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Heteropolymer filaments of vimentin and desmin in vascular smooth muscle tissue and cultured baby hamster kidney cells demonstrated by chemical crosslinking

(intermediate filament/10-nm filament/cytoskeleton/protein disulfide bridge/muscle differentiation)

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ABSTRACT Certain smooth muscle cells of blood vessel walls as well as cultured baby hamster kidney cells contain simultaneously two different intermediate-sized filament (IF) proteins, desmin and vimentin. We have examined the question of the occurrence of both proteins in the same IF by chemically crosslinking the single cysteine group present in each of them. Oxidative crosslinking of filaments present in cytoskeletal preparations with cupric ion complexes of 1,10-phenanthroline resulted in formation of three types of dimers: vimentin–vimentin, desmin–desmin, and vimentin–desmin. These dimers were separated by $NaDodSO_4$/polyacrylamide gel electrophoresis and characterized by binding of specific antibodies, by one- and two-dimensional gel electrophoresis of monomers obtained after cleavage of the disulfide bond by thiol agents, and by mapping of radioiodinated tryptic peptides. The demonstration of heterodimers of vimentin and desmin in vascular smooth muscle tissue of cow and chicken and in baby hamster kidney cells shows that the two proteins can be integrated in the same IF and can be nearest neighbors, oriented with their cysteine residues in a mirror-image symmetry. The possible existence of heteropolymer IF in other cell types is discussed.

Intermediate-sized filaments (IF) are a group of structurally similar, but biochemically and immunologically diverse, filaments found in cytoskeletons of vertebrates. Five classes of IF that have different constituent proteins have been distinguished and shown to exhibit cell type specificity (1–3): desmin in various muscle cells, vimentin in diverse mesenchymally derived cells, proteins related to epidermal keratin (cytokeratins) in epithelial cells, neurofilaments in neuronal cells, and glial filaments in astrocytes. In most tissues, the location of these IF filament classes appears to be mutually exclusive (2–5), but cell cultures often show a tendency to express not only their cell type-specific IF class but also vimentin filaments. For example, many cultured epithelia-derived cells express vimentin IF in addition to cytokeratin IF (6, 7). Coexpression of vimentin with glial filaments has been reported in cultured astrocytes or glioma cells (8–10), as well as in astroglia *in situ* (10–12). Vimentin can also occur simultaneously with desmin during myogenic differentiation (13, 14) and is permanently expressed in cultured baby hamster kidney (BHK-21) cells (15–17), a line probably derived from an embryonal vascular smooth muscle cell type (18). Coexpression of vimentin with desmin is not restricted to cells cultured *in vitro* but also has been demonstrated in tissues. In certain cells of vascular smooth muscle tissue, vimentin is expressed alone whereas, in other cells, it is expressed together with desmin, varying in cells of different locations within the vascular bed (18–22). Coexistence of vimentin and desmin has also been reported for Z lines of developing and mature cross-striated muscle, but this is still subject to debate (2–5, 13–15).

The simultaneous occurrence of two types of IF proteins in the same cell leads to the question whether these proteins are located in different filaments, as has been shown for vimentin and cytokeratin IF in cultured epithelial cells (6, 7), or whether they are incorporated into the same IF. Formation of heteropolymer IF seems to be especially likely for vimentin and desmin in view of the similarity of these two proteins in terms of their amino acid sequences (23), their response to antimitotic drugs (3, 14, 24), and filament disassembly and reassembly *in vitro* (25–27). Recently, the reconstitution *in vitro* of IF from mixtures of vimentin and desmin and the solubility characteristics of reconstituted and native BHK-21 IF have been interpreted as indicating the presence of both proteins in the same filaments (27). In this study, we have used chemical crosslinking to examine the molecular relationships in IF of native cytoskeletons.

MATERIALS AND METHODS

Cell Culture and Tissues. BHK-21/C13 cells were grown on 8.5-cm plastic Petri dishes to near confluency (see ref. 28). Aortas from cow and chicken were used directly or frozen and processed as described (4, 21).

Preparation of Cytoskeletons. BHK-21 cells were extracted with Triton X-100 and high-salt buffers as described (6, 26). Extracted cytoskeletons or purified and washed IF (26) were used in crosslinking experiments. The 1,10-phenanthroline–cupric ion complex (Cu-P) was sometimes directly included in the Triton/high-salt buffer (final concentration, 72 μM); in these cases, L-1-tosylamide-2-phenylethyl chloromethyl ketone (Sigma) replaced EDTA as protease inhibitor. Cytoskeletal material from each Petri dish of cells was used directly or stored at −20°C until required. Cytoskeletons of whole aortic tissue or stripped *tunica media* from bovine aorta were prepared as described (19, 21). In some cases, cytoskeletons were prepared in the presence of 50 mM iodoacetamide.

Throughout preparation and subsequent crosslinking of cytoskeletal material, buffers [40 mM Tris·HCl (pH 7.5)] with ionic strengths that maintain filament integrity were used (see refs. 17, 25–27).

Crosslinking. Prior to crosslinking, cytoskeletal pellets from BHK-21 cells were sometimes digested for 20 min with 250 units of micrococcal nuclease (Worthington). Cytoskeletal material from aortic walls was homogenized until a fine suspension

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Abbreviations: IF, intermediate-sized filament(s); Cu-P, cupric ion–bis(1,10-phenanthroline) complex; BHK, baby hamster kidney.

was obtained. The crosslinker used was Cu-P, which catalyzes the air oxidation of sulfhydryl groups (29). Stock solutions (1.8 and 3.6 mM) were prepared just before use by mixing copper sulfate and 1,10-phenanthroline (Sigma) at a mol/mol ratio of 1:2, and most experiments were done with 1:50 dilutions. Copper can also form a complex with Tris·HCl, even when EDTA and 1,10-phenanthroline are present, and catalyze oxidation of sulfhydryl groups (30). The use of phosphate buffers did not alter the distribution or extent of crosslinked products formed. Crosslinking was terminated by addition of iodoacetamide or *N*-ethylmaleimide (final concentrations, 50 and 32 mM, respectively). Samples were prepared for gel electrophoresis by dilution with equal volumes of sample buffer/20% (vol/vol) glycerol/10% (wt/vol) $NaDodSO_4$.

Gel Electrophoresis. $NaDodSO_4$/polyacrylamide gel electrophoresis was according to Laemmli (31) but in the complete absence of reducing agents. To characterize the crosslinked products, gel tracks were cut out, incubated for 30 min in sample buffer (31)/10% (vol/vol) 2-mercaptoethanol, applied horizontally to the top of another gel, and subjected to electrophoresis in the second dimension. In some experiments, the phosphate-based gel electrophoresis system of Weber and Osborn (32) was used. Gels were scanned after staining with Serva blau R (Serva) at 550 nm using a Gilford 240 spectrophotometer equipped with linear gel transport. Two-dimensional gel electrophoresis was according to O'Farrell (33). Homo- and heterodimers of vimentin and desmin were prepared by excising the appropriate bands from the stained gels, and proteins were electrophoretically eluted from the gel slices. Transfer of proteins from gels to nitrocellulose paper was carried out electrophoretically (34). Protein bands reacting with the specific antibodies were detected by using ^{125}I-labeled protein A (New England Nuclear) and subsequent autoradiography at −80°C.

Antibodies. The vimentin antibodies used were raised in guinea pigs against human vimentin (28). Desmin antibodies were from guinea pigs as described (35) or were rabbit antibodies against human desmin (generously supplied by E. Rungger-Brändle, Department of Pathology, University of Geneva, Switzerland).

Peptide Mapping. The procedure of Elder *et al.* (36) using trypsin digestion of radioiodinated proteins was used. Approximately 0.3 mCi of ^{125}I (1 Ci = 3.7×10^{10} becquerels; Radiochemical Centre, Amersham) was added to each gel piece. The proteins were digested by using 50 μg of trypsin (ketone treated; 217 units/mg; Millipore).

RESULTS

Crosslinking. IF are insoluble in the buffers used in this study, including high-salt/Triton buffers (1–3, 15–17, 25–27). A typical time course for the crosslinking of BHK-21 cytoskeletons by using Cu-P is shown in Fig. 1*a*. Three crosslinked products, two major and one minor, are visible as new polypeptide bands, and this triplet pattern of crosslinked products does not change over a 10- to 40-min reaction period. The percentage of the three crosslinked products in relation to the total vimentin and desmin recovered, as determined from densitometric scans (data not shown), is in the range of 15–20. Moreover, after prolonged exposure to Cu-P, no additional major crosslinked products are detected. The same products are seen when Cu-P is directly included in the Triton/high-salt buffer used for the initial lysis and extraction as when purified IF (26) are used.

When cytoskeletons from aortic tissue of cow and chicken are crosslinked with Cu-P, a similar triplet of bands of high molecular weight products appears (Fig. 1 *b* and *c*). The ratio of the three products in each case approximately reflects the ratio of

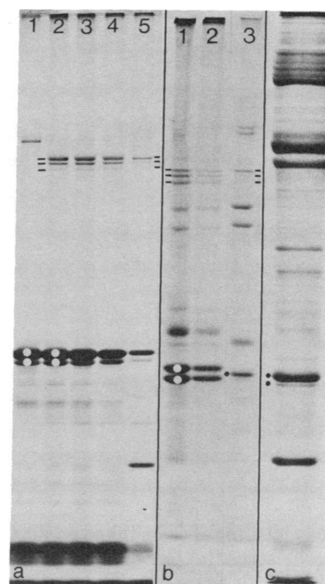

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoresis of crosslinked IF proteins. Horizontal bars mark the triplet of crosslinked products, and the white and black dots indicate vimentin (upper band) and desmin (lower band). In *a* and *b*, linear polyacrylamide gradients (6–15%) were used in the separating gel. In *c*, the separating gel contained 7.5% acrylamide. (*a*) Lanes: 1–4, time course of Cu-P (36 μM)-catalyzed crosslinking in purified IF from BHK-21 cells; 1, control, no Cu-P added; 2, 10 min; 3, 20 min; 4, 40 min; 5, products from crosslinked cytoskeletons of BHK-21 cells obtained by including Cu-P (36 μM) in the Triton/high-salt extraction buffer. Densitometric scanning of similar gels has shown the ratio of the three crosslinked products to be 3.0 (upper band):1.0 (middle):0.3 (lower), which corresponds to the total vimentin/desmin ratio of 4.5 in this BHK-21 cell culture (see refs. 16, 26). (*b*) Lanes: 1 and 2, crosslinked products from cytoskeletons of bovine aortic *tunica media* as induced by 72 μM Cu-P after 10 min (lane 1) and after autoxidation (lane 2) showing a triplet band ratio of 1:1:1; 3, Cu-P (72 μM)-catalyzed crosslinking of IF proteins from chicken aorta after 10 min of incubation showing a ratio of crosslinked products of 7.5:1.0:0.5. The black dot denotes chicken vimentin. (*c*) Cytoskeletal material from chicken aorta run under fully reducing conditions, showing the high ratio of monomeric vimentin to desmin in this tissue.

total vimentin and desmin in these cells and tissues. A striking feature of the aortic material from both species, not seen to this extent in BHK-21 cells, is the relatively high endogenous content of such crosslinked proteins in the control samples, which appears to result from autoxidation, as it is not seen in samples prepared in the presence of 50 mM iodoacetamide.

The molecular weights of the crosslinked products were difficult to determine in Tris glycine-based gel systems, which can result in overestimates of molecular weight of crosslinked polypeptides (37, 38). By using phosphate buffer-based gel electrophoresis (32), we found molecular weights in the 135,000–140,000 range for the crosslinked IF proteins. This indicates the formation of dimeric crosslinked polypeptides, in agreement with the known cysteine content (one; ref. 23) of both vimentin (mammalian, M_r, 57,000) and desmin (M_r, 54,000).

Immunological Characterization of the Crosslinked Products. The formation of a triplet of crosslinked products from two proteins can be interpreted as the formation of two homodimers containing only one protein each and one heterodimer containing both proteins. We have identified these triplet bands, after transfer to nitrocellulose paper, by labeling with antibodies specific for either vimentin or desmin (Fig. 2). The top and middle bands react with vimentin antibodies whereas the bottom and the middle bands react with desmin antibodies. Hence the

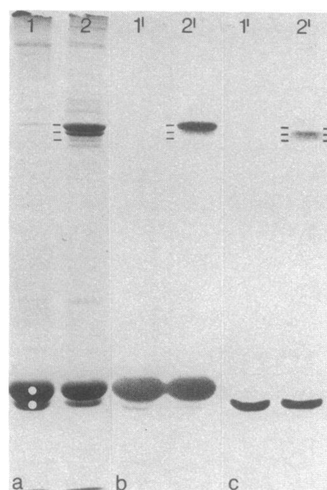

FIG. 2. Antibody labeling of crosslinked products (horizontal bars) from BHK-21 cytoskeletons. (*a*) Gel used for protein blot transfer to nitrocellulose stained with Serva blau R. Lanes: 1, control; 2, crosslinked samples (72 μM Cu-P, 10 min of incubation). (*b* and *c*) Autoradiograms of the protein blot after labeling with antibodies specific for vimentin (*b*) and desmin (*c*) and subsequent reaction with ^{125}I-labeled protein A. In *b* and *c*, lanes 1′ and 2′ are equivalent to lanes 1 and 2 in *a*, respectively. When shorter exposures are used for the vimentin antibody labeling shown in *b*, two weak but distinct bands are resolved that correspond to the top and middle bands. The reaction of the vimentin antibodies with a minor component (bottom part of *b*) is not with the desmin band but with a slightly faster migrating degradation product of vimentin (see refs. 14, 15, 19, 21).

middle band contains both desmin- and vimentin-specific determinants.

Biochemical Characterization of the Crosslinked Products. The advantage of using Cu-P as a crosslinker is the reversibility of the disulfide links on exposure to a reducing environment, which allows direct demonstration of the constituent monomers on subsequent gel electrophoresis. Electrophoresis of the triplet of crosslinked products separated in the first dimension shows, in a second dimension (Fig. 3), after cleavage of disulfide bonds, three combinations of polypeptide spots all clearly displaced from the diagonal expected for monomeric polypeptides. Homodimers of vimentin and desmin yield one spot each in the molecular weight position of the specific constituent monomer, whereas the middle component is unequivocally identified as a heterodimer by the appearance of two spots, one with the molecular weight of vimentin and the other with a molecular weight identical to that of desmin. To show more clearly the identity of the component monomers, we have also excised the individual bands of the triplet and analyzed the constituent monomers obtained on reduction by subsequent one- and two-dimensional gel electrophoresis. As shown in Fig. 4 for the heterodimer, the two IF protein monomers are detected in nearly equal amounts. Two-dimensional analysis further shows that the heterodimer contains the same set of isoelectric variants of vimentin and desmin (Fig. 4*c*) as the original cytoskeletal material (Fig. 4*d*), thus indicating that phosphorylated (39, 40) molecules are also included in such heterodimers—i.e., in heteropolymer IF.

We have also established the identity of each of the crosslinked products by peptide mapping of the excised polypeptide bands of the triplet (Fig. 5). The maps of tryptic peptides from radioiodinated crosslinked proteins show identity of the top band with monomeric vimentin and of the bottom band with monomeric desmin, but show common and different peptides

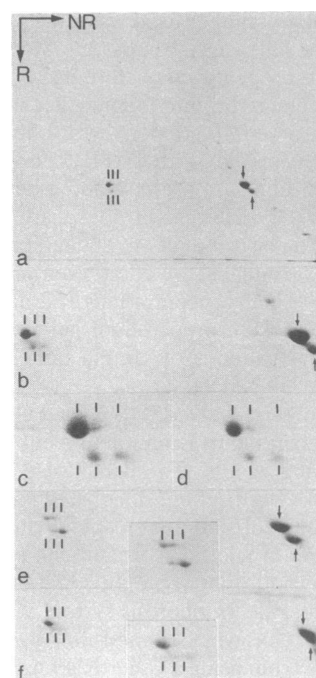

FIG. 3. Cleavage of crosslinked products of IF proteins by electrophoresis in a second dimension under reducing conditions. Polypeptides crosslinked and separated in one dimension under nonreducing conditions (NR) and then cleaved with 2-mercaptoethanol and run under reducing conditions (R) will be displaced from the expected diagonal of monomeric proteins. Cleavage products are marked by vertical bars and monomeric vimentin (upper spot) and desmin are indicated by arrows. (*a–d*) BHK-21 cells (*a*, survey; *b* and *c*, enlargements of relevant areas); (*d*) another preparation, with better spot separation, showing the presence of both vimentin and desmin in the middle band of the original triplet of crosslinked products. A similar pattern has been obtained for IF from *tunica media* of bovine aorta (*e*) and from chicken aorta (*f*). (*Insets*) Interesting areas of *e* and *f* shown at higher magnification.

of both proteins in the middle band component, identifying the latter as a heterodimer (Fig. 5*c*).

Controls. "Collision complexes" (41) resulting from interactions of protein monomers with other monomers or with filaments could distort the interpretation of the formation of crosslinked products. To prevent monomer or oligomer dissociation from filaments, buffers having ionic strengths known to maintain IF integrity were used throughout the preparation and subsequent crosslinking of cytoskeletal material or purified IF. Analysis of supernatant and pelletable material (150,000 × *g*, 20 min) after crosslinking showed no detectable IF protein in the supernatant. Furthermore, when IF preparations at different degrees of dispersion were compared, the extent and ratio of crosslinked products did not change. This latter control experiment also shows that the possibility of formation of interfilament cross-links is minimal, a possibility that is eliminated by the absence of formation of heterodimers when separate and homogeneous preparations of vimentin IF (isolated from RVF-SM cells; ref. 26) and desmin IF (from chicken gizzard) are mixed and then crosslinked, even when crosslinking of gizzard desmin was as much as 40% of total desmin.

DISCUSSION

Desmin and vimentin have been localized, by immunofluorescence microscopy, to fibrillar arrays of BHK-21 cells (15, 18, 28) and certain tracts of smooth muscle cells of the *tunica media* of aorta tracts of rat, cow, and chicken (19, 21, 22). Steinert *et*

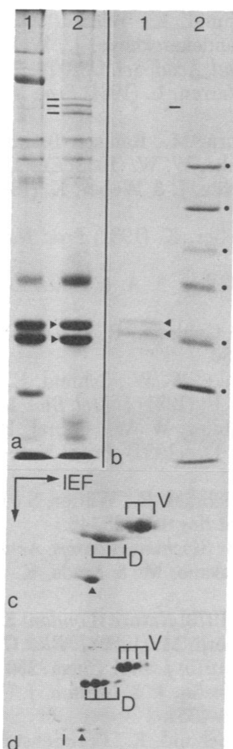

FIG. 4. Characterization of the heterodimer from crosslinked IF proteins of bovine aortic cytoskeletons by one- and two-dimensional gel electrophoresis. (*a*) Lanes: 1, purified IF prepared with 50 mM iodoacetamide in the extraction and washing buffers, showing the complete lack of autoxidation products (arrowheads, monomeric vimentin and desmin); 2, crosslinked products (horizontal bars) after 10 min of incubation with 72 μM Cu-P. (*b*) Lanes: 1, reelectrophoresis of excised and eluted middle-band protein after cleavage with 2-mercaptoethanol, showing the presence of equal amounts of vimentin and desmin (arrowheads) and a trace of uncleaved heterodimer (horizontal bar); 2, reference proteins (dots) are, from top to bottom, β-galactosidase (M_r, 120,000), phosphorylase a (94,000), transferrin (76,000), bovine serum albumin (68,000), glutamate dehydrogenase (55,000), and actin (42,000). (*c*) Two-dimensional gel electrophoresis (33). The eluted heterodimer was cleaved by reduction (IEF, isoelectric focusing; vertical arrow, second dimension electrophoresis in the presence of $NaDodSO_4$). Note the isoelectric variants of vimentin (V) and desmin (D) found in the heterodimer, which are also found in the respective homodimers (data not shown). Skeletal muscle α-actin (arrowhead) was added as marker. (*d*) Native cytoskeletal proteins from the same region of bovine aorta, showing the various isoelectric forms of vimentin and desmin. Endogenous vascular smooth muscle α-type actin (arrowhead); vertical bar, γ-type actin.

al. (27) have recently shown that purified desmin and vimentin can form copolymers *in vitro*. In these experiments, IF have been obtained that display solubility characteristics compatible with the presence of both IF proteins in the same filament. These authors have also suggested, from the specific mode of ionic-strength dependence of IF disintegration in BHK-21 cytoskeletons, that such IF may contain mixtures of desmin and vimentin. Our crosslink experiments on native cytoskeletons indicate that desmin and vimentin can occur as subunit proteins in the same IF, both in cultured BHK-21 cells and in smooth muscle tissues from avian and mammalian blood vessels. Artificial redistribution and rearrangement of IF protein molecules as a result of the production of soluble collision complexes and inter-IF crosslinking has been excluded by our controls. Therefore, we conclude that heteropolymer IF of desmin and vimentin occur in the living cell.

Within IF containing both desmin and vimentin, various ar-

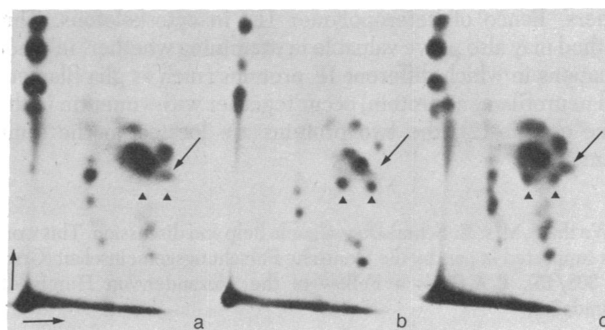

FIG. 5. Maps of radioiodinated tryptic peptides, detected by autoradiography, of the crosslinked products of IF proteins from bovine aorta *tunica media* (horizontal arrow, direction of electrophoresis; vertical arrow, direction of chromatography; see ref. 36). For simpler orientation, diagnostic peptides from vimentin (arrow) and desmin (arrowheads) are indicated. The top band of the triplet of the crosslinked products has a map (*a*) identical to that of monomeric vimentin, the map of the bottom band (*b*) is identical to that of monomeric desmin, and tryptic peptides characteristic of both vimentin and desmin are found in the middle band (*c*).

rangements could exist, such as mosaics of blocks of homopolymers of either protein or random distributions of the two proteins throughout an IF. While our data do not rule out the possible occurrence of homopolymer "islands" within heteropolymer IF, they clearly show that desmin and vimentin frequently occur as nearest neighbors, allowing crosslinking of the cysteine residues.

The ready formation of desmin–vimentin heterodimers as result of autoxidation or Cu-P-catalyzed oxidation also allows the conclusion that the only cysteine residue present in both IF proteins, which is located at position 141 from the carboxy terminus (23), is exposed on the surface of the molecule. In addition, our data show that within IF adjacent desmin and vimentin molecules are frequently, if not always, oriented in a mirror-image symmetry, with their cysteine residues close together, allowing the formation of disulfide bridges of 2.08 Å (42) within structurally intact IF. Although the formation of intermolecular disulfide bridges is compatible with IF structure, it is important to note that disulfide bridges are not necessary for maintenance of IF structure and for IF assembly. IF are resistant to treatment with thiol agents and it has been shown that IF assembly from monomers takes place in the presence of high concentrations of reducing agents (25–27, 43–45).

The demonstration of the existence of heteropolymer IF in the cells studied does not exclude the additional occurrence of some homopolymer IF in the same cells. In fact, double-label immunofluorescence microscopy of BHK-21 cells (ref. 20; unpublished data) shows that fibrillar arrays stained with vimentin antibodies often extend to cytoplasmic regions in which no desmin antibody staining is observed. This indicates that IF containing vimentin but not, or exceedingly little, desmin can occur in these cells in addition to IF that stain with antibodies to both desmin and vimentin.

Different types of smooth muscle cells contain different types of IF (4, 5, 18–22, 24). By immunofluorescence microscopy, various types of smooth muscle cells can be distinguished: one type, which is abundant in vascular walls, is positive for vimentin only; another type, found in smooth muscles, is positive only for desmin. It remains to be seen whether heteropolymer IF of desmin and vimentin are characteristic of a special route of smooth muscle differentiation and cell culture lines derived therefrom (see ref. 18). We suggest the use of oxidative crosslinking by Cu-P for the unequivocal demonstration of hetero-

dimers, hence of heteropolymer IF, in cytoskeletons. This method may also prove valuable in examining whether, in other situations in which different IF proteins (such as glia filament and neurofilament protein) occur together with vimentin in the same cell (3–12), the two proteins are located in the same filaments.

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