The 47-kD Lens-specific Protein Phakinin Is a Tailless Intermediate Filament Protein and an Assembly Partner of Filensin

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Abstract. In previous studies we have characterized a lens-specific intermediate filament (IF) protein, termed filensin. Filensin does not self-assemble into regular IFs but is known to associate with another 47-kD lens-specific protein which has been suggested to represent its assembly partner. To address this possibility, we cloned and sequenced the cDNA coding for the bovine 47-kD protein which we have termed phakinin (from the greek φακος = phakos = lens). The predicted sequence comprises 406 amino acids and shows significant similarity (31.3% identity over 358 residues) to type I cytokeratins. Phakinin possesses a 95-residue, non-helical domain (head) and a 311 amino acid long α-helical domain punctuated with heptad repeats (rod). Similar to cytokeratin 19, phakinin lacks a COOH-terminal tail domain and it therefore represents the second known example of a naturally tailless IF protein. Confocal microscopy on frozen lens sections reveals that phakinin colocalizes with filensin and is distributed along the periphery of the lens fiber cells. Quantitative immunoblotting with whole lens fiber cell preparations and fractions of washed lens membranes suggest that the natural stoichiometry of phakinin to filensin is ~3:1. Under in vitro conditions, phakinin self-assembles into metastable filamentous structures which tend to aggregate into thick bundles. However, mixing of phakinin and filensin at an optimal ratio of 3:1 yields stable 10-nm filaments which have a smooth surface and are ultrastructurally indistinguishable from "mainstream" IFs. Immunolabeling with specific antibodies shows that these filaments represent phakinin/filensin heteropolymers. Despite its homology to the cytokeratins, phakinin does not coassemble with acidic (type I), or basic (type II) cytokeratins. From these data we conclude that filensin and phakinin are obligate heteropolymers which constitute a new membrane-associated, lens-specific filament system related to, but distinct from the known classes of IFs.

THE eye lens represents a transparent and avascular tissue which contains two cell populations: (*a*) cuboidal epithelial cells organized as a monolayer at the anterior surface of the organ; and (*b*) elongated lens fiber cells (LFCs)[1] arranged in concentric layers and extending from the anterior to the posterior surface of the organ. The LFCs originate from the lens epithelium. Upon further differentiation, they acquire a hexahedral shape, develop an extensive system of intercellular junctions, accumulate crystallins, and lose their nuclei. The cytoskeleton and the membrane–skeleton of the cortical LFCs contain certain "generic" components, such as actin, vimentin, fodrin, and protein 4.1 (Ramaekers et al., 1980; Granger and Lazarides, 1984; Aster et al., 1984*a*,*b*, 1986). However, LFCs also contain some unique cytoskeletal structures, the so-called "beaded-chain filaments" (BFs) (Maisel and Perry, 1972).

We have previously characterized filensin, a 110-kD protein associated with the plasma membrane of the LFCs in mammalian lens (Merdes et al., 1991; Brunkener and Georgatos, 1992). Exchange of antibodies has shown that filensin is antigenically similar to CP115, a 115-kD polypeptide thought to be a component of the BFs in bovine lens (FitzGerald and Gottlieb, 1989; and our own unpublished observations). cDNA cloning and sequencing of the mammalian (Gounari et al., 1993) and avian (Remington, 1993) analogues of filensin has revealed that it represents a remote member of the intermediate filament (IF) family with an unusually short rod domain.

Purified filensin assembles into short 10-nm fibrils which are morphologically distinct from "mainstream" IFs but resemble the structures formed by isolated neurofilament-M and -H subunits (Merdes et al., 1991). The same protein

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

binds under in vitro conditions to the IF subunit vimentin and to a 47-kD lens-specific cytoskeletal protein (Merdes et al., 1991). Binding of filensin to vimentin involves the COOH-terminal domain of the latter and does not result in copolymerization (Merdes et al., 1991; and our own unpublished observations). However, there are indications that filensin may coassemble with the 47-kD protein. This is because the avian form of filensin (formerly CP95) has been shown to copurify with a 49-kD BF protein (CP49) which probably represents the avian analogue of the 47-kD polypeptide (Ireland and Maisel, 1984).

Sequencing of a partial cDNA clone coding for the murine analogue of CP49 has already provided hints that this protein shares sequence homology with IF proteins (Hess et al., 1993). To obtain more information, we have determined the primary structure of the entire 47-kD protein, which we have termed phakinin. Sequence analysis reveals that phakinin lacks a tail domain but contains a canonical IF-rod domain showing significant similarity to type I cytokeratins. Furthermore, in vitro reconstitution and in situ studies suggest that phakinin forms obligate heteropolymers with filensin. Phakinin does not copolymerize with acidic or basic cytokeratins and does not conform to any of the known types of IF proteins.

Materials and Methods

Protein Chemical Procedures

Phakinin was purified from bovine lenses essentially as described by Ihl (1989). In brief, cortical regions of adult lenses were excised and homogenized in 2.5 mM NaP_i, pH 7.5, 1 mM EDTA, 10 mM β-mercaptoethanol, 0.5 mM PMSF at 4°C. The homogenate was spun at 12,100 *g* and the pellet washed with homogenization buffer containing 1% Triton X-100. The detergent-insoluble material was extracted with 6 M urea, 20 mM Tris-acetate, pH 7.5, 5 mM DTT, 1 mM EDTA, and 1 mM PMSF. The clarified extract was loaded onto a DEAE-cellulose column (DE 53, Whatman, Maidstone, GB) and the bound material was eluted with a gradient of 0–100 mM KCl in the 6 M urea buffer. Phakinin-containing fractions (eluted at ~40 mM KCl) were pooled and further chromatographed in a hydroxylapatite column (Bio Rad Laboratories, Richmond, CA). The column was eluted with a gradient of 0–125 mM KP_i in 6 M urea buffer without EDTA. The isolation of bovine filensin has been described in Merdes et al. (1991). Purified cytokeratins 8 and 18 were kindly provided by M. Hatzfeld (Max Planck Institute for Biophysical Chemistry, Göttingen, F.R.G.).

Immunochemical Methods

Polyclonal antibodies were prepared by immunizing rabbits with electrophoretically pure phakinin. For affinity-isolation of the antibodies, phakinin was subjected to SDS-PAGE, transferred onto nitrocellulose filters, and incubated with the anti-phakinin antiserum. The bound antibodies were eluted with 200 mM glycine-HCl, pH 2.3, and rapidly neutralized with base. The construction of the anti-filensin-peptide antibodies has been described (Gounari et al., 1993). The antibodies were characterized by immunoblotting and indirect immunofluorescence assays. For quantitative immunoblotting, the cortical region of bovine lens was dissected and either boiled in 4 × Laemmli sample buffer (Laemmli, 1970), or homogenized and further extracted with isotonic buffer (see Merdes et al., 1991). Increasing amounts of these preparations, in parallel with known quantities of purified filensin and phakinin, were analyzed by SDS-PAGE and immunoblotted with anti-filensin and anti-phakinin antibodies and ^{125}I-protein A. The blots were exposed on x-ray film, the bands corresponding to the two proteins excised, and the radioactivity associated with each band measured by γ counting. A "standard curve" was constructed, correlating the amount of radioactivity in each band with the amount of protein loaded on the gel. The relative amounts of filensin and phakinin in the lens preparations were calculated from this plot.

Cloning and Sequencing of Phakinin cDNAs

A calf lens λgt11 cDNA library was provided by H. Bloemendal (Nijmegen, NL). Screening of 10^6 plaques was performed with an anti-phakinin antiserum (see above), preabsorbed against *E. coli* lysates and diluted 1:200, as described in Sambrook et al. (1989). First strand cDNA for PCR was prepared from total lens RNA using AMV reverse transcriptase (Boehringer, Mannheim, F.R.G.) according to the conditions recommended by the supplier. The PCR step was carried out using the downstream primer 5'TGCAGCTGGCATTTGTGCGAC 3' (47K4) in combination with the 5'CGGACGACTTTAAGGAGAGGTACG 3' (47K1) upstream primer.

Sequence Analysis and Multiple Alignment

Database searches were performed with the FASTA version 1.6c (Pearson and Lipman, 1988). The SWISSPROT sequence database release 24 was used (Bairoch and Boeckmann, 1991). A multiple alignment of 80 complete rod domains of IF proteins, including filensin and phakinin, was constructed using the PILEUP of the GCG sequence analysis software package version 7.1 (Devereux et al., 1984). The parameters for gap open (3.1) and gap elongation (0.1) were used. The amino acid comparison matrix was the normalized Dayhoff matrix (Gribskov and Burgess, 1986). Secondary structure prediction of bovine phakinin was performed according to Chou and Fasman (1978).

Indirect Immunofluorescence and Immunoelectron Microscopy

Indirect immunofluorescence microscopy was performed as specified in Merdes et al. (1991). Double immunolabeling was performed using the rabbit anti-filensin antibody aFL 2 (Brunkener and Georgatos, 1992; Gounari et al., 1993) and the anti-phakinin mAb aCP49 (kindly provided by P. Fitz-Gerald, University of California, Davis, CA). Vimentin was stained with the mAb 7A3 (Papamarcaki et al., 1991). For whole mount immunoelectron microscopy, the filaments were decorated on the grid after fixation with 8% formaldehyde in 250 mM Hepes, pH 7.4, for 1 min, and blocking with 0.5% gelatin in PBS, for 15 min. First, anti-filensin antibody (aFL-2, see Gounari et al., 1993) was applied at a dilution of 1:40 for 15 min. After three washes with blocking buffer, the samples were further incubated with protein A-gold (5 nm). Unbound protein A-gold was washed off on three drops of buffer, and the samples were fixed for 1 min with 1% glutaraldehyde, to block free IgG-binding sites (modified from Slot and Geuze, 1985). After quenching with 20 mM glycine in PBS, affinity purified polyclonal anti-phakinin antibody (approximate concentration 200 μg/ml) was applied for 15 min. After washing, protein A-gold (14 nm) was applied as above. The specimens were washed several times and negatively stained with 2% uranyl acetate.

In Vitro Assembly

Purified phakinin was dialyzed from 6 M urea to 170 mM KCl, 20 mM Tris/HCl, pH 7.4, 0.1 mM EGTA, 1 mM $MgCl_2$, 1 mM DTT, and 0.5 mM PMSF (isotonic buffer), on ice, for 1–2 h. Dialysis was continued at 37°C, in the same buffer, for various periods of time. Heteropolymers of phakinin and filensin were reconstituted as follows. First, the two proteins were equilibrated independently in 9 M urea, 20 mM Tris/HCl, pH 7.4, 0.1 mM EGTA, 1 mM $MgCl_2$, 1 mM DTT, and 0.5 mM PMSF. Then, they were mixed at different ratios and dialyzed against isotonic buffer. The specimens were applied to collodium/carbon coated copper grids, negatively stained with 2% uranyl acetate, and visualized in a Philips EM 301 microscope. Assembly of cytokeratins 8 and 18 and coassembly experiments with cytokeratins and phakinin involved dialysis of urea-solubilized preparations against 50 mM Tris-Cl, pH 7.5, and 5 mM EDTA, at room temperature. For recycling filensin/phakinin heteropolymers, purified filensin and phakinin were first equilibrated in the 9 M urea buffer. The proteins were then mixed at different ratios and reconstituted in isotonic buffer, as described above. Filament formation was monitored by negative staining and EM. Finally, the heteropolymers were pelleted at 109,000 *g* for 53 min, solubilized in 9 M urea, and dialyzed again against isotonic buffer. The recycling procedure was repeated three times. Aliquots of the pellets and supernatants were analyzed by SDS-PAGE.

Results

Isolation of Phakinin cDNA Clones

Probing of a calf lens expression library with the anti-phakinin serum yielded 57 positive clones. Analysis of these isolates revealed several overlapping clones from which we deduced a continuous 708-bp nucleotide sequence (PK1). PK1 contained an initiation codon at position 82–84, followed by an open reading frame coding for 208 amino acids. The last 112 amino acids of the translated PK1 sequence showed significant sequence homology to IF proteins (see below). Upstream of the first ATG there were three in frame termination codons at positions 23–25, 32–34, and 59–61.

Two other clones (both identical and termed PK14) overlapped at their 5′ ends with a stretch of PK1 located between nucleotides 315–363. However, the 3′ end of the PK14 clones diverged from the 3′ sequence of PK1. When we translated the divergent region of PK14 and compared it with sequences of the SWISSPROT database, we noticed that the predicted sequence EAEQQLQAREHLLSHKCQLQTDVASYHALL-DREESS showed significant homology to the coil 2b region of IF proteins. Immediately after this region, the clone PK14 had an in-frame termination codon, followed by two more termination codons further downstream. Because Northern blot analysis with a PK1-cDNA probe ruled out the presence of alternatively spliced phakinin mRNAs, we reasoned that the PK14 clones contained an internal deletion. To obtain the missing sequence, we used a PCR approach using a sense oligonucleotide primer from the 3′ of the PK1 sequence and an antisense primer corresponding to sequences of the divergent part of the PK14 clones (see Materials and Methods). This PCR step yielded a 642-bp product (PK2) which covered the sequence missing from PK1 and PK14. The assembled cDNA sequence comprised 1373 bp, of which 1218 bp represented coding region. The predicted sequence of phakinin was confirmed by microsequencing tryptic peptides obtained from purified protein (see below). The calculated molecular weight of phakinin amounted to 45.5 kD, in reasonable agreement with the apparent molecular weight estimated by SDS-PAGE (47 kD).

Northern blot analysis using RNA from various bovine tissues (not shown; for relevant information see Hess et al., 1993) indicated that phakinin is exclusively expressed in the lens but not in other organs or a cell line derived from bovine lens epithelial cells (Ramaekers et al., 1980).

Sequence Analysis and Homology of Phakinin to Intermediate Filament Proteins

The predicted amino acid sequence of phakinin (Fig. 1 *B*) was compared to the Swissprot protein database (see Materials and Methods). The first 80 entries with significant homology to it were IF proteins. Phakinin showed highest homology to type I cytokeratins (the first 14 entries in the homology chart) and, by alignment with the proteins of this class, could be divided into two distinct regions (head and rod domain, respectively; see Fig. 1 *A*). The bovine phakinin sequence exhibited 79.9% identity (at the amino acid level) with the partial sequence of the murine 49-kD protein (Hess et al., 1993; see introduction). From this we conclude that phakinin is the bovine homologue of murine CP49.

The head domain of phakinin consisted of 95 amino acids and showed no significant sequence similarity to other protein sequences available in the database. Secondary structure prediction suggested that this region may contain multiple β turns between amino acids 24 and 69. The segment with the predicted turns contained six aromatic residues arranged at positions 25, 33, 40, 55, 59, and 75 (Fig. 1 *B*). Stacking of the phenyl rings of these residues may stabilize the structure of the protein in this region (see Burley and Petsko, 1985).

The rod domain of phakinin comprised 311 amino acids, had the classical features of an IF rod (for an alignment see

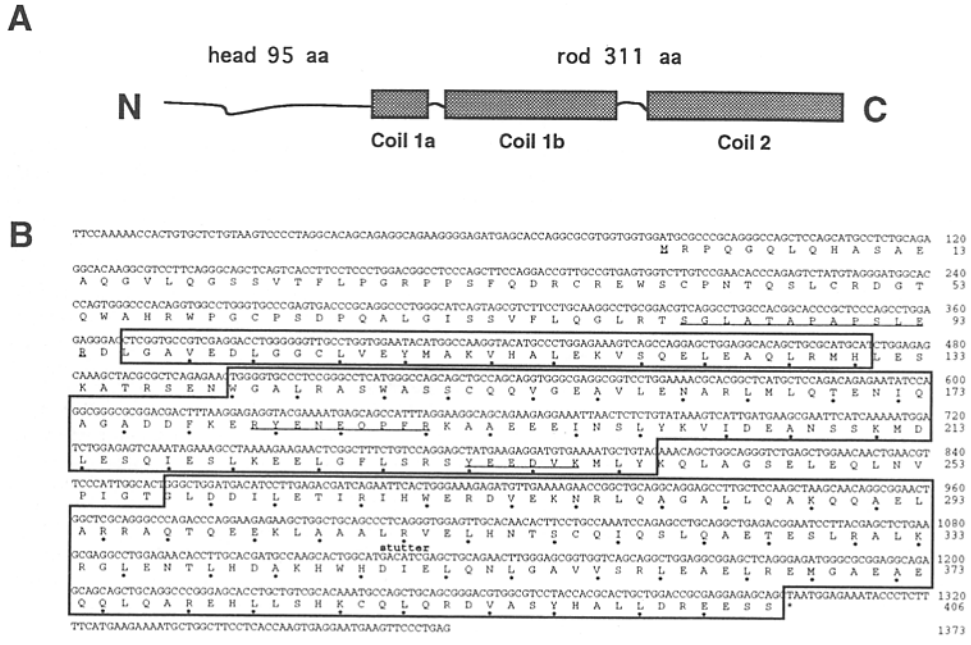

Figure 1. (*A*) Predicted domain structure of bovine phakinin. The drawing shows the two domains of phakinin (head to rod, respectively). (*B*) Nucleotide and predicted amino acid sequence of bovine phakinin. The first methione, as well as the peptide sequences obtained by analysis of purified bovine phakinin, are underlined. In the rod domain, the a and d positions of the heptad repeats are indicated by dots, while the three helical domains are enclosed in boxes. *Stutter* denotes the heptad repeat stutter in the region of coil 2. An asterisk denotes the in-frame termination codon at the 3′ of the cDNA. These sequence data are available from EMBL/GenBank/DDBJ under accession number X75160.

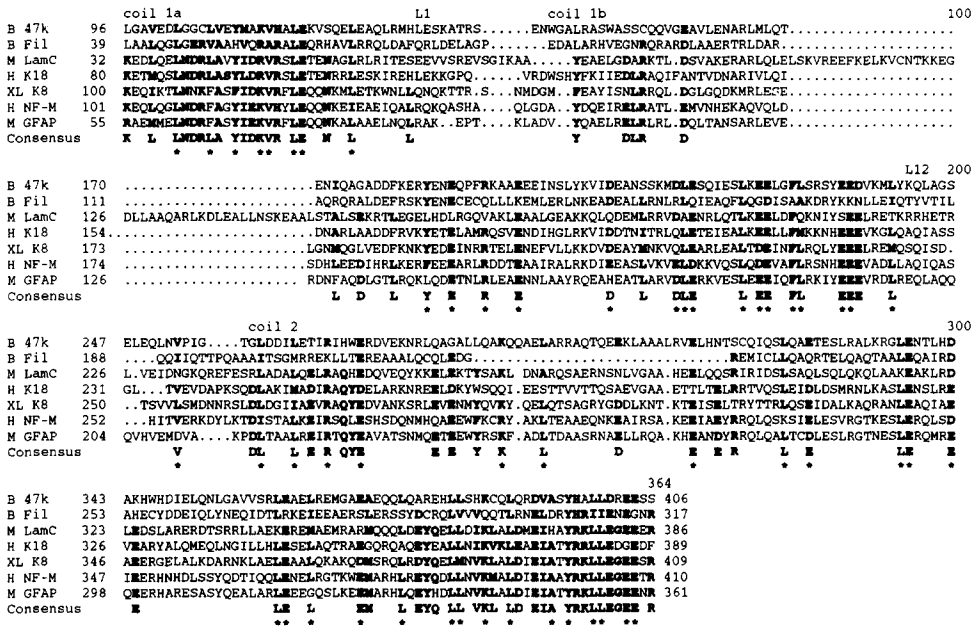

Figure 2. Sequence alignment of bovine phakinin with other IF proteins in the region of the rod domain. The rod domains of 78 IF proteins, as well as those of filensin and phakinin, were aligned as described in Materials and Methods. Representative examples of this alignment are shown here. *B 47k*, bovine phakinin; *B fil*, bovine filensin (Gounari et al., 1993); *M LamC*, mouse lamin C (Riedel and Werner, 1989); *H K18*, human cytokeratin 18 (Oshima et al., 1986); *XL K8*, *Xenopus laevis* keratin 8 (Franz and Franke, 1986); *H NF-M*, human neurofilament M protein (Myers et al., 1987); and *M GFAP*, mouse glial fibrillary acidic protein (Balcarek and Cowan, 1985). Positions which share the same residues in 2/3 of the 80 entries were included in the consensus. Amino acid positions of the consensus sequence also found in phakinin are indicated by asterisks; identities and conservative substitutions within the consensus are shown in bold.

Fig. 2) and showed the highest sequence similarity to human cytokeratin 18 (32.5% identity over an alignment length of 308 amino acids). By secondary structure prediction and alignment to other IF sequences, the rod could be divided into three α-helical subdomains: coil 1A (35 amino acids), coil 1B (100 amino acids), and coil 2 (149 amino acids), possessing the characteristic heptad repeated pattern of coiled-coil proteins. The coil 2 region also exhibited the characteristic "stutter" between amino acids 347 and 351, found in the corresponding regions of IF proteins. The helical subdomains were joined together by two non-helical linkers, L1 (10 amino acids, linking coil 1A to coil 1B) and L 12 (17 amino acids, linking coil 1B to coil 2). A termination codon, located immediately after the last amino acid of the coil 2 subdomain, indicated that phakinin is a completely tail-less If protein.

Location of Phakinin In Situ

To localize phakinin, we examined lens cryosections by indirect immunofluorescence and confocal microscopy. As shown in Fig. 3, *A* and *B*, phakinin was codistributed with filensin along the periphery of the lens fiber cells. In comparison, vimentin could be localized at both the periphery of the LFCs and the cytoplasm (Fig. 3, *C* and *D*), consistent with previously published results (Ramaekers et al., 1982; Merdes et al., 1991), which show that the *trans*-cytoplasmic vimentin network is anchored to the plasma membrane. These data suggested that phakinin and filensin may form a copolymer in situ.

In Vitro Assembly Properties of Phakinin

To examine the polymerization competence of phakinin, we isolated it from lens urea extracts using standard chromatorphic techniques (for an SDS-PAGE profile see Fig. 4 *F*, lane *I*). Phakinin was slightly contaminated with a 40-kD degradation product cross-reactive with anti-phakinin antibodies. The purified protein was dialyzed against an isotonic buffer at 0°C. Under these conditions, phakinin formed thin filamentous structures (mean diameter 7–8 nm, Fig. 4 *B*) resembling assembly intermediates of IFs. Upon prolonged dialysis, or after raising the temperature to 37°C, the filaments aggregated and became densely packed (Fig. 4 *A*). The phakinin aggregates were clearly different from the short 10-nm fibrils assembled from purified filensin after dialysis against the same assembly buffer (Fig. 4 *C*).

Ligand blotting experiments had previously shown that phakinin binds to filensin (Merdes et al., 1991). We therefore investigated whether the two proteins could coassemble. Dialysis of a mixture of phakinin and filensin (at a roughly equimolar ratio) against isotonic buffer yielded long filaments (Fig. 4 *D*). These assemblies were markedly different from the filensin fibrils and the phakinin aggregates formed under the same conditions from each protein alone. Since these filaments could be decorated with anti-phakinin and anti-filensin antibodies (see below), we concluded that the two proteins were able to copolymerize. However, closer inspection of the assembly mixtures by EM revealed the presence of numerous rod-like particles (Fig. 4 *D*, *arrows*) which coexisted with the filensin/phakinin filaments. We therefore suspected that one of the two components was not fully incorporated into the polymer. To improve the incorporation of the subunits into filaments, or remove the unincorporated material, we subjected the mixture of phakinin and filensin to cycles of polymerization and depolymerization, as explained in Materials and Methods. After recycling, the heterotypic filaments had a diameter of ~10 nm and a smooth surface (Fig. 4 *E*). Closer inspection of the recycled material showed that the incorporation of subunits into the copolymer was better; however, there were still some rod-like fibrils which probably cosedimented with the heteropolymers.

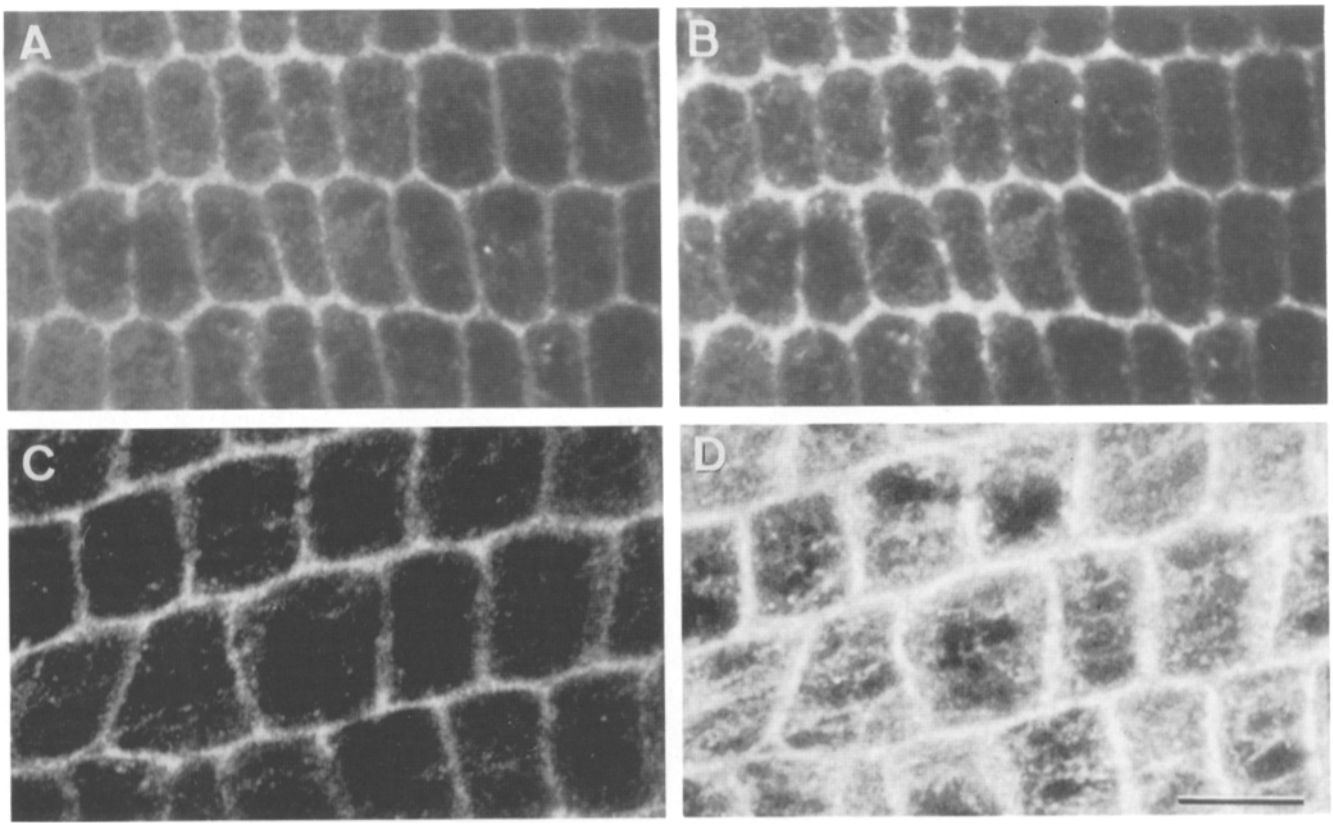

Figure 3. Double-labeling immunofluorescence microscopy on frozen lens sections. *A* and *B* correspond to a section decorated with anti-filensin and anti-phakinin antibodies, respectively. *C* and *D* correspond to a section decorated with anti-filensin and anti-vimentin antibodies, respectively. Note the colocalization of filensin and phakinin along the periphery of the lens fiber cells. Bar, 10 μm.

To better define the optimal conditions for in vitro reconstitution of the copolymers, we mixed phakinin and filensin in several different ratios and analyzed the products by EM. As it could be seen in Fig. 5 *A*, when the two proteins were mixed in a 1:1 stoichiometry, apart from heteropolymeric 10-nm filaments, there was an abundance of short fibrils scattered in the fields. These fibrils represented filensin oligomers and could be decorated with anti-filensin antibodies (not shown). The same was observed in samples containing a phakinin to filensin ratio of 2:1 (Fig. 5 *B*). However, when the ratio was increased to 3:1, there were no detectable filensin oligomers, the phakinin/filensin filaments grew longer and formed dense networks (Fig. 5 *C*). Labeling with anti-filensin and anti-phakinin antibodies revealed that the reconstituted assemblies contained both proteins over their entire length (Fig. 5 *H*). Finally, mixing the two proteins in ratios higher than 3:1, yielded primarily aggregates which were identical to the phakinin homopolymers (compare Fig. 5, *D* and *E* with Fig. 4 *A*). These titration experiments suggest that phakinin and filensin incorporate fully into the heteropolymer when mixed in a 3:1 molar ratio.

To examine the relevance of these in vitro results to the in vivo situation, we next determined the relative abundance of phakinin and filensin in the eye lens. To accomplish this, we analyzed homogenates and washed plasma membranes from cortical LFCs by quantitative immunoblotting. We used cortical LFCs instead of total lens because the central part of the organ (nucleus) is almost devoid of intact filensin and phakinin and contains exclusively their degradation products (Merdes, A., F. Gounari, and S. D. Georgatos, unpublished observations; see also FitzGerald, 1988). As demonstrated in Table I, the molar ratio of phakinin to filensin varied from

Table I. Determination of the In Vivo Abundance of Phakinin and Filensin in Cortical LFCs by Quantitative Immunoblotting

	Sample 1	Sample 2	Sample 3	Mean
Homogenates boiled in SDS-sample buffer:				
Volume of homogenate	10 μl	20 μl	40 μl	
Phakinin	1.70 μg	3.48 μg	7.80 μg	
Filensin	1.13 μg	2.16 μg	4.53 μg	
Molar ratio Pha./Fil.	2.74:1	2.94:1	3.14:1	2.9:1
LFC membranes washed with isotonic buffer:				
Amount of protein	6.90 μg	13.70 μg	27.50 μg	
Phakinin	0.78 μg	2.90 μg	6.10 μg	
Filensin	0.40 μg	1.49 μg	2.80 μg	
Molar ratio Pha./Fil.	3.56:1	3.55:1	3.97:1	3.7:1

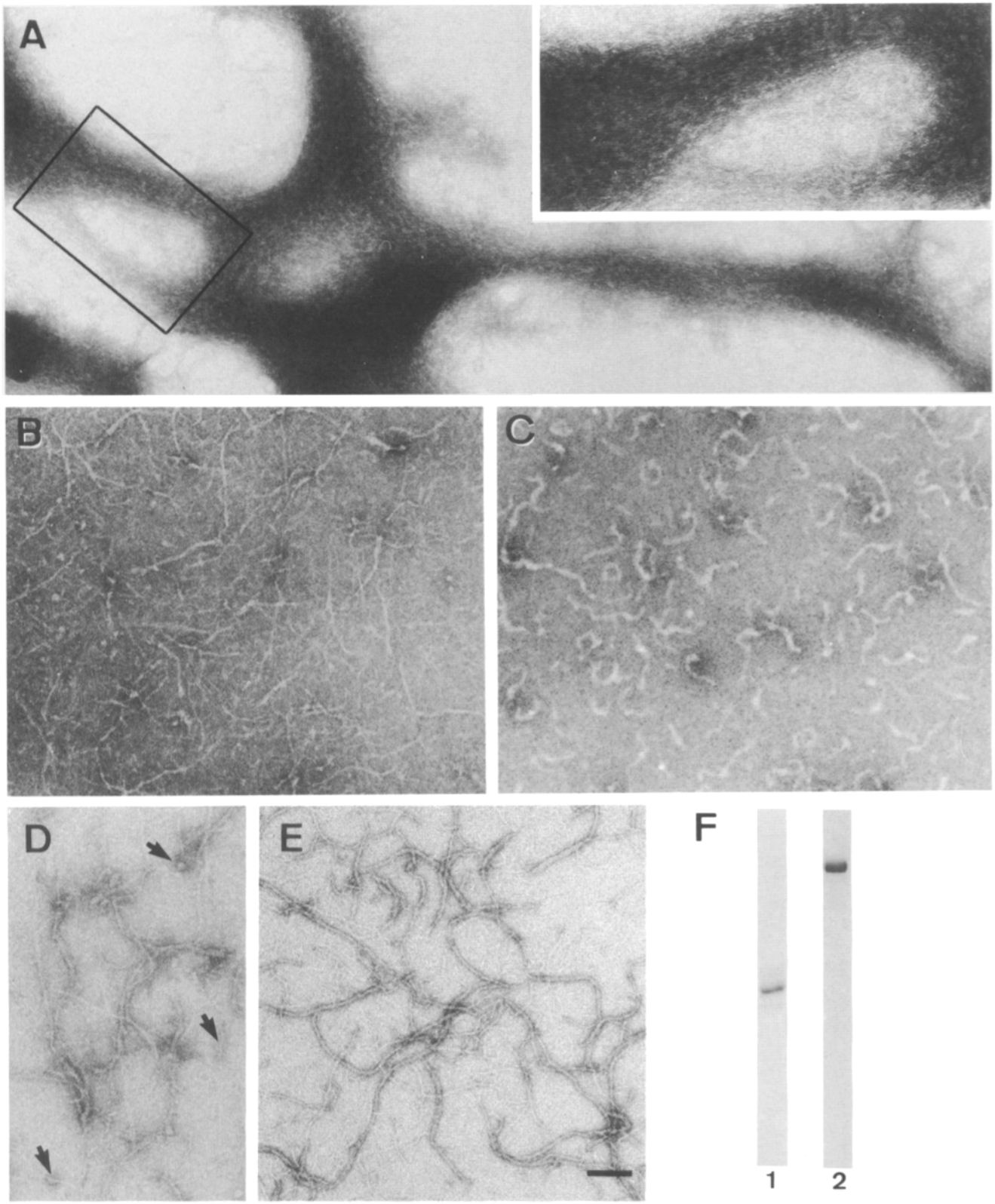

Figure 4. Assembly properties of phakinin and filensin. (*A*) Isolated phakinin reconstituted in isotonic buffer at 37°C. Note the aggregates formed under these conditions. The inset shows a higher magnification of the area included in the frame, revealing the fibrous texture of the aggregates. (*B*) Metastable phakinin fibers (~7–8 nm in diameter) obtained when phakinin is reconstituted in isotonic salt at 0°C. (*C*) Purified filensin, reconstituted as in *A*. (*D*) Mixing of phakinin and filensin at an equimolar ratio and reconstitution as in *A*. Note the existence of long filaments which are different from the filensin fibrils and the phakinin aggregates. Also note the short, rod-like particles in the background (*arrows*) which represent unincorporated material. (*E*) Mixing of phakinin and filensin as in *D* and recycling of the preparation as specified in Materials and Methods. Note that the morphological appearance of the phakinin/filensin heteropolymers is more homogeneous after recycling. (*F*) SDS-PAGE profile of purified phakinin (lane *1*) and filensin (lane *2*) used in the coassembly studies. The phakinin preparation contains traces of endogenous degradation products which could not be completely removed by the methods used. Bar, 100 nm.

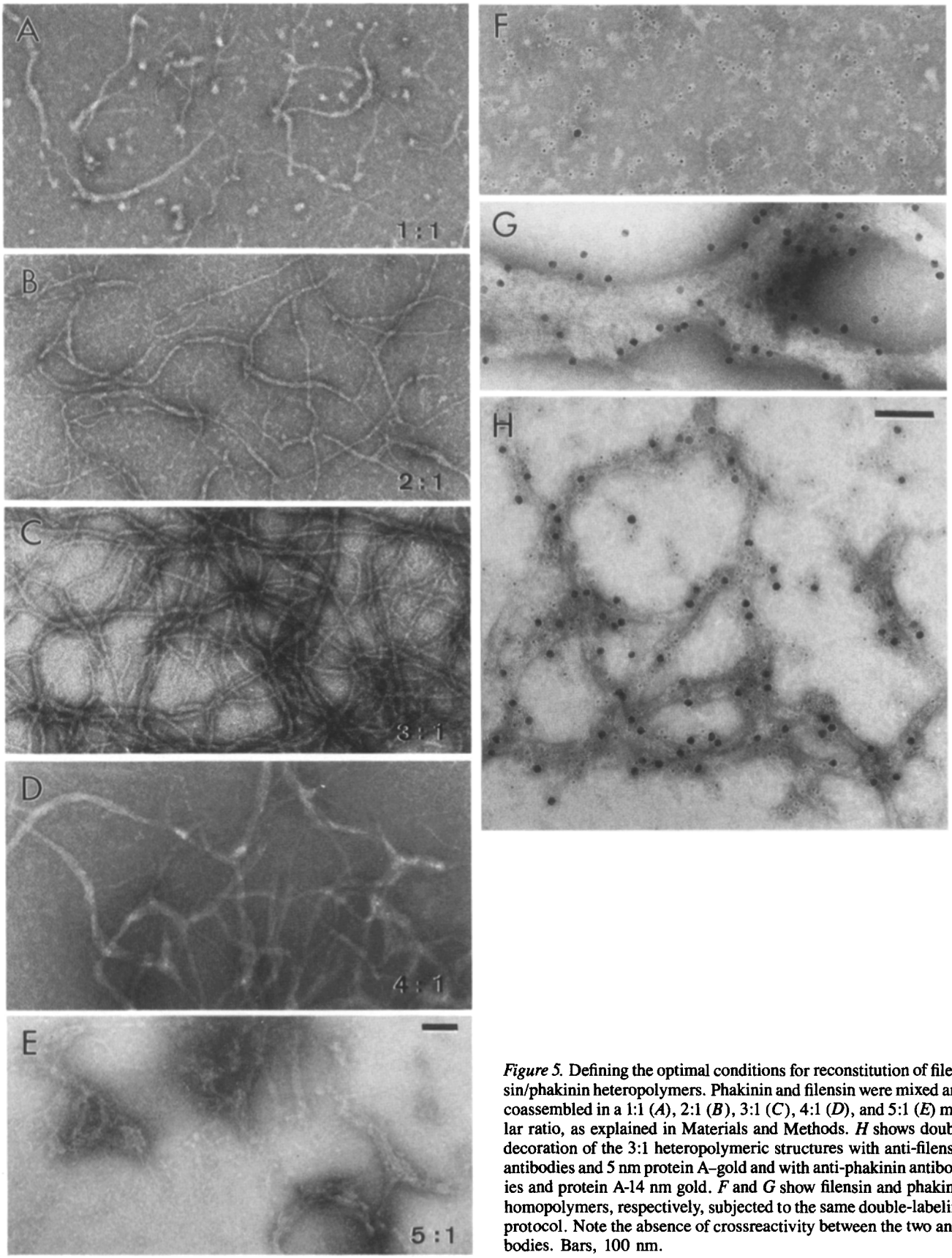

Figure 5. Defining the optimal conditions for reconstitution of filensin/phakinin heteropolymers. Phakinin and filensin were mixed and coassembled in a 1:1 (*A*), 2:1 (*B*), 3:1 (*C*), 4:1 (*D*), and 5:1 (*E*) molar ratio, as explained in Materials and Methods. *H* shows double decoration of the 3:1 heteropolymeric structures with anti-filensin antibodies and 5 nm protein A–gold and with anti-phakinin antibodies and protein A-14 nm gold. *F* and *G* show filensin and phakinin homopolymers, respectively, subjected to the same double-labeling protocol. Note the absence of crossreactivity between the two antibodies. Bars, 100 nm.

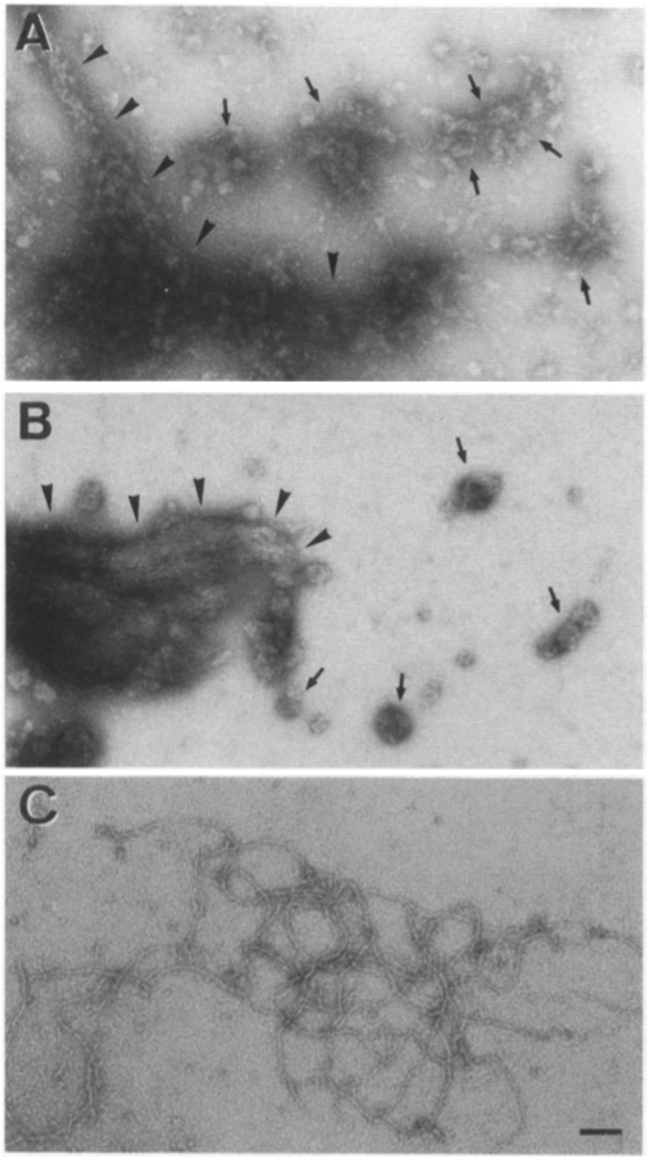

Figure 6. Phakinin does not coassemble with cytokeratins. Equimolar mixtures of phakinin and cytokeratin 18 (*A*) or cytokeratin 8 (*B*) were codialyzed from 9 M urea into 50 mM Tris-Cl, pH 7.5, and 5 mM EDTA. The proteins do not co-polymerize into filaments, but assemble into separate structures which resemble the characteristic phakinin bundles (*arrowheads*) and the "globular" cytokeratin aggregates (*arrows*) formed by each protein alone (data not shown). Mixtures of the two cytokeratin proteins (*C*), or phakinin and filensin (not shown) form extended filamentous structures when assembled under the conditions described above. Bar, 100 nm.

2.9:1 (in whole LFCs) to 3.7:1 (in isotonic buffer-washed membranes). Thus, within experimental error, the stoichiometry of phakinin and filensin in vivo was similar to the one determined by in vitro reconstitution experiments.

Phakinin Does Not Coassemble with Cytokeratins

Due to the similarity of phakinin with the type I cytokeratins, we also examined whether phakinin can form hybrid IFs with type II or type I cytokeratins. When phakinin was codialyzed with an equimolar amount of purified cytokeratin 8 (type II) or cytokeratin 18 (type I) under conditions supporting cytokeratin assembly (50 mM Tris, pH 7.5, + 5 mM EDTA [Hatzfeld and Weber, 1990]; or 10 mM Tris, pH 7.2, + 10 mM β-mercaptoethanol [Coulombe et al., 1990]), no regular IFs were assembled (Fig. 6, *A* and *B*). Instead, the cytokeratins and phakinin formed separate aggregates which were identical to those formed by each protein alone. The same results were obtained when we combined the phakinin and the cytokeratins in a 3:1 molar ratio (not shown). From these experiments we concluded that phakinin does not complement type II or type I cytokeratins.

In addition, we tested whether phakinin can assemble with the other lens IF-protein vimentin (not shown). Similar to cytokeratins, vimentin failed to copolymerize with phakinin and did not bind to it in ligand-blotting assays.

Discussion

We have cloned and sequenced the cDNA coding for phakinin, a novel IF protein exclusively expressed in the eye lens. Our results extend the previously published observations of Hess et al. (1993) and show that phakinin (also termed CP49) is an IF protein. Unlike filensin, phakinin contains a canonical rod domain of 311 amino acids and shows a clear structural kinship to type I cytokeratins. However, phakinin does not functionally complement type II or type I cytokeratins.

An unusual feature of this protein is the complete lack of a tail domain. Whereas other tailless intermediate filament proteins (such as cytokeratin 19; Bader et al., 1986) contain at least 10 amino acids after the end of the rod domain, the physical COOH terminus of phakinin coincides with the end of coil 2. When reconstituted into isotonic buffers, phakinin self-assembles into metastable filamentous structures which further aggregate into thick bundles. A similar peculiarity has been noted among some IF proteins which have been rendered tailless by proteolysis (Kaufmann et al., 1985; Nakamura et al., 1993), or have been mutagenized at the tail domain (Kouklis et al., 1993). This may reflect a role of the COOH-terminal domain in the lateral packing of IFs (for a discussion see Kouklis et al., 1991, 1993; for other views see Eckelt et al., 1992).

When combined with its natural partner (filensin), phakinin forms regular 10-nm filaments. If the slight contamination of phakinin by one of its degradation products does not influence its in vitro behavior, one may conclude that phakinin and filensin incorporate into the heteropolymer in a 3:1 molar ratio. One may speculate that phakinin represents the "core polymer" in these assemblies, whereas filensin constitutes the "business end" of the heterotypic filaments. This provisional interpretation is supported by two sets of observations: (*a*) phakinin could form (albeit transiently) some long filamentous structures by itself, whereas filensin polymerizes only into short fibrils; and (*b*) filensin (but not phakinin) has the capacity to bind to vimentin IFs (Merdes et al., 1991). However, vimentin does not co-polymerize with either of these two components.

From the data described here, we conclude that filensin and phakinin, similarly to the cytokeratins, constitute obligate heteropolymers. However, we find it rather unlikely that these lens proteins form coiled-coil heterodimers similar to those formed by the cytokeratins (Hatzfeld and Weber,

1990; Steinert, 1990; Coulombe and Fuchs, 1990). First, the lens IF proteins do not seem to associate in a 1:1 ratio, as it would be expected from a coiled-coil heterodimer. Second, whereas both proteins can be efficiently cross-linked into homodimers, so far we have not detected specific heterodimers under the same conditions (Merdes, A., and S. D. Georgatos, unpublished observations). Third, "native" immunoprecipitation of in vitro–translated filensin does not yield coimmunoprecipitation of phakinin (Gounari et al., 1993), as one would expect from stable and strongly associated (hetero)dimers. Finally, the filensin rod (279 amino acids) is missing 29 amino acids in the first half of the coil 2 region (Gounari et al., 1993); therefore, a parallel and in-register arrangement with the phakinin rod (311 amino acids) would not be physically possible.

Several reports have proposed that filensin and phakinin homologues are the components of a lens-specific structure called "beaded filament" (BF) (Ireland and Maisel, 1984; Alcala and Maisel, 1985; FitzGerald and Gottlieb, 1989; Lieska et al., 1991). However, other reports have suggested that the backbone of BFs consists of actin (Bloemendal, 1977; Bradley et al., 1979*a*,*b*), whereas the beads represent α crystallins or clusters of ribosomes (for an overview see Benedetti et al., 1981). We favor the first interpretation, although the heteropolymers reconstituted from purified filensin and phakinin are smooth and nonbeaded. The only reasonable explanation for the absence of "beads" would be that crude BF preparations isolated from lens tissue contain (specifically or nonspecifically) bound elements. Alternatively, the "beading" may not be detectable under our assembly conditions and it may require more precise mass/unit length measurements in order to become apparent. In either case, the sequence data and the assembly properties of filensin and phakinin make it clear that these two proteins form a new type of lens-specific IFs.

This work is dedicated to B. Santos.

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Note Added in Proof. In a recent report (Orii, H., K. Agata, K. Sawada, G. Eguchi, and H. Maisel. 1993. *Curr. Eye Res.*), a partial sequence of chick CP49 has been described which possesses 68% identity to bovine phakinin and seems to be the chick homologue of this protein.

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