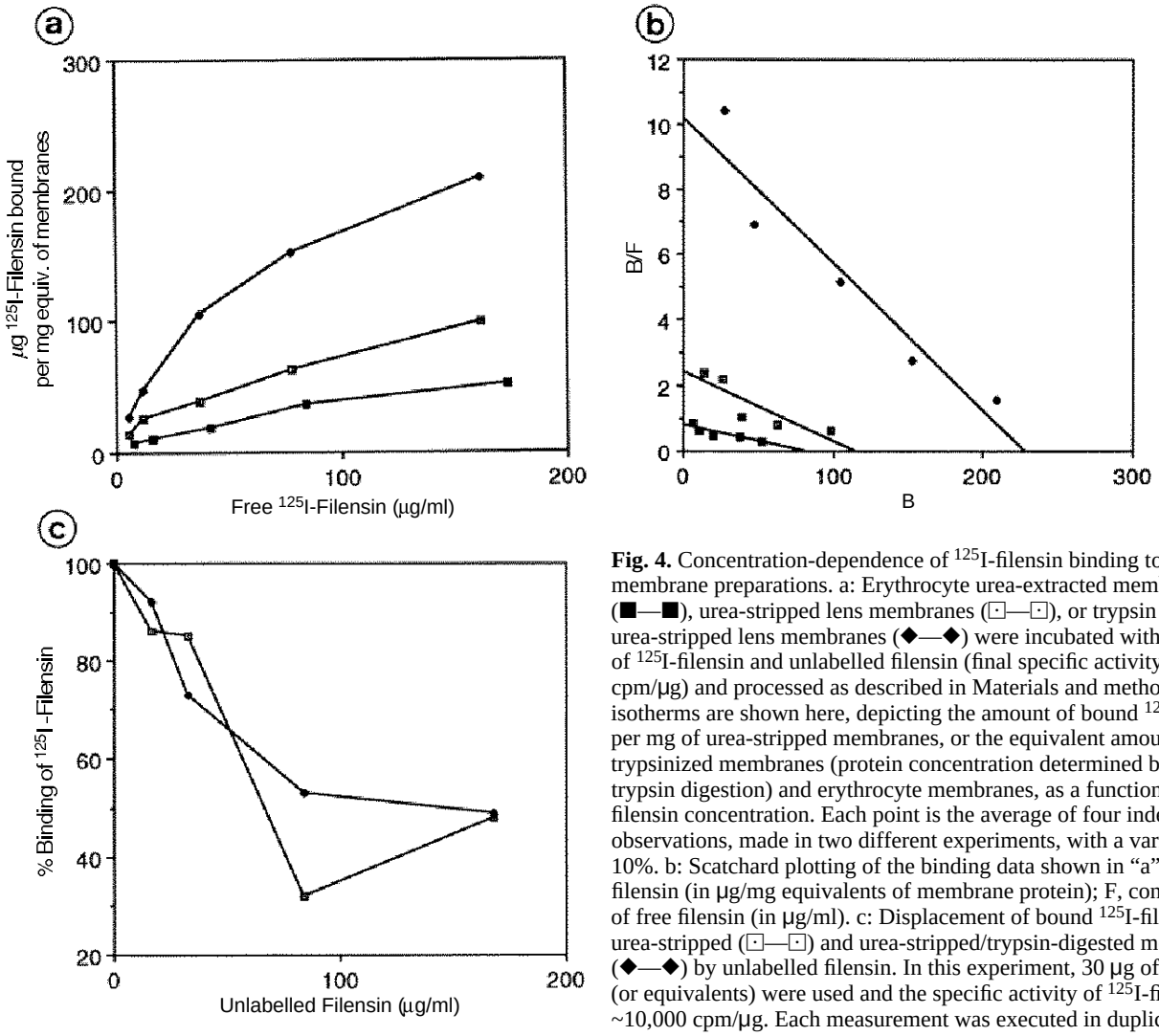

Fig. 4. Concentration-dependence of ^{125}I-filensin binding to different membrane preparations. a: Erythrocyte urea-extracted membranes (■—■), urea-stripped lens membranes (⊡—⊡), or trypsin digested urea-stripped lens membranes (◆—◆) were incubated with a mixture of ^{125}I-filensin and unlabelled filensin (final specific activity ~2,000 cpm/µg) and processed as described in Materials and methods. Binding isotherms are shown here, depicting the amount of bound ^{125}I-filensin per mg of urea-stripped membranes, or the equivalent amount of trypsinized membranes (protein concentration determined before trypsin digestion) and erythrocyte membranes, as a function of the free filensin concentration. Each point is the average of four independent observations, made in two different experiments, with a variation of < 10%. b: Scatchard plotting of the binding data shown in "a". B, bound filensin (in µg/mg equivalents of membrane protein); F, concentration of free filensin (in µg/ml). c: Displacement of bound ^{125}I-filensin from urea-stripped (⊡—⊡) and urea-stripped/trypsin-digested membranes (◆—◆) by unlabelled filensin. In this experiment, 30 µg of membranes (or equivalents) were used and the specific activity of ^{125}I-filensin was ~10,000 cpm/µg. Each measurement was executed in duplicate.

kDa peripheral membrane protein of the lens cells (see Merdes et al., 1991, and Introduction), we found it important to examine whether filensin binding to urea-stripped membranes can be facilitated by the 47 kDa component. Data presented in Fig. 3d and e indicate that addition of stoichiometric quantities of purified 47 kDa protein (SDS-PAGE profile shown in Fig. 3a, lane 47) do not increase the binding of filensin to the urea-stripped membranes (compare lanes FL+N, FL+47+N, and FL+47).

To assess the concentration dependence of the binding, we performed the cosedimentation assay using increasing quantities of ^{125}I-filensin. In these experiments, we also used an unrelated membrane system (rabbit erythrocyte urea-stripped inside-out-vesicles) as a means to measure the general affinity of filensin for non-lens intrinsic membrane components. As demonstrated in Fig. 4a, and consistent with the previous data, the radiolabelled filensin binds differently to the non-trypsinized and the trypsinized lens membranes. The binding to the former appears to be low, whereas the binding to the latter seems to be much higher with a clear trend towards saturation. Finally, the net binding to the erythrocyte membranes is low, but measurable. From the binding isotherms (Fig. 4a) and the corresponding Scatchard plots (Fig. 4b), it can be calculated that filensin binds to the trypsin-treated membranes with a K_d of 2×10^{-7} M and the maximum binding corresponds to approximately 226 µg/mg of membrane protein equivalent (for details see legend to Fig. 4). The corresponding K_d for the binding to the non-trypsinized membranes is approximately 4×10^{-7} M, but the maximum binding corresponds to only 116 µg/mg of membrane protein equivalent. Finally, the binding of filensin to the erythrocyte membranes appears to be one order of magnitude lower in affinity than the binding to the lens cell membranes. From these data, it would seem that filensin binds with comparable affinity to the non-trypsinized and the trypsinized membranes, which, however, show a two-fold difference in their filensin-binding capacities. (The experimentally determined values of these parameters should be considered only approximate because, despite the minimal variability in the measurements, segments of the Scatchard plots appear to deviate from linearity.)

To assess the specificity of the binding of ^{125}I-filensin, we performed the cosedimentation assay with a fixed amount of probe and increasing quantities of unlabelled purified filensin. Results shown in Fig. 4c demonstrate that

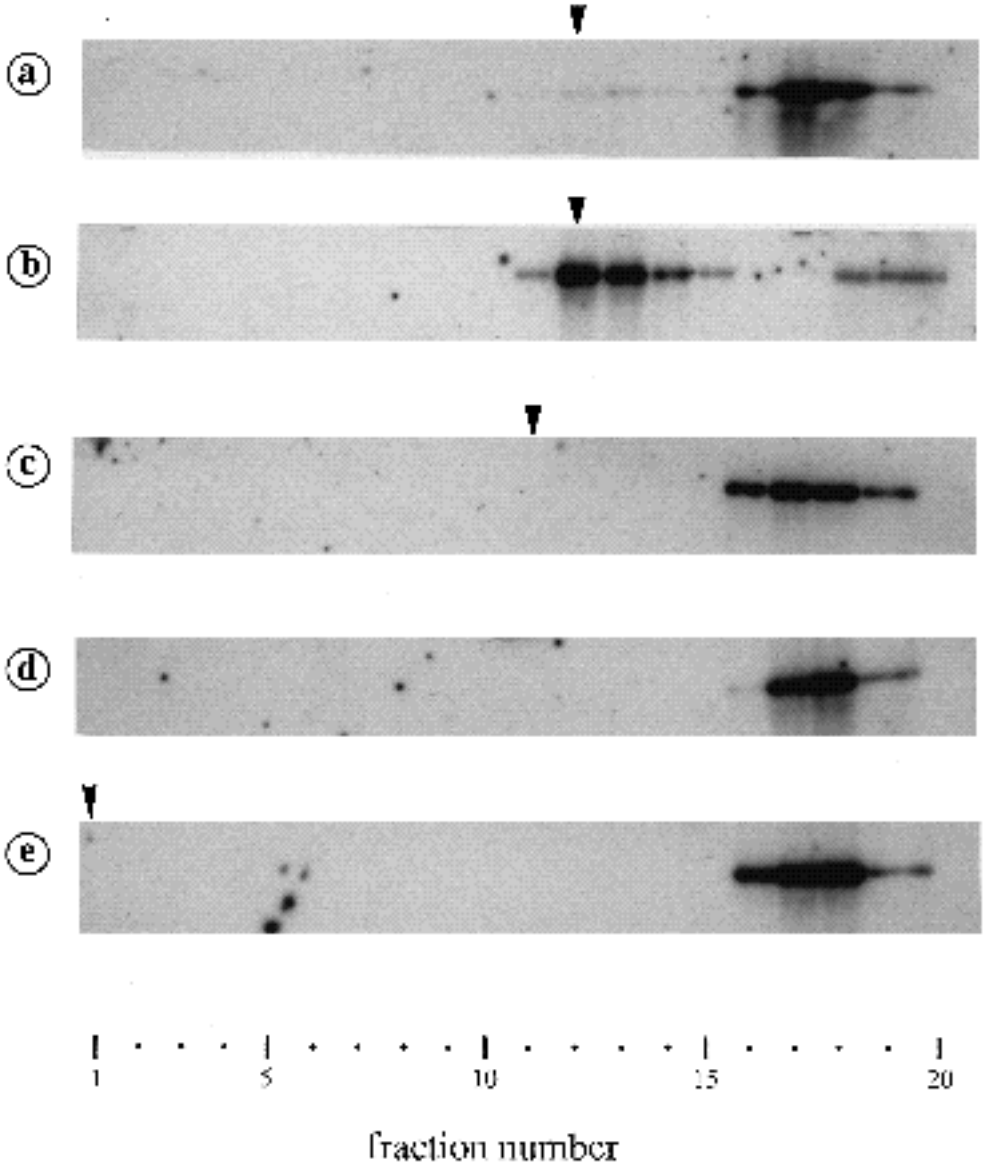

Fig. 5. Binding of filensin to different membrane preparations and membrane lipid vesicles, as detected by a flotation assay. Twenty five μg of filensin were coincubated with 75 μg of urea-stripped lens membranes (panel a), the equivalent amount of urea-stripped/trypsin-digested lens membranes (panel b), 75 μg of urea-extracted erythrocyte vesicles (panel c), 500 μg of vesicles reconstituted from total lens lipids (panel e), or assay buffer alone (panel d) in a final volume of 150 μl. After incubation, the samples were diluted to 600 μl by adding a concentrated sucrose solution, loaded onto sucrose step-gradients and centrifuged. The gradients were fractionated, and 7% of each fraction analyzed by SDS-PAGE, or SDS-PAGE followed by transfer onto nitrocellulose filters. The gels were stained with Coomassie blue to monitor the migration of the membranes (not shown), whereas the corresponding blots were probed with polyclonal anti-filensin antibodies (diluted 1:400). The gallery shows parts of the blots in the area of 100 kDa. The numbers refer to fraction numbers, whereas the arrowheads indicate the peaks containing the floated membranes or lipid vesicles. (The position of the lipid vesicles is based on microscopic identification of vesicular structures done in a similar gradient; see Fig. 6.)

unlabelled filensin can displace the radioactive tracer from both the trypsinized and the non-trypsinized membranes. Due to polymerization of filensin at concentrations equal or higher to 100 μg/ml (see Merdes et al., 1991), the apparent displacement of the radiolabelled filensin does not exceed the value of ~50%. However, the fact that similar concentrations of unlabelled filensin are needed for ~50% displacement of the bound ^{125}I-filensin from the trypsinized and the non-trypsinized membranes is consistent with the previous data and indicates, again, that filensin binds to the two preparations with similar affinities.

In summary, the combined binding data suggest that filensin can associate directly to intrinsic components of the lens membrane independently of the 47 kDa protein. From the same results, it also seems plausible that the 51 kDa degradation product of filensin interferes with the association of intact filensin with the lens membrane by masking or occupying potential filensin-binding sites.

The binding of filensin is probably mediated by a trypsin-resistant integral membrane protein

To evaluate the specificity of the filensin-membrane interactions by another method, we examined the binding of filensin to various membrane preparations and to vesicles reconstituted from lens lipids using a flotation assay. This involved coincubation of purified filensin with the different substrates, addition of a “heavy sucrose” solution to the reaction mixtures, and loading of each sample at the bottom of a sucrose step-gradient. After centrifugation and fractionation of the gradients, aliquots were electrophoresed and the migration of filensin was assessed by probing the corresponding blots with anti-filensin antibodies. Representative results obtained by this method are depicted in Fig. 5 and show that the trypsinized lens membranes bind substantial quantities of filensin, as indicated by the “shift” in its migration and the cofractionation with the floated membranes. Consistent with the previous data, filensin binding

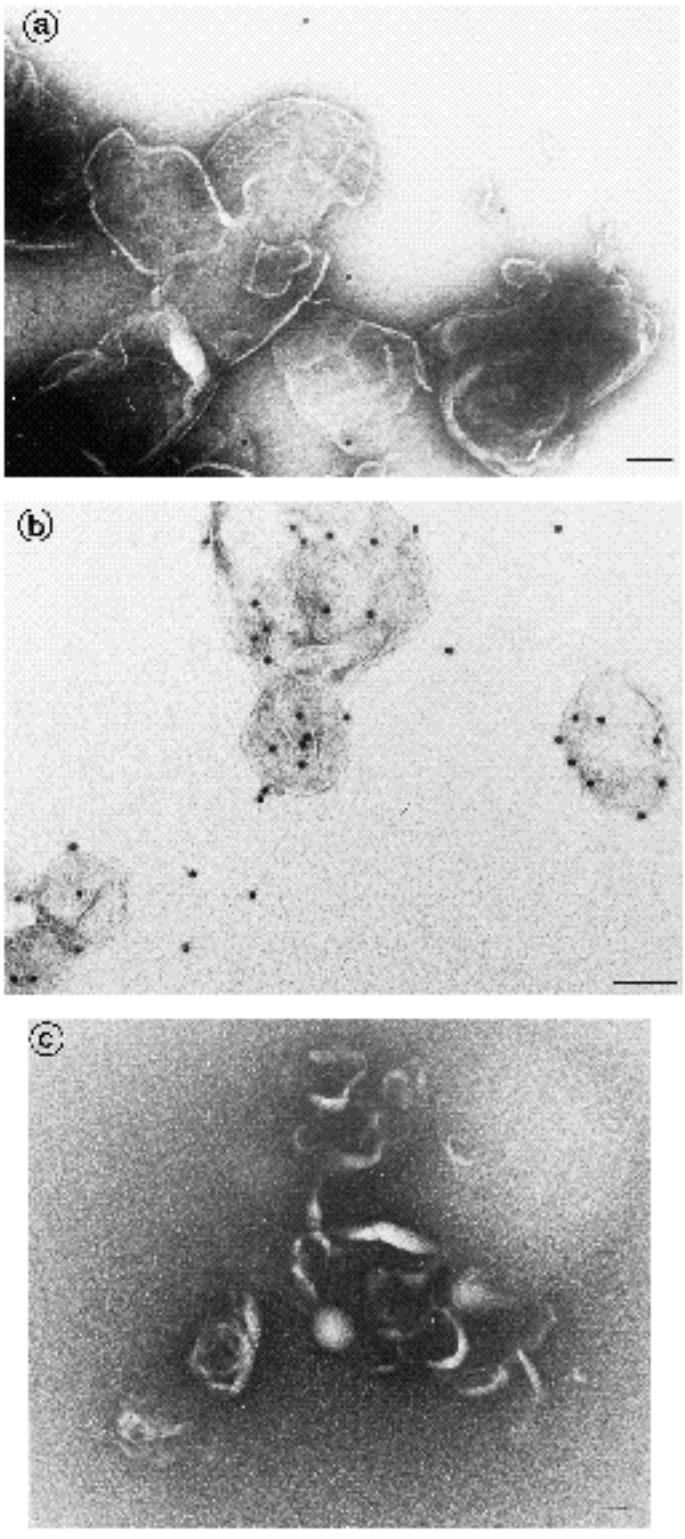

Fig. 6. Binding of filensin to floated membrane and lipid vesicle preparations, as detected by whole mount immunoelectron microscopy and negative staining. Trypsinized membranes (a and b), or vesicles reconstituted from total lens lipids (c) were incubated either with buffer (a), or with purified filensin (b, c) and floated as described in Fig. 5. The peak fraction of floated membranes and the top fraction containing the lipid vesicles were applied to carbon-coated EM grids and stained with affinity-purified anti-FL 2 rabbit antibodies (1 μg/ml) and 14 nm gold-Protein A (for details see Materials and methods). In the end of each incubation all samples were stained with uranyl acetate and visualized in the electron microscope. Bars correspond to 100 nm.

to non-trypsinized membranes is low (however, notice that, at the concentration tested here, filensin binding to trypsinized membranes is predicted to be approximately three-fold higher than the binding to non-trypsinized membranes; Fig. 4a and legend to Fig. 5B). Binding to erythrocyte vesicles and to lens lipid vesicles is not detected under these conditions. From this we conclude that the low amounts of filensin that cosediment with erythrocyte membranes in direct sedimentation assays (Fig. 4a) probably include some self-pelleting material which is not physically bound to these membranes.

When portions of the floated fractions are examined by whole-mount immunoelectron microscopy and negative staining (for details see Materials and methods), one sees that filensin-incubated trypsinized membranes are heavily decorated by anti-FL 2 antibodies and Protein A-gold (Fig. 6b). Consistent with the biochemical data, trypsinized membranes incubated with buffer alone and filensin-incubated lens lipid vesicles are not decorated by the antibodies (Fig. 6a and 6c). Assessment of filensin binding to non-trypsinized membranes by the above method is complicated because, as it might be expected, the antibodies heavily decorate the 51 kDa filensin degradation product (data not shown).

To titrate our analysis, we also examined the binding of ^{125}I-filensin to lens membranes treated in several different ways. Fig. 7a demonstrates that binding of filensin to trypsinized membranes is lowered by heat-treatment (column Th), but not by NEM-treatment of these membranes (column Ta). After digestion of urea-extracted lens membranes with thrombin, which totally removes the 51 kDa filensin degradation product (Fig. 2, lanes 5), their filensin-binding capacity remains low (Fig. 7b, column TH). This suggests that thrombin also cleaves a putative filensin "receptor" found in the membranes. Digestions with chymotrypsin or V8 protease, which also remove the filensin peptide from the membranes (Fig. 2, lanes 3 and 5), result in a moderate increase in the filensin-binding capacity of the membranes (Fig. 7b, columns CH and V8), probably because some filensin-binding sites are spared. Analysis of the pellets and fractions of the supernatants by SDS-PAGE and autoradiography confirms that these differences in the binding are not due to filensin degradation from residual protease activity in the various membrane preparations (data not shown).

Taken together, these results suggest that filensin may specifically associate with an integral membrane protein of the lens cells which is trypsin-resistant but sensitive to thrombin and other proteinases.

Discussion

Filensin behaves as a hydrophilic peripheral membrane protein

In this study we have systematically examined the solubil-

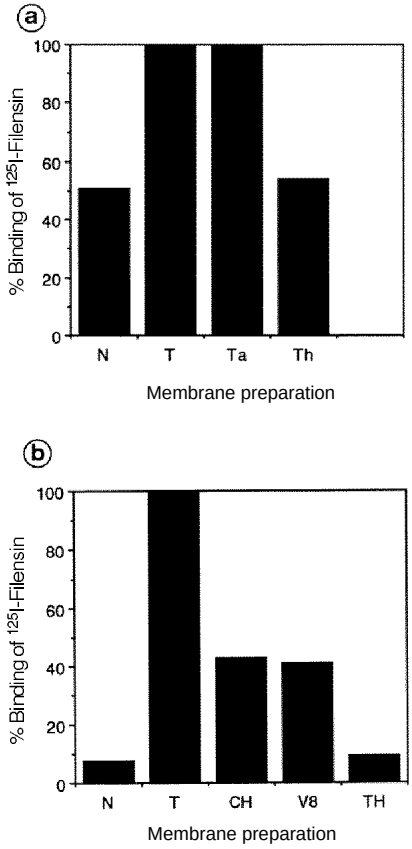

Fig. 7. Binding of ^{125}I-filensin to lens membranes digested with different proteases, or subjected to chemical and physical treatments. Urea-stripped lens membranes, treated as described below, were incubated (in triplicate) with ^{125}I-filensin and then sedimented. The amount of bound radiolabelled filensin was determined by γ-counting. The histograms depict% binding, with the values corresponding to the trypsin-treated membranes taken as 100%. The variation in these measurements was < 10%. a: Assays done with 25 μg of membranes (or equivalents) and ~23 μg/ml of ^{125}I-filensin (specific activity=2,000 cpm/μg). N, non-treated membranes; T, trypsin-digested membranes; Ta, trypsin-digested membranes treated with NEM; Th, trypsin-digested membranes, heated for 10 min at 95°C. b: Assays done with 23 μg of membranes (or equivalents) and ~34 μg/ml of ^{125}I-filensin (specific activity, 2,000 cpm/μg). N, non-treated membranes; T, trypsin-digested membranes; CH, chymotrypsin-treated membranes; V8, V8 protease-treated membranes; TH, thrombin-digested membranes. In all cases, the binding to equivalent quantities of erythrocyte membranes has been subtracted.

ity and the membrane-binding properties of filensin under in vitro conditions. Employing controlled extraction with various agents, we have confirmed that filensin is strongly associated with the LFC membrane (Merdes et al., 1991). Moreover, by assessing its partitioning to Triton X-114 and its behavior after hydroxylamine treatment of lens cell membranes at alkaline pH, we have obtained results arguing against the possibility that filensin is anchored to the membrane via a covalently-bound fatty acid moiety. This point is strongly supported by two other lines of evidence. First, filensin does not appear to bind significantly to vesicles reconstituted from lens lipids (this report). Second, the so far analyzed cDNA sequence reveals that filensin lacks motifs which are usually required for certain types of fatty-acylation (F. Gounari, A. Merdes, R. Quinlan, J. Hess, P., FitzGerald, C. Ouzounis and S. D. Georgatos, manuscript in preparation).

Filensin and a 51 kDa COOH-terminal filensin degradation product associate with intrinsic elements of the lens cell membranes

Surprising as it may be, results obtained by two different assay methods and quantitative binding measurements indicate that isolated filensin binds more avidly to trypsinized lens membranes than to non-trypsinized membranes. Because all binding assays have been performed in the presence of excess carrier protein (gelatin), and because filensin binding to trypsinized membranes shows a clear trend to saturation, artificial adsorption of filensin to "sticky" membrane surfaces generated by proteolysis can be safely ruled out. This is further supported by the fact that proteinases more "promiscuous" than trypsin (e.g., chymotrypsin) do not result in such an increase of the filensin-binding capacity. The difference in the binding capacities of trypsinized and non-trypsinized membranes seems to be due to the presense of an endogenous filensin degradation product which remains bound to the urea-stripped membranes and interferes with the binding of exogenously added filensin. The relatively low abundance of the filensin-related peptide in the lens membranes did not allow us to obtain more information about its identity by microsequencing techniques. However, because of its cross-reaction with polyclonal (affinity-purified) anti-filensin antibodies and anti-peptide antibodies recognizing authentic filensin sequences, we find it very likely that this protein represents indeed a filensin fragment. It remains to be examined why the 51 kDa species is not entirely extractable by urea or alkali, whereas the parent molecule is. One intriguing possibility may be that the 51 kDa product is further modified and that it associates with membrane components in more than one way.

At a first glance, it may seem paradoxical that a trace amount (i.e., undetectable with Coomassie blue staining) of the 51 kDa peptide which remains bound to the urea-stripped membranes can mask approximately 50% of the filensin-binding sites. However, one reasonable explanation for this could be that the membrane-binding capacities do not exactly correspond to the number of filensin-binding sites because a certain percentage of filensin is expected to be oligomeric or even polymeric under the isotonic conditions of the assays (see also Merdes et al. 1991). Thus, even a small quantity of the 51 kDa product may be sufficient to block most of the filensin-binding sites in non-trypsinized membranes.

In the same context, it is interesting to discuss another observation. Analyzing different parts of the lens tissue, we have recently found that intact filensin, although very abundant in the lens cortex, is totally absent in the region of the lens nucleus. However, when membranes from the LFCs which populate the nuclear region of the lens are isolated and probed with affinity-purified anti-FL 2 antibodies, we can readily detect the 51 kDa filensin degradation product (A. Merdes and S. D. G., unpublished observations). One

interpetation can therefore be that the 51 kDa peptide represents a naturally occurring processed form of filensin, which is generated by proteolytic cleavage of the parent polypeptide as the LFCs age and accumulate in the nuclear region. This processed form of filensin, presumably because it contains the membrane-binding domain of the original molecule, could still attach to the lens cell membrane. Age and position-associated proteolytic processing of lens membrane components has been previously observed, as in the case of the integral membrane protein MP70, which is found intact in the lens cortex and proteolytically cleaved to MP38 in the lens nucleus (Kistler and Bullivant, 1987).

Taking into account all the binding data, one may speculate that purified filensin binds specifically to an integral membrane protein which is trypsin-resistant, but thrombin and heat-sensitive. The idea that filensin can associate with intrinsic components of the lens cell membrane is consistent with previous observations, showing that a 100 kDa polypeptide and other proteins synthesized by in vitro translation of lens RNA bind to exogenously added urea-stripped membranes (Ramaekers et al., 1982). In any case, it is reasonable to assume that if a proteinaceous filensin "receptor" does exist in vivo it will probably be a low abundance species (since filensin self-assembles, even a low number of membrane attachment sites would suffice to dock a much greater number of filensin molecules to the membrane). Attempts to identify such a filensin-binding membrane protein by chemical cross-linking, affinity chromatography and ligand blotting have been so far unsuccessful. Future experiments, based on recombinant DNA approaches, may allow a better understanding of the filensin-membrane interactions and elucidation of its in vivo targeting to the plasma membrane.

Finally, the low (but measurable) binding of filensin to urea-extracted erythrocyte membranes may either represent "background", or be biochemically meaningful. Given that the lens cell membrane shares a number of components with the erythrocyte membrane (for example, the anion transporter, or band 3, molecule; Allen et al., 1987), the binding of filensin to the latter may involve an interaction with a protein homologous to its physiological lens membrane "receptor".

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This work is dedicated to Elias Brountzos.

References

Allen, D. P., Low, P. S., Dola, A. and Maisel, H. (1987). Band 3 and ankyrin homologues are present in the eye lens: evidence for all major erythrocyte membrane components in the same non-erythroid cell. *Biochem. Biophys. Res. Comm.* **149**, 266-275.

Aster, J. C., Brewer, G. J., Hanash, S. M. and Maisel, H. (1984a). Band 4.1-like proteins of the bovine lens. *Biochem. J.* **224**, 609-616.

Aster, J. C., Brewer, G. J. and Maisel, H. (1986). The 4.1-like proteins of the bovine lens: spectrin-binding proteins closely related in structure to the RBC protein 4.1. *J. Cell Biol.* **103**, 115-122.

Aster, J. C., Welsh, M. J., Brewer, G. J. and Maisel, H. (1984b). Identification of spectrin and protein 4.1-like proteins in mammalian lens. *Biochem. Biophys. Res. Commun.* **119**, 726-734.

Bennett, V. (1983). Proteins involved in membrane-cytoskeleton association in human erythrocytes: spectrin, ankyrin and band 3. *Methods Enzymol.* **96**, 313-324.

Berger, M. and Schmidt, M. F. G. (1984). Cell-free acid acylation of Semliki Forest viral polypeptides with microsomal membranes from eucaryotic cells. *J. Biol. Chem.* **259**, 7245-7252.

Bloemendal, H. (1981). The lens proteins. In *Molecular and Cellular Biology of the Eye Lens* (ed. H. Bloemendal), pp. 1-47. John Wiley & Sons, New York.

Fitzgerald, P. G. and Gottlieb, W. (1989). The Mr 115 kd fiber cell-specific protein is a component of the lens cytoskeleton. *Curr. Eye Res.* **8**, 801-811.

Folch, J., Lees, M. and Sloane Stanley, G. H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226**, 497-509.

Georgatos, S. D. and Marchesi, V. T. (1985). The binding of vimentin to human erythrocyte membranes: a model system for the study of intermediate filament-membrane interactions. *J. Cell Biol.* **100**, 1955-1961.

Georgatos, S. D., Weaver, D. C. and Marchesi, V. T. (1985). Site specificity in vimentin-membrane interactions: intermediate filament subunits associate with the plasma membrane via their head domains. *J. Cell Biol.* **100**, 1962-1967.

Georgatos, S. D., Weber, K., Geisler, N. and Blobel, G. (1987). 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. *Proc. Nat. Acad. Sci. U.S.A.* **84**, 6780-6784.

Granger, B. L. and Lazarides, E. (1984). Membrane skeletal protein 4.1 of avian erythrocytes is composed of multiple variants that exhibit tissue-specific expression. *Cell* **37**, 595-607.

Ireland, M. and Maisel, H. (1984). A cytoskeletal protein unique to lens fiber cell differentation. *Exp. Eye Res.* **38**, 637-645.

James, G. and Olson, E. N. (1990). Fatty acylated proteins as components of intracellular signaling pathways. *Biochemistry* **29**, 2623-2634.

Kistler, J. and Bullivant, S. (1987). Protein processing in lens intercellular junctions: cleavage of MP70 to MP38. *Invest. Ophthalmol. Vis. Sci.* **28**, 1687-1692.

Kistler, J. and Bullivant, S. (1989). Structural and molecular biology of the eye lens membranes. *CRC Crit. Rev. Biochem. Mol. Biol.* **24**, 151-181.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Magee, A. I., Gutierrez, L., McKay, I. A., Marshall, C. J. and Hall, A. (1987). Dynamic fatty acylation of p21 N-ras. *EMBO J.* **6**, 3353-3357.

Maisel, H. and Perry, M. M. (1972). Electron microscope observations on some structural proteins of the chick lens. *Exp. Eye Res.* **14**, 7-12.

Maisel, H. (1985). In *The Ocular Lens* (ed. H. Maisel). Marcel Dekker, Inc., New York.

Merdes, A., Brunkener, M., Horstmann, H. and Georgatos, S. D. (1991). Filensin: a new vimentin-binding, polymerization-competent, and membrane-associated protein of the lens fiber cell. *J. Cell Biol.* **115**, 397-410.

Ramaekers, F. C. S., Dunia, I., Dodemont, H. J., Benedetti, E. L. and Bloemendal, H. (1982). Lenticular intermediate-sized filaments: biosynthesis and interaction with plasma membrane. *Proc. Nat. Acad. Sci. U.S.A.* **79**, 3208-3212.

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