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Contributions of the structural domains of filensin in polymer formation and filament distribution

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SUMMARY

Filensin and phakinin constitute the subunits of a heteropolymeric, lens-specific intermediate filament (IF) system known as the beaded-chain filaments (BFs). Since the rod of filensin is four heptads shorter than the rods of all other IF proteins, we decided to examine the specific contribution of this protein in filament assembly. For these purposes, we constructed chimeric proteins in which regions of filensin were exchanged with the equivalent ones of vimentin, a self-polymerizing IF protein. Our in vitro studies show that the filensin rod domain does not allow homopolymeric filament elongation. However, the filensin rod is necessary for co-polymerization of filensin with phakinin and seems to counteract the inherent tendency of the latter protein to homopolymerize into large, laterally associated filament bundles. Apart from the rod domain, the presence of an authentic or substituted tail domain in filensin is also essential for co-assembly with the naturally tail-less phakinin and formation of extended filaments in vitro. Finally, transfection experiments in CHO and MCF-7 cells show that the rod domain of filensin plays an important role in de novo filament formation and distribution. The same type of analysis further suggests that the end-domains of filensin interact with cell-specific, assembly-modulating factors.

Key words: Intermediate filament, Beaded filament, Swapping mutant

INTRODUCTION

The superfamily of intermediate filament (IF) proteins includes more than 50 polypeptides in humans. The members of this family share several structural features. A typical IF protein consists of highly variable NH_2-terminal head and COOH-terminal tail domains and a largely α-helical central domain (rod) with conserved size and organization. The rod domain is subdivided into four helical segments (coils 1a, 1b, 2a and 2b) and is involved in coiled-coil interactions. Beyond the α-helical coiled-coil dimer, there are three distinct oligomeric species, i.e. the protofilament, the protofibril and the half-filament. The formation of these oligomers involves multiple interactions between NH_2-terminally or COOH-terminally half-staggered dimers, or between unstaggered dimers (for recent review see Heins and Aebi, 1994). Further polymerization seems to involve complex interactions in which the rod as well as the end-domains contribute self-association sites (Traub and Vorgias, 1983; Kaufmann et al., 1985; Albers and Fuchs, 1987, 1989; Lu and Lane, 1990; Raats et al., 1990; Hatzfeld and Weber, 1991; Hermann et al., 1992; Hoffmann and Hermann, 1992; Kouklis et al., 1991). The various rod subdomains participate to a different extent in higher order interactions. While α-helix disrupting point mutations in the central part of the type I keratin rod domain are tolerable, even mild alterations in the conserved end-segments of the rod have profound effects in filament stability in vivo and in vitro (Hatzfeld and Weber, 1991; Letai et al., 1992). The involvement of the rod extremities in IF organization and stability is further emphasized by the fact that mutations in these sequences lead to human diseases (for recent review see Fuchs and Weber, 1994). The critical role of coil 1a and coil 2b in filament assembly is probably due to the NH_2-COOH-terminal overlap between adjacent subunits during IF elongation (Heins et al., 1993; Heins and Aebi, 1994). This type of overlap represents the fourth type of interaction in addition to the three lateral interactions mentioned above.

The contribution of the variable end domains in IF assembly has been studied intensively. Truncation, mutagenesis, or competition with small peptide mimics of the head domain of desmin, vimentin, keratins, or neurofilament-light subunit (NF-L) has been shown to have deleterious effects on filament formation both in transfected cells, and in vitro (Raats et al., 1991; Hermann et al., 1992; Hofmann and Hermann, 1992; Wilson et al., 1992; Heins et al., 1993). Thus, the current notion is that the head domain of IF proteins promotes lateral associ-

ation of protofilaments or protofibrils into 10 nm filaments (for recent review see Heins and Aebi, 1994). In contrast, the involvement of the tail domain in this process has remained somewhat controversial. COOH-terminally truncated vimentin and cytokeratin subunits have been shown to assemble into normal-looking IFs under in vitro conditions (Hatzfeld and Weber, 1990; Eckelt et al., 1992; Wilson et al., 1992), but it is not entirely clear whether these filaments contain the normal number of protofilaments per filament cross section. Tail-less GFAP does not assemble (Quinlan et al., 1989) and fully or partially truncated desmin (Kaufmann et al., 1985), vimentin (Shoeman et al., 1990; Kouklis et al., 1993) and NF-L (Nakamura et al., 1993; Heins et al., 1993) laterally aggregate into thick bundles. Transfection studies show that an intact tail is required for normal desmin, NF-M and vimentin assembly in vivo (Wong and Cleveland, 1990; Raats et al., 1991), while similar studies with tail-less cytokeratins have yielded conflicting results suggesting that the tail domain may (Lu and Lane, 1990) or may not (Bader et al., 1991) be essential for IF assembly in living cells.

We and others have previously characterized and cloned cDNAs encoding two new IF proteins, phakinin and filensin, specific to the fiber cells of the eye lens (Merdes et al., 1991, 1993; Brunkener and Georgatos, 1992; Masaki and Watanabe, 1992; Gounari et al., 1993; Hess et al., 1993; Remington, 1993). Phakinin and filensin conform to the general structural principles of IF proteins and readily co-assemble into heteropolymeric filaments known as beaded filaments - BFs (Maisel and Perry, 1972; Merdes et al., 1993; reviewed by Georgatos et al., 1994). These proteins have structural peculiarities which distinguish them from other IF proteins. Phakinin is completely tail-less (Merdes et al., 1993; Hess et al., 1993), while filensin has a rather short rod domain lacking four heptad repeats in coil 2 (Gounari et al., 1993; Remington, 1993). Phakinin and filensin do not form IFs on their own; in vitro the former self-assembles into thick helical bundles, while the latter forms short fibrils (Merdes et al., 1991, 1993).

To examine more rigorously the role of filensin in filament formation, we studied the properties of filensin swapping mutants in vitro and in vivo. The results of this study show that the filensin rod domain plays a pivotal role in filament structure, whereas the end-domains probably regulate filament network formation in vivo.

MATERIALS AND METHODS

Construction of bacterial and eukaryotic expression vectors

A previously isolated 2.5 kb bovine filensin cDNA was ligated into the *Eco*RI polylinker site of the pT7-7 bacterial expression vector (Studier et al., 1990) and expressed in *Escherichia coli* (BL21). The filensin synthesized from this construct has an NH_2-terminal addition corresponding to the amino acid sequence MARIPARGGA. This construct was designated FFF and was used as the backbone for the generation of the swapping mutants. Constructs made in this study are designated by three letters, referring to the three structural domains of IF proteins (head, rod and tail). 'V', 'F' and 'P' indicate segments derived from vimentin, filensin, and phakinin, respectively, while 'X' denotes absence of a domain. The head, rod and tail domains of the hamster vimentin were amplified by PCR using the upstream oligonucleotides 5′GCATGAATTCATATGTCTACCAGGTCCGTGTCCTCGTCCTCTTA3′, 5′CGAGTTCAAGAACGCGGCCGCCAACGAGAAG3′, and 5′GAGGAGAGCAGGATGAGCTCGCCTCTTCCCAACT3′; and the downstream oligonucleotides 3′CTTCTCGTTGGCGGCCGCGTTCTTGAACTC5′, 3′AGTTGGGAAGAGGCGAGCTCATCCTGCTCTCCTC5′, and 3′TGCAGTCGACTTATTCAAGGTCATCATGATGC5′, respectively. PCR amplification with these primers introduced an upstream *Nde*I site and a downstream *Not*I site flanking the ends of the vimentin head, an upstream *Not*I site and a downstream *Sac*I site flanking the vimentin rod, and an upstream *Sac*I site and a downstream *Sal*I site flanking the vimentin tail. To generate the VFF construct, the vimentin head was ligated at the unique *Nde*I-*Not*I sites of FFF. The FVF mutant was constructed by cloning the vimentin rod in the *Not*I-*Sac*I sites of FFF. Similarly, FFV was made by ligating the vimentin tail at the *Sac*I-*Sal*I sites of FFF. The double swapping mutant VFV was constructed on the backbone of FFV by ligation of the vimentin head at the *Nde*I-*Not*I sites. To obtain the tail-less filensin (FFX), the palindromic oligonucleotide 5′TAACTGAGTAGGATCCTACTCAGTTA3′ (terminator oligo) was inserted into the blunted *Sac*I site at the end of the filensin rod. A bovine phakinin cDNA (Merdes et al., 1993) was used to prepare suitable constructs (PPX) for expression of this protein in bacteria. We should note here that the previously reported sequence of phakinin (Merdes et al., 1993) contained two sequencing errors which alter the frame in the 5′ region of the cDNA. The corrected sequence has been deposited in the EMBL data bank (file X75160. EMNEW). To generate the PPX vector, an *Nde*I site was engineered by amplification with the upstream oligonucleotide 5′GGGAATTCCATATGAGCACCAGGCGCGTG3′ in the translation initiation codon of the phakinin cDNA. The resulting fragment was inserted between the *Nde*I-*Hin*dIII sites of the pT7-7 vector.

Eukaryotic expression constructs were generated by subcloning the coding sequences from the corresponding bacterial expression constructs into the PSVK3 vector (Pharmacia, Uppsala, Sweden). Thus, FFF was inserted into PSVK3 as an *Eco*RI fragment. A *myc* tag (Evan et al., 1985) was added to the PPX eukaryotic expression vector as an *Nco*I-*Nde*I insertion, upstream and in frame with the phakinin translation initiation codon. FVF was inserted into PSVK3 as an *Eco*RI fragment. FFX was produced by excision of the 1.5 kb *Sac*I fragment at the 3′ of the FFF cDNA and insertion of the terminator oligo in the blunted *Sac*I site. FFV was cloned into PSVK3 as a 1.1 kb *Eco*RI-*Sal*I fragment. To optimize eukaryotic expression the linker GAATTCGCCACTATG (Kozak linker) was inserted into the *Nde*I sites of VFF and VFV before subcloning into PSVK3 as a 2.6 kb *Eco*RI and 1.3 kb *Eco*RI-*Sal*I fragment, respectively. A *myc* tag (Evan et al., 1985) was added to the PPX eukaryotic expression vector as an *Nco*I-*Nde*I insertion, upstream and inframe with the phakinin translation initiation codon. Recombinant DNA manipulations were performed essentially as described by Sambrook et al. (1989).

Bacterial expression and purification of proteins

Logarithmically growing cultures (500 ml, A_{600}=1) were induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Biofinex, Praroman, Switzerland). After another 3 hours at 37°C, the bacteria were harvested by low-speed centrifugation, the cell pellet resuspended in 1/10 of the original volume in 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 2 μg/ml antipapain and 2 μg/ml aprotinin (lysis buffer) and lysed by sonication at 0°C. The lysates were sedimented at 12,000 ***g*** for 15 minutes at 4°C and the pellet was washed with 1% Triton X-100 in lysis buffer. The Triton-insoluble material was washed with 1 M urea and 1% Triton X-100 in lysis buffer. The washed pellet was solubilized in 8 M urea, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 2 μg/ml antipapain and 2 μg/ml aprotinin (urea-Tris extraction buffer) and centrifuged at 12,000 ***g*** for 45 minutes at 18°C to remove insoluble material. The clarified urea extract was chromatographed in DEAE-cellulose

(DE53, Whatman, Maidstone, GB) using a 1-150 mM NaCl gradient in urea-Tris extraction buffer. Fractions enriched for the protein of interest were pooled and further chromatographed in a hydroxylapatite column (Bio-Rad Laboratories, Richmond, CA). The column was eluted with a gradient of 10-100 Na_3PO_4 in 7 M urea, 10 mM Na_3PO_4, pH 7.5, 1 mM DTT, 1 mM PMSF (urea-phosphate buffer).

Phakinin was purified either from bacterial lysates (prepared essentially as above), or from fresh bovine lenses obtained from a local slaughterhouse. The lenses were washed three times with ice-cold homogenization buffer (155 mM NaCl, 20 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, 2 μg/ml leupeptin and 2 μg/ml pepstatin) and homogenized in a Waring blender at 4°C. The homogenate was first cleared from debris by spinning at 1,000 rpm for 5 minutes at 4°C in a tabletop centrifuge. After centrifugation for 30 minutes at 12,000 ***g***, 4°C, the pellet was resuspended in 600 mM KCl, 50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 0.1 mM EGTA, 1 mM PMSF and recentrifuged at 12,000 ***g*** for 30 minutes at 4°C. The insoluble material was extracted first with 0.5 % Triton X-100 in homogenization buffer and then with low salt buffer (10 mM Tris-HCl, pH 7.5, 1 mM PMSF, 1 mM DTT). The pellet was extracted with 6 M urea, 10 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM DTT and 1 mM PMSF (6 M urea buffer) and centrifuged at 12,000 ***g*** for 60 minutes at 18°C. The clarified supernatant was loaded onto a DEAE-cellulose column. Bound material was eluted with a gradient of 0-100 mM KCl in the 6 M urea buffer. Phakinin-enriched fractions were pooled, loaded onto a Superdex-200 gel filtration column (Pharmacia, Uppsala, Sweden) and the column eluted with 6 M urea buffer. Fractions containing purified phakinin were identified by SDS-PAGE in 12.5% polyacrylamide gels.

In vitro assembly experiments

In vitro assembly of FFF, FFX, FVF, VFV, FFV and VFF was performed at protein concentrations of 0.2-0.4 mg/ml at room temperature. The purified proteins were stored in 8 M urea, 10 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM DTT, 2 μg/ml aprotinin, 2 μg/ml antipapain, 2 μg/ml pepstatin, 2 μg/ml leupeptin and 0.5 mM PMSF (urea buffer). To effect assembly, the proteins were dialyzed against solutions of decreasing urea concentrations, containing 160 mM KCl, 20 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 1 mM DTT and 0.5 mM PMSF (isotonic buffer). The FFX, VFV and FFV mutant proteins were also assembled in low ionic strength buffer, containing 2-10 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 1 mM DTT and 0.5 mM PMSF. Heteropolymers of phakinin and each of the other proteins were reconstituted as follows. Proteins (except phakinin) were first dialyzed to 4 M urea buffer for 2 hours at room temperature, followed by dialysis against isotonic buffer, overnight at 4°C. Polymerized material was pelleted at 400,000 ***g*** for 40 minutes at 18°C, solubilized in 6 M urea, mixed with phakinin and dialyzed against isotonic buffer containing 1 mM $MgCl_2$, for 3 hours, on ice. Assembly of phakinin/VFV, phakinin/FFX and phakinin/FFV was induced by dialysis against low ionic strength buffer.

Electron microscopy

Samples were observed by negative staining or glycerol spraying/rotary metal shadowing. To negatively stain in vitro reconstituted polymers, 10 μl aliquots of each specimen were applied to collodium/carbon-coated copper grids. After washing with distilled water, the material was stained with 2% uranyl acetate and air-dried. The grids were then examined in a Philips 400 or 301 electron microscope. For glycerol spraying/rotary metal shadowing, a 20 μl aliquot of each sample was mixed with glycerol to a final concentration of 30% and sprayed onto pieces of freshly cleaved mica. The mica pieces were placed on the table of a high-vacuum evaporation-machine (Balzers BAE 080) for drying and rotary-shadowed with platinum/carbon (using an electron beam source) at an elevation angle of ~3° (Fowler and Aebi, 1983).

Whole-mount immunoelectron microscopy was performed using the rabbit anti-filensin antibody aFL-2 (Gounari et al., 1993) and the anti-phakinin antibody mAb 49 (kindly provided by Dr P. G. FitzGerald).

Light microscopy

Indirect immunofluorescence microscopy was performed as described by Merdes et al. (1991). Specimen preparation for confocal microscopy was essentialy the same except that prior to mounting the samples ~20 μm 'feet' were made on the coverslips. Double immunolabeling was done using various anti-filensin antibodies (Gounari et al., 1993), the mAb 9E10 against *myc* (a gift from S. Fuller, EMBL, Heidelberg, FRG), the mAb 7A3 recognizing vimentin (Papamarkaki et al., 1991), and an anti-keratin 8 mAb (kindly provided by M. Osborn, Max Planck Institute for Biophysical Chemistry, Göttingen, FRG).

Transfection of cultured cells

CHO (Chinese hamster ovary cells) were obtained from the American tissue culture collection. MCF-7 mouse mammary carcinoma cells were obtained from W. W. Franke (German Cancer Research Center, Division of Cell Biology, Heidelberg, FRG). Clones of SW13 human adrenal carinoma cells were provided by R. Evans (University of Colorado, Health Science Center, Denver, CO). Transfections were carried out according to the calcium phosphate precipitation method, essentially as described by Wingler et al. (1979). In short, 1×10^5 cells were plated onto 6 cm tissue culture plates containing sterile coverslips. On the following day the cells were refed and 4-8 hours later 0.5 ml of freshly prepared precipitate containing 10 μg DNA was applied. The precipitate was washed off after 16-20 hours and the cells were allowed a further 24 hour incubation before processing for immunofluorescence.

Other methods

Polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) was performed according to the method of Laemmli (1970). Protein concentrations were measured using a Bio-Rad kit (Bio-Rad, Richmond, CA).

RESULTS

Design, expression and purification of recombinant proteins

Using the pT7-7 vector expressing filensin in bacteria as a backbone and the hamster vimentin cDNA (Hatzfeld et al., 1992) as a template, we engineered one deletion and four swapping mutants. In each of the latter, segments encoding structural domains of filensin (head, rod or tail) were exchanged with the analogous segments of vimentin. The resulting constructs were designated VFF, FVF, FFV, and VFV, depending on the exchanged domain (for details see Materials and Methods). The deletion mutant corresponded to an 'exactly tail-less' filensin form (FFX), as a termination codon was introduced immediately after the triplet coding for the last amino acid residue of the rod domain. (For a schematic representation of the constructs see Fig. 1A.)

Recombinant plasmids were characterized by sequencing and used to transform *E. coli* (BL21). After induction the overexpressed proteins were extracted from inclusion bodies by 8 M urea. Wild-type and mutant proteins were isolated from urea extracts by ion exchange, gel filtration and hydroxylapatite chromatography (for details see Materials and Methods). Electrophoretic profiles of the bacterially-produced polypeptides are shown in Fig. 1B.

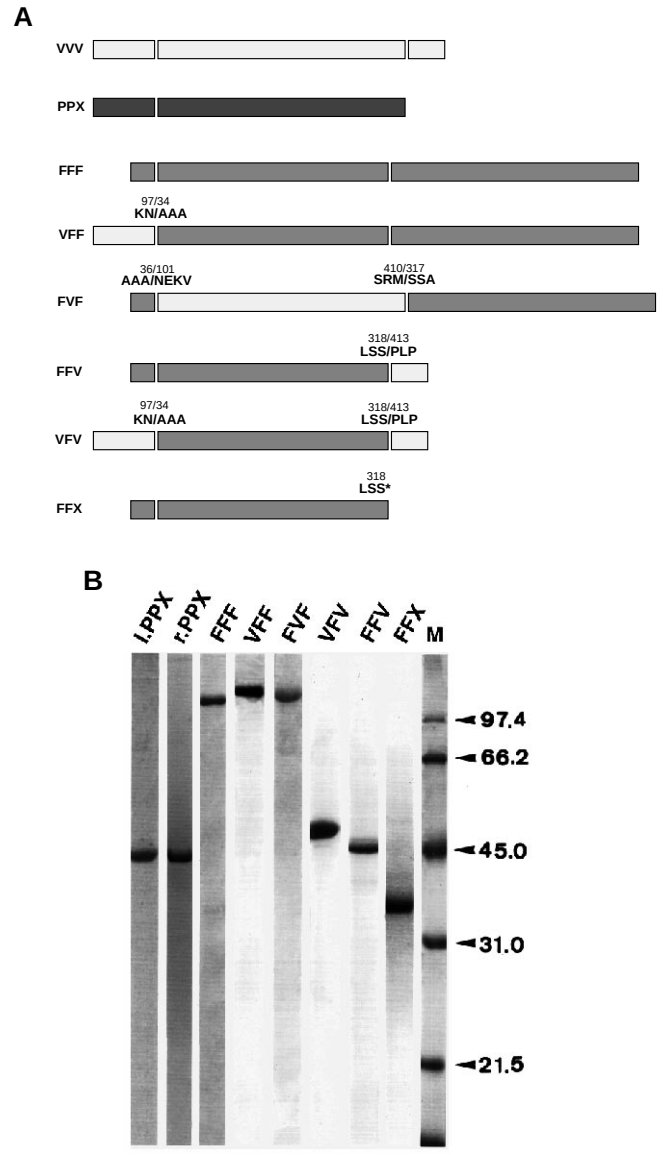

Fig. 1. (A) Stick diagram of wild-type and mutant proteins used in this study. Constructs are designated by three capital letters, in the order head/rod/tail domain. 'V' denotes vimentin; 'F' denotes filensin; 'P' indicates phakinin, and 'X' signifies absence of a domain. The residues at the borders of exchanged domains are indicated using the one letter amino acid code. Numbers in each junction correspond to the positions of the amino acids in the domains that were joined together. More details on construction strategies are described in Materials and Methods.
(B) Electrophoretic profiles of purified bovine phakinin and bacterially-expressed proteins. Molecular mass values of markers (M) are given in kDa. l. PPX: phakinin purified from bovine lenses; r. PPX: recombinant phakinin purified from *E. coli*. FFF, VFF, FVF, VFV, FFV and FFX represent purified bacterial filensin and filensin mutants (for nomenclature see A, and Materials and Methods).

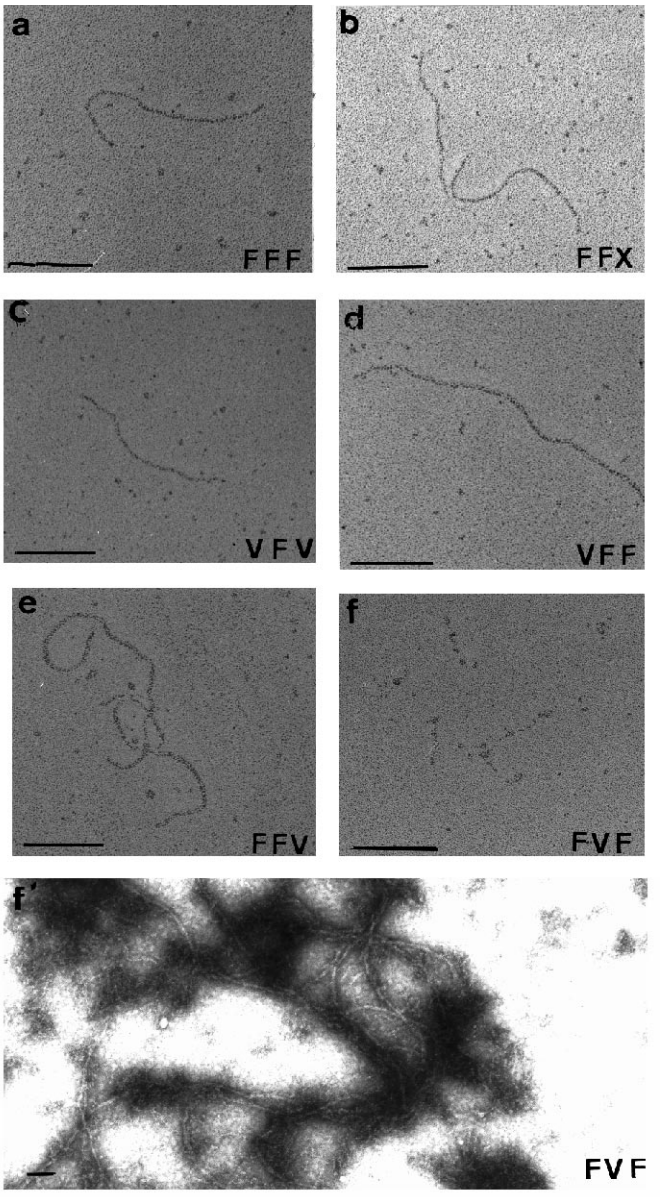

Fig. 2. Self-assembly of wild-type and mutant filensin under in vitro conditions. (a-f) Rotary shadowing images of the indicated proteins (for terminology see Fig. 1 and Materials and Methods). (f′) A negatively-stained profile of the same preparation shown in f. Note the formation of short fibrils by FFF, VFF, FFV, VFV, and FFX and the formation of long 10 nm filaments by FVF. Mutants FFX, FFV and VFV have been assembled in low salt buffer, whereas all other proteins were reconstituted in isotonic buffer (for details see Results). Bars, 100 nm.

Role of the rod and the end-domains of filensin in filament formation

To examine the contribution of each domain of filensin in co-assembly with phakinin and filament formation, we performed in vitro assembly studies using lens phakinin and a collection of filensin mutants. Before proceeding with the co-assembly experiments, we found it crucial to compare the self-assembly properties of each mutant to those of wild-type filensin. These experiments involved analysis by both negative staining and rotary shadowing. Both techniques gave comparable results, with one exception explained below.

When reconstituted into isotonic medium, wild-type filensin polymerized into short fibrils which lacked the diagnostic 21 nm axial periodicity of IFs (Fig. 2a). The same type of structures were produced by all mutants containing a filensin-type rod, i.e. VFF, FFV, VFV and FFX (Fig. 2b-e). However, unlike

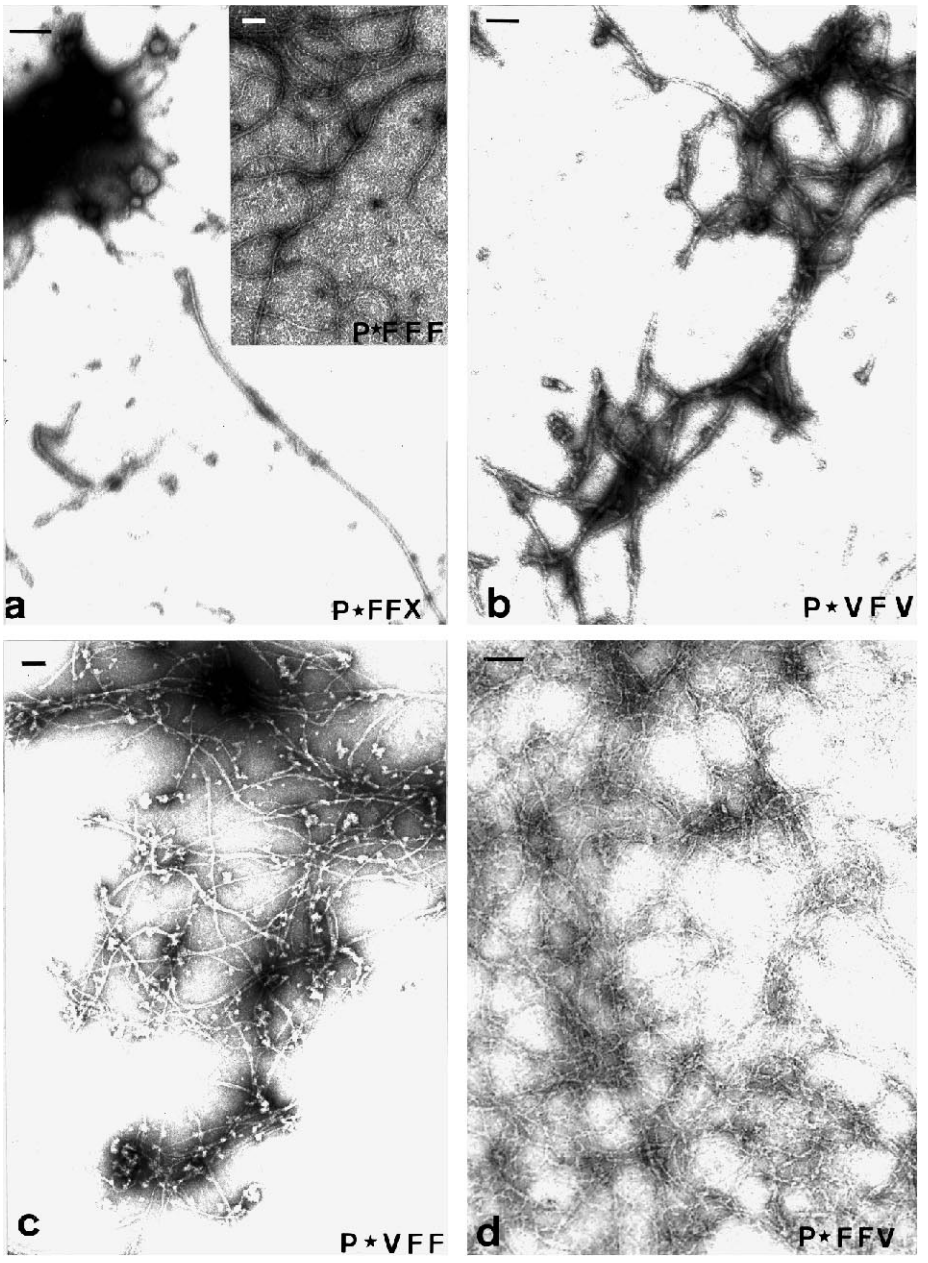

Fig. 3. Co-assembly of lens phakinin with various filensin mutants under in vitro conditions. (a) P and FFX (inset P and FFF); (b) P and VFV; (c) P and VFF; (d) P and FFV. The specimens were negatively-stained as described in Materials and Methods. Note that, despite some irregularities, all mutants except FFX co-polymerize with phakinin into long, extended filaments. Mutants FFX, FFV and VFV have been co-assembled with phakinin in low salt buffer, whereas FFF, VFF and phakinin were reconstituted in isotonic buffer (for details see Results). Bars, 100 nm.

wild-type filensin and VFF, the mutants FFV, VFV and FFX could form such fibrils only under conditions of low ionic strength (2-10 mM Tris-HCl). Under isotonic conditions, the latter three proteins formed globular particles or large aggregates (data not shown). A likely interpretation for this maybe that the 'extended' configuration of filensin fibrils is dependent on ionic interactions between the rod and the tail domain (for relevant information see Kouklis et al., 1991; McCormick et al., 1993). In contrast to other mutants, the hybrid protein FVF polymerized into long filaments (Fig. 2f′). These filaments were very fragile and extensively fragmented upon spraying in glycerol and processing for rotary shadowing (Fig. 2f). Breakage upon spraying in glycerol was not observed with the polymers assembled from other filensin mutants as similar structures were observed by negative staining and rotary shadowing. Taken together, the results suggest that the rod domain of filensin exerts a negative influence on polymer elongation. This is evident from the fact that all mutants containing a filensin-type rod (similarly to wild-type filensin) yield short fibrils rather than long filaments, whereas the FVF mutant which carries a vimentin-type rod is able to elongate.

To examine whether the filensin rod plays a role in assembly partner recognition, we tested whether the mutant VFV interacts with phakinin. This mutant was selected because vimentin (i.e. VVV) does not associate in any manner with phakinin under in vitro conditions (Merdes et al., 1991, 1993). As shown in Fig. 3b, VFV co-assembled with phakinin, forming micrometer-long filaments. Although these filaments had a somewhat rough surface in comparison to the wild-type filaments (Fig. 3a, inset), we can still conclude that the filensin rod confers to the model protein VFV an ability to co-polymerize with phakinin. Consistent with this interpretation, the mutant VFF also co-assembled with phakinin, yielding long filamentous structures (Fig. 3c). However, it should be pointed

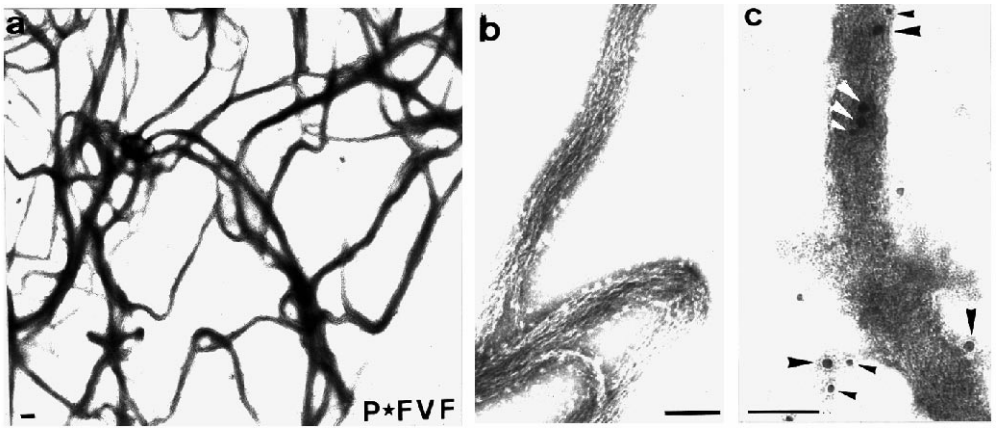

Fig. 4. Co-assembly of phakinin and FVF in vitro. Electron micrographs of in vitro reconstituted phakinin/FVF heteropolymers assembled in isotonic buffer are shown. (a) Low power view; (b) high magnification of a similar specimen; (c) a specimen similar to that shown in a and b, decorated by anti-filensin antibodies/10 nm Protein A-gold (small arrowheads) and anti-phakinin antibodies/15 nm Protein A-gold (large arrowheads). Notice the formation of thick, long, branched bundles with a diameter of approximately 50 nm. Bars, 100 nm.

out that, as in the case of VFV, phakinin/VFF filaments had an atypical morphology. Apparently, the vimentin head and tail domains that had been attached to the mutants do not interfere with partner recognition and filament assembly, but do affect the surface properties of heteropolymeric assemblies.

To find out whether the end-domains of filensin play a role in partner recognition, we examined the ability of the FVF mutant to complement phakinin. As shown in Fig. 4a,b, mixing of FVF and phakinin resulted in the formation of thick bundles. These structures consisted of tightly packed sub-filaments and were reminiscent of the bundles formed by phakinin alone (Merdes et al., 1993; Goulielmos et al., 1995). In these mixing experiments FVF-type filaments (see Fig. 2f) were never detected in the background. From this it could be inferred that FVF and phakinin had co-polymerized. However, to confirm this in a more definitive manner, we performed immunoelectron microscopy. As shown in Fig. 4c, the bundles assembled in the presence of phakinin and FVF were doubly-decorated by specific anti-filensin and anti-phakinin antibodies.

Collectively, the co-assembly data described above suggest that the rod and (one or both of) the end-domains of filensin are involved in partner recognition. However, each domain of filensin seems to contribute differently in the formation of the co-polymer: whereas the filensin rod binds to phakinin and seems to counteract the tendency of phakinin to grow into thick bundles (e.g. as in the phakinin/VFV co-polymer), the end-domains associate with phakinin without modifying its assembly behavior (e.g. as in the phakinin/FVF co-polymer).

Knowing that filensin and phakinin co-polymerize and taking into account that phakinin is naturally tail-less, we found it interesting to investigate whether the tail domain of filensin is necessary for heteropolymeric filament architecture. For this purpose, we examined whether the tail-less FFX mutant could form IFs when combined with phakinin. Fig. 3a shows that mixing of FFX and phakinin yielded predominantly large aggregates with filamentous projections that were distinct from the structures assembled from each protein alone. Only on rare occasions could isolated longer filaments be observed. Modifying the assembly conditions did not change at all the appearance of the final products, suggesting that the absence of a tail domain results in irreversible collapse of the co-polymer. That FFX interacts with phakinin could be anticipated from the fact that this hybrid protein contains the rod domain of filensin which is clearly enough for co-polymer formation (see above). However, this result was somewhat surprising in view of other data suggesting that pairs of tail-less keratins form normal-looking IFs (Hatzfeld and Weber, 1990; Bader et al., 1991). To differentiate whether the collapse of the co-polymer was due to the complete absence of tails or to the absence of essential sites present specifically in the tail domain of filensin, we performed the same type of experiment using the mutant FFV. As illustrated in Fig. 3d, FFV and phakinin co-assembled into filaments which, despite their atypical features, were extended and non-bundled. From these experiments it seems reasonable to infer that the tail domain of filensin, although not as essential for the formation of co-polymer as the rod domain, plays an important role in filament architecture.

Assembly and distribution of heteropolymeric filaments in vivo

To find out which domains of filensin play a role in filament assembly and filament distribution in vivo, we proceeded with transfection experiments. Previous studies (Goulielmos et al., 1996; to be reported elsewhere) had shown that full-length filensin and *myc*-tagged phakinin assemble de novo in a variety of lens and non-lens cells, forming either radial networks, or submembranous ‘sheets’ of laterally-associated filaments. Consistent with these results, co-transfection of CHO cells with phakinin and FFF yielded radial filament networks (Fig. 5a) which co-localized with the endogenous vimentin IFs (data not shown). In contrast, when MCF-7 cells were co-transfected with the same combination of constructs, arrays of fibers were seen to emanate from peripheral sites located near the plasma membrane (Fig. 5b; for more details see Goulielmos et al. 1996). The peripherally-disposed fibers appeared ‘stretched’ and did not co-localize with endogenous keratin filaments (data not shown). This phenotype was seen in roughly 30% of the cells, whereas the remainder of the cells contained filaments as well as large aggregates that stained for both phakinin and FFF. To address the role of the filensin end-domains in de novo assembly, we co-transfected cells with phakinin and VFV-encoding plasmids. These experiments yielded extended filament networks in CHO cells and peripheral aggregates mixed with filaments in MCF-7 cells (Fig. 5c,d). Although the filaments seen in MCF-7 cells appeared to be compromised, their overall distribution was reminiscent of that of phakinin/FFF. Contrasting this phenotype were the results obtained by co-transfection of phakinin and FVF-encoding plasmids. While in CHO cells this combination formed radial,

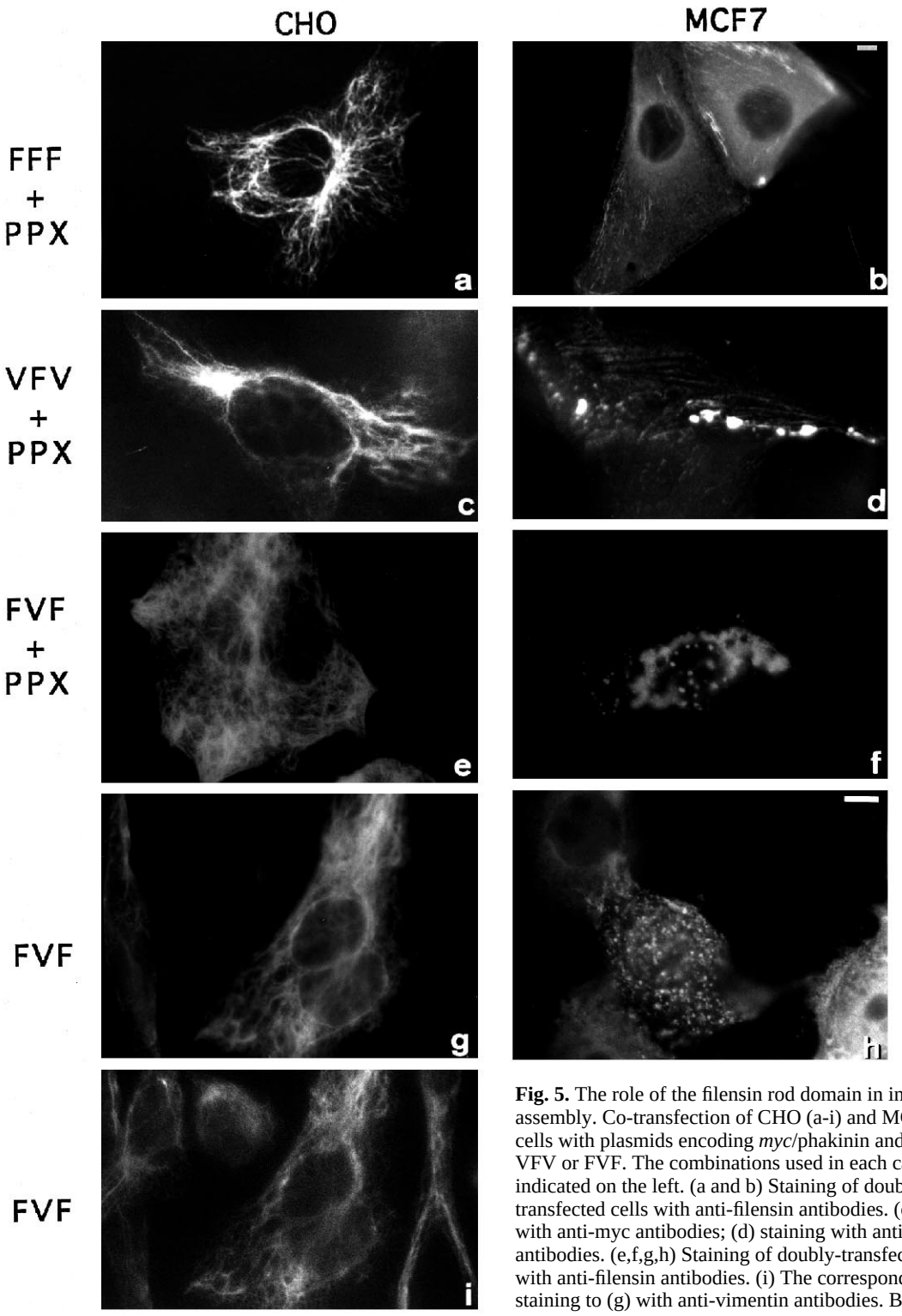

Fig. 5. The role of the filensin rod domain in in vivo assembly. Co-transfection of CHO (a-i) and MCF-7 (b-h) cells with plasmids encoding *myc*/phakinin and FFF, VFV or FVF. The combinations used in each case are indicated on the left. (a and b) Staining of doubly-transfected cells with anti-filensin antibodies. (c) Staining with anti-myc antibodies; (d) staining with anti-vimentin antibodies. (e,f,g,h) Staining of doubly-transfected cells with anti-filensin antibodies. (i) The corresponding staining to (g) with anti-vimentin antibodies. Bars, 2 μm.

vimentin co-localizing networks (Fig. 5e), in MCF-7 cells only aggregates could be observed (Fig. 5f). The behavior of FVF/phakinin in CHO cells can be explained by the affinity of FVF for the endogenous vimentin. Indeed, single transfections of FVF into CHO cells resulted in assembly of radial networks (Fig. 5g) which fully co-localized with endogenous vimentin (data not shown). In combination, these experiments suggest that the rod domain of filensin plays an important role in filament assembly and distribution in vivo.

Since the structures formed by VFV/phakinin were compromised, we examined the role of individual filensin end-domains in the presence of a filensin rod. Thus, we first co-transfected the two cell lines with phakinin and FFV. Transfected CHO cells contained aggregates ending in filamentous extensions

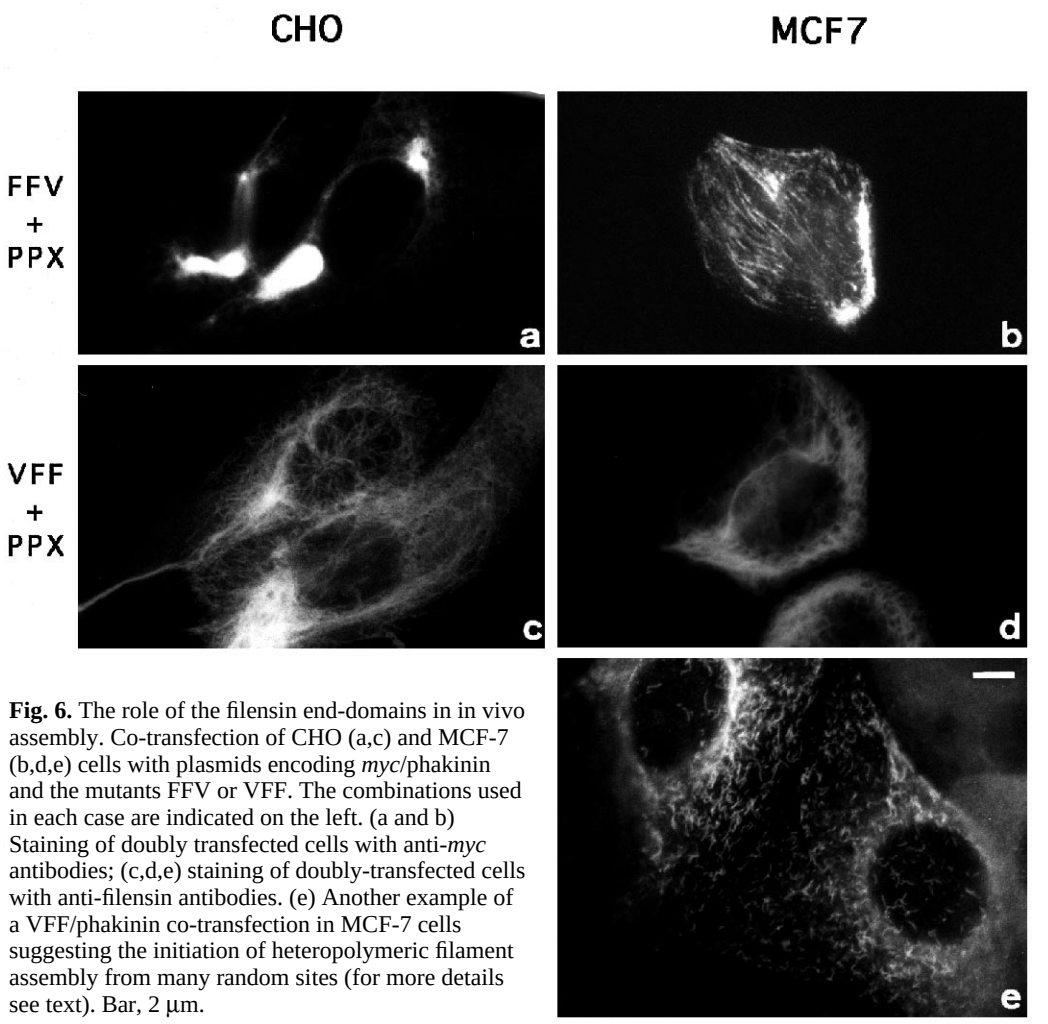

Fig. 6. The role of the filensin end-domains in in vivo assembly. Co-transfection of CHO (a,c) and MCF-7 (b,d,e) cells with plasmids encoding *myc*/phakinin and the mutants FFV or VFF. The combinations used in each case are indicated on the left. (a and b) Staining of doubly transfected cells with anti-*myc* antibodies; (c,d,e) staining of doubly-transfected cells with anti-filensin antibodies. (e) Another example of a VFF/phakinin co-transfection in MCF-7 cells suggesting the initiation of heteropolymeric filament assembly from many random sites (for more details see text). Bar, 2 μm.

(Fig. 6a), while MCF-7 cells contained peripheral filamentous structures similar to those formed by wild-type filensin/phakinin (Fig. 6b). Co-transfection with phakinin and VFF gave well-extended, vimentin co-localizing networks in CHO cells and radial networks in MCF-7 cells (Fig. 6c,d). Interestingly, the filaments assembled in MCF-7 cells appeared to grow from random cytoplasmic sites and areas close to the nucleus (Fig. 6e). To examine whether the radial pattern of distribution of phakinin/VFF in MCF-7 cells was 'dominant' or 'recessive' with respect to the peripheral localization of phakinin/FFV and phakinin/VFV, we performed triple transfections (i.e. phakinin/VFF plus either FFV, or VFV). As shown in Fig. 7a,b, the VFF protein (detected by an anti-peptide antibody specific for the tail domain of filensin) was incorporated into peripherally-disposed filamentous structures. These structures also contained phakinin and FFV or VFV as indicated by double-labeling experiments with anti-*myc* and anti-vimentin tail antibodies (not shown). Co-transfections of IF-deficient SW13 cells with phakinin and VFF, FFV or VFV gave results comparable with those described for MCF-7 cells, while cells co-transfected with phakinin and FFX exclusively contained aggregates (data not shown). Finally, single transfections with all other filensin mutants yielded spherical particles, indistinguishable from those formed by wild-type filensin alone (data not shown).

These experiments clearly show that heteropolymer distribution in vivo is heavily influenced by the chemical nature of the NH_2- and COOH-terminal regions. In this respect, it should be noted that the cellular host into which the various hybrid proteins are transfected also affects polymer distribution. From these data we conclude that the end-domains of filensin are probably involved in a variety of interactions with cell-specific factors.

DISCUSSION

Contribution of the filensin rod domain in filament formation

The basic corollary of this study is that the rod domain of filensin determines the length and surface morphology of filamentous assemblies. Crosslinking approaches (Geisler et al., 1992; Steinert et al., 1993a,b,c) have established that rod-rod interactions are important in the assembly of the tetrameric IF building blocks which consist of the anti-parallel, staggered (N-N) or (C-C) tetramers and the antiparallel, in-register (NC-CN) tetramer. Rod-rod interactions also mediate the head-to tail interactions of adjacent protofilaments in the longitudinal direction and may influence filament elongation (for recent review see Heins and Aebi, 1994). Knowing this, it seems plausible to suggest that the

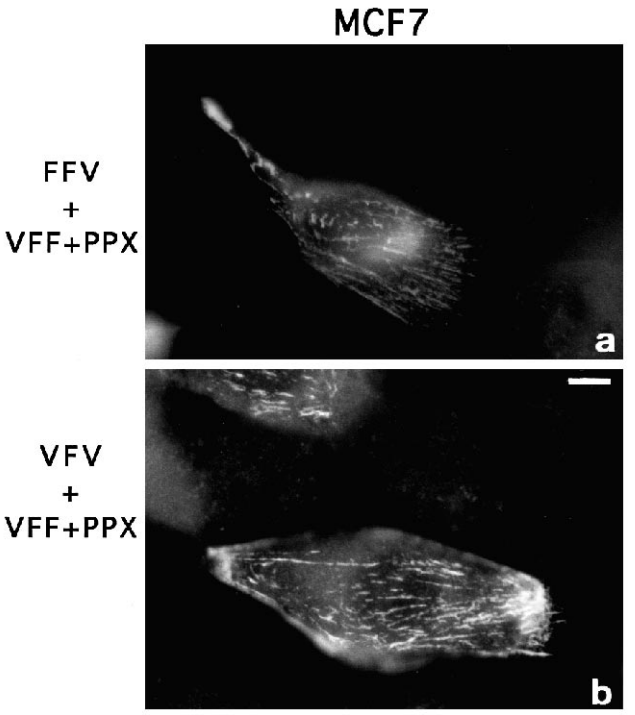

Fig. 7. Filament network distribution in MCF-7 cells co-transfected with VFF/phakinin and other filensin mutants: triple transfection of MCF-7 cells with plasmids encoding myc/phakinin/VFF and either FFV, or VFV. The combinations used in each case are indicated on the left. (a and b) Staining of the triply transfected cells with anti-filensin antibodies recognizing the tail of filensin (anti-FL2; for more information see Gounari et al., 1993; Merdes et al., 1993). Bar, 2 μm.

29 amino acid truncation in the coil 2b of the filensin rod domain prevents filensin filament elongation. Although such a truncation would not interfere with the formation of NC-CN type and N-N type tetramers, it would certainly lower the stability of the C-C type tetramer (which requires an overlap in the missing region). Destabilization or under-representation of staggered C-C tetramers may physically interfere with filament elongation. The lack of axial repeats in filensin polymers may reflect an atypical mode of assembly. However, it is also possible that even 'normal' assembly of the rod-truncated filensin would not yield a visible axial periodicity.

Co-assembly of the filensin mutants with phakinin indicates that the filensin rod, but not the vimentin rod, counteracts the inherent tendency of the tail-less phakinin to assemble into thick filament bundles. Moreover, results presented elsewhere suggest that heterotypic filensin/phakinin interaction may occur at the level of dimers (Goulielmos et al., 1996). The organization of putative heterodimers presents a structural puzzle. Although the coils 1a, 1b, and 2a, of the filensin and phakinin rod domains are likely to form a coiled-coil, the region of coil 2b that is present in phakinin and absent in filensin may loop out. If this were correct, one could imagine that the phakinin loop is involved in lateral interactions between filensin/phakinin heterodimers and phakinin-phakinin homodimers.

Role of filensin domains in heteropolymeric filament assembly in vivo

As shown above, transfection experiments with various filensin mutants yielded different filament phenotypes, depending on construct combination and host cell type. Before making an attempt to molecularly interprete these phenotypes, it is important to note here that domain exchanges between filensin and vimentin led to different modes of assembly. Inspecting numerous specimens, it appeared to us that whereas phakinin/FFF or VFV or FFV assemble predominantly from cell surface-associated sites, phakinin/VFF initiate from many randomly distributed sites in MCF-7 cells. In contrast, almost all filensin mutants assemble onto or in co-alignment with pre-existing vimentin filaments in CHO cells. Looking at these data from a broader perspective, we note the existence of multiple, alternative modes of IF formation in vivo. It is likely that each of these assembly pathways involves domain-specific interactions between IF proteins and cell-specific factors (for a relevant discussion see Georgatos and Maison, 1995).

While swapping of filensin end-domains with the corresponding vimentin end-domains has only minimal effects on the surface morphology of heteropolymeric filaments formed in vitro, the same exchange of domains dramatically affects filament network formation and network organization in vivo. This can be interpreted in a number of ways. For instance, the presence of a filensin-type tail may be important for the formation of an extended filament network in CHO cells, as this COOH-terminal region may interact with endogenous vimentin IFs. Conversely, the presence of a filensin-type head domain may have a greater influence in filament organization in MCF-7 cells. In either case, one should take into account that the filensin rod is necessary for heteropolymeric filament formation in vitro and in vivo. This is clearly shown by the in vitro polymerization studies and transfection experiments with the VFV and the FVF mutants presented above.

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REFERENCES

Albers, K. and Fuchs, E. (1987). The expression of mutant epidermal keratin cDNAs transfected in simple epithelial and squamous cell carcinoma cells. *J. Cell Biol.* **105**, 791-806.

Albers, K. and Fuchs, E. (1989). Expression of mutant keratin cDNAs in epithelial cells reveals possible mechanisms for initiation and assembly of intermediate filaments. *J. Cell Biol.* **108**, 1477-1493.

Bader, B. L., Magin, T. M., Freudenmann, M., Stumpp, S. and Franke, W. W. (1991). Intermediate filaments formed de novo from tail-less cytokeratins in the cytoplasm and in the nucleus. *J. Cell Biol.* **115**, 1293-1307.

Brunkener, M. and Georgatos, S. D. (1992). Membrane-binding properties of filensin, a cytoskeletal protein of the lens fiber cells. *J. Cell Sci.* **103**, 709-718.

Eckelt, A., Herrmann, H. and Franke, W. W. (1992). Assembly of a tail-less mutant of the intermediate filament protein, vimentin, in vitro and in vivo. *Eur. J. Cell Biol.* **58**, 319-330.

Evan, G. I., Lewis, G. K., Ramsay, G. and Bishop, J. M. (1985). Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol. Cell Biol.* **5**, 3610-3616.

Fowler and Aebi, U. (1983). Preparation of single molecules and supramolecular complexes for high-resolution metal shadowing. *J. Ultrastruct. Res.* **83**, 319-334.

Fuchs, E. and Weber, K. (1994). Intermediate filaments: Structure, dynamics, function, and disease. *Annu. Rev. Biochem.* **63**, 345-382.

Geisler, N., Schunemann, J. and Weber, K. (1992). Chemical cross-linking indicates a staggered and antiparallel protofilament of desmin intermediate

filaments and characterizes one higher-level complex between protofilaments. *Eur. J. Biochem.* **207**, 841-852.

Georgatos, S. D., Gounari, F. and Remington, S. (1994). The beaded intermediate filaments and their potential functions in eye lens. *BioEssays* **16**, 413-418.

Georgatos, S. D. and Maison, C. (1996). Integration of intermediate filaments into cellular organelles. *Int. Rev. Cytol.* **164**, 91-138.

Goulielmos, G., Gounari, F., Remington, S., Müller, S., Häner, M., Aebi, U. and Georgatos, S. D. (1995). Filensin and phakinin form a novel type of beaded intermediate filaments and co-assemble *de novo* in cultured cells. *J. Cell Biol.* (in press).

Gounari, F., Merdes, A., Quinlan, R., Hess, J., FitzGerald, P. G., Ouzounis, C. A. and Georgatos, S. D. (1993). Bovine filensin possesses primary and secondary structure similarity to intermediate filament proteins. *J. Cell Biol.* **121**, 847-853.

Hatzfeld, M. and Weber, K. (1990). Tailless keratins assemble into regular intermediate filamens in vitro. *J. Cell Sci.* **97**, 317-324.

Hatzfeld, M. and Weber, K. (1991). Modulation of keratin intermediate filament assembly by single amino acid exchanges in the consensus sequence at the C-terminal end of the rod domain. *J. Cell Sci.* **99**, 351-362.

Hatzfeld, M., Dodenmont, H., Plessmann, U. and Weber, K. (1992). Truncation of recombinant vimentin by ompT. Identification of a short motif in the head domain necessary for assembly of type III intermediate filament proteins. *FEBS Lett.* **302**, 239-242.

Heins, S., Wong, P. C., Muller, S., Godie, K., Cleveland, D. W. and Aebi U. (1993). The rod domain of NF-L determines neurofilament architecture, whereas the end domains specify filament assembly and network formation. *J. Cell Biol.* **123**, 1517-1533.

Heins, S. and Aebi, U. (1994). Making heads and tails of intermediate filament assembly, dynamics and networks. *Curr. Opin. Cell Biol.* **6**, 25-33.

Hermann, H., Hofmann, I. and Franke, W. W. (1992). Identification of a nonapeptide motif in the vimentin head domain involved in intermediate filament assembly. *J. Mol. Biol.* **223**, 637-650.

Hess J. F., Casselman, J. T. and FitzGerald, P. G. (1993). cDNA analysis of the 49 kDa lens fiber cell cytoskeletal protein: a new lens specific member of the intermediate filament family? *Curr. Eye Res.* **12**, 77-88.

Hofmann, I. and Herrmann, H. (1992). Interference in vimentin assembly in vitro by synthetic peptides derived from the vimentin head domain. *J. Cell Sci.* **101**, 687-700.

Kaufmann, E., Weber, K. and Geisler, N. (1985). Intermediate filament forming ability of desmin and vimentin derivatives lacking either the amino-terminal 67 or the carboxy-terminal 27 residues. *J. Mol. Biol.* **185**, 733-742.

Kouklis, P. D., Papamarcaki, T., Merdes, A. and Georgatos, S. D. (1991). A potential role for the COOH-terminal domain in the lateral packing of type III intermediate filaments. *J. Cell Biol.* **114**, 773-786.

Kouklis, P. D., Hatzfeld, M., Brunkener, M., Weber, K. and Georgatos, S. D. (1993). In vitro assembly properties of vimentin mutagenized at the β-site tail motif. *J. Cell Sci.* **106**, 919-928.

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

Letai, A., Coulombe, P. and Fuchs, E. (1992). Do the ends justify the mean? Proline mutations at the ends of the keratin coiled-coil rod segment are more disruptive than internal mutations. *J. Cell Biol.* **116**, 1181-1195.

Lu, X. and Lane, E. B. (1990). Retrovirus-mediated transgenic keratin expression in cultured fibroblasts: Specific domain functions in keratin stabilisation and filament formation. *Cell* **62**, 681-696.

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

Masaki, S. and Watanabe, T. (1992). cDNA sequence analysis of CP95: Rat lens fiber cell beaded-filament structural protein shows homology to cytokeratins. *Biochem. Biophys. Res. Commun.* **186**, 190-198.

McCormick, M. B., Kouklis, P., Syder, A. and Fuchs, E. (1993). The roles of the rod end and the tail in vimentin IF assembly and IF network formation. *J. Cell Biol.* **122**, 395-407.

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.

Merdes, A., Gounari, F. and Georgatos, S. D. (1993). The 47-kD lens-specific protein phakinin is a tailless intermediate filament protein and an assembly partner of filensin. *J. Cell Biol.* **123**, 1507-1516.

Nakamura, Y., Takeda, M., Aimoto, S., Hariguchi, S., Kitajima, S. and Nishimura, T. (1993). Acceleration of bovine neurofilament L assembly by deprivation of acidic tail domain. *Eur. J. Biochem.* **212**, 565-571.

Papamarkaki, T., Kouklis, P., Kreis, T. E. and Georgatos, S. D. (1991). The 'lamin b-fold'. *J. Biol. Chem.* **226**, 21247-21251.

Quinlan, R. A., Moir, R. D. and Stewart, M. (1989). Expression in *Escherichia coli* of fragments of glial fibrillary acidic protein: characterization, assembly properties and paracrystal formation. *J. Cell Sci.* **93**, 71-83.

Raats, J. M. H., Henderik, J. B. J., Verdijk, M., van Oort, F. L. G., Gerards, W. L. H., Ramaeckers F. S. C. and Bloemendal H. (1991). Assembly of carboxy-terminally deleted desmin in vimentin-free cells. *Eur. J. Cell Biol.* **56**, 84-103.

Remington, S. G. (1993). Chicken filensin: A lens fiber cell protein that exhibits sequence similarity to intermediate filament proteins. *J. Cell. Sci.* **103**, 709-718.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Shoeman, R. L., Mothes, E., Kesselmeier, C. and Traub, P. (1990). Intermediate filament assembly and stability in vitro: Effect and implications of the removal of head and tail domains of vimentin by the human immunodeficiency virus type I protease. *Cell Biol. Int. Rep.* **14**, 583-594.

Steinert, P. M., Marekov, L. N. and Parry, D. A. D. (1993a). Conservation of the structure of keratin intermediate filaments: Molecular mechanism by which different keratin molecules integrate into preexisting keratin intermediate filaments during differentiation. *Biochemistry* **32**, 10046-10056.

Steinert, P. M., Marekov, L. N., Fraser, R. D. B. and Parry, D. A. D. (1993b). Keratin intermediate filament structure. Crosslinking studies yield quantitative information on molecular dimensions and mechanism of assembly. *J. Mol. Biol.* **230**, 436-452.

Steinert, P. M., Marekov, L. N. and Parry, D. A. D. (1993c). Diversity of intermediate filament structure. *J. Biol. Chem.* **258**, 24916-24925.

Studier, F. W., Rosenberg, A. H., Dunn, J. J. and Dubendorfl, J. W. (1990). Use of T4 RNA polymerase to direct expression of cloned genes. *Meth. Enzymol.* **185**, 60-89.

Traub, P. and Vorgias, C. E. (1983). Involvement of the N-terminal polypeptide of vimentin in the formation of intermediate filaments. *J. Cell Sci.* **63**, 43-67.

Wilson, A. K., Coulombe, P. A. and Fuchs, E. (1992). The roles of K5 and K14 head, tail and R/K L L E G E domains in keratin filament assembly in vitro. *J. Cell Biol.* **119**, 401-414.

Wingler, M., Sweet, R., Sim, G. K., Wold, B., Pellicer, A., Lacy, E., Maniatis, T., Silverstein, S. and Axel, R. (1979). T9 transformation of mammalian cells with genes from prokaryotes and eukaryotes. *Cell* **16**, 777-785.

Wong, P. C. and Cleveland, D. W. (1990). Characterization of dominant and recessive assembly defective mutations in mouse neurofilament NF-M. *J. Cell Biol.* **111**, 1987-2004.

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