Properties of the Desmin Tail Domain: Studies Using Synthetic Peptides and Antipeptide Antibodies

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Abstract. Intermediate filament (IF) proteins have a common structural motif consisting of an α-helical rod domain flanked by non-α-helical amino-terminal head and carboxy-terminal tail domains. Coiled-coil interaction between neighboring rod domains is thought to generate the backbone of the 10-nm filament. There must also be other interactions between subunits to bring them into alignment and to effect elongation of the filament, but these are poorly understood. To examine the involvement of the tail domain in filament structure and stabilization, we have studied the interaction between a synthetic peptide corresponding to residues 442–450 of avian desmin, and authentic desmin protein. The potential importance of this region lies in its hydrophilic nature and its high degree of homology among the Type III IF proteins and cytokeratins 8 and 18. The peptide, D442-450, binds to a 27-residue region between lys-436 and leu-463, the carboxy terminus. The presence of the peptide during assembly causes the filaments to appear much more loosely packed than normal desmin IF. We have also generated polyclonal antibodies against this peptide and attempted to localize this portion of the tailpiece along desmin IFs by immunological procedures. By immunoblotting, we found that anti-D442-450 antibodies recognize desmin and only those proteolytic fragments that contain the tailpiece. In contrast, the antibodies do not label any structure in adult gizzard smooth muscle and skeletal muscle myofibrils in immunofluorescence experiments during which conventional antidesmin antibodies do. At the ultrastructural level, anti-D442-450 antibodies label free desmin tetramers but not desmin IFs. These results show that, as part of an assembled IF, the epitope of anti-D442-450 is inaccessible to the antibodies, and suggest that either the tailpiece of an IF protein may not be entirely peripheral to the filament backbone, or the interaction between end domains during assembly masks this particular region of the IF molecule.

INTERMEDIATE filaments (IFs)[1] are a major class of cytoskeletal elements found in nearly all eukaryotic cells, whose functions have not been fully elucidated. More than 30 different proteins are known to be capable of forming 8–10-nm IFs; all are located in the cytoplasm except the lamins, which form IF-like networks within the nuclear lamina. IF proteins can be divided into six distinct types based on their amino acid sequence, and their distribution roughly corresponds to the cell type origin of the protein (Steinert and Liem, 1990; Stewart, 1990). IF proteins share a common structural theme consisting of a highly conserved α-helical rod domain, flanked by a non-α-helical amino-terminal (headpiece) and carboxy-terminal (tailpiece) domains of variable size and sequence. Because the length of the rod region is virtually constant, it is thought that the rods stack side by side to form the IF backbone. In current models of IF assembly two monomers interact via their rod domains, in a parallel, in-register manner to form a coiled-coil dimer. Two dimers align side by side to form a tetramer (Geisler et al., 1982; Ip et al., 1985*a*; Quinlan et al., 1986) and further association of tetramers, both laterally and in an end-to-end fashion, ultimately leads to the formation of a 10-nm-wide filament (reviewed by Steinert and Roop, 1988; Robson, 1989; Stewart, 1990). Hence, IF proteins of different molecular masses can form nearly identical 10-nm filaments by virtue of having structurally homologous rod domains.

While this general model explains satisfactorily how a diverse family of proteins can form morphologically near-identical filaments, it raises several intriguing questions concerning the contribution of the terminal domains to the assembly of IFs. A corollary of the hypothesis is that the end domains of IF proteins are located outside the filament backbone. Yet, removal of the headpiece, either by limited chymotryptic digestion (Kaufmann et al., 1985) or by deletion at the DNA level (van den Heuvel et al., 1987; Albers and Fuchs, 1989), renders an IF protein assembly-incompetent. What, then, is the mechanism by which the headpiece exerts its influence on assembly while remaining peripheral?

A second question concerns the tailpiece. Although dele-

1. *Abbreviations used in this paper*: EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; IF, intermediate filament; RSA, rabbit serum albumin.

tion studies have shown that at least portions of the tailpiece are unessential for IF assembly, all type III IF proteins and cytokeratins 8 and 18 contain a highly homologous stretch of 10–15 residues that corresponds to the most hydrophilic region of the tailpiece (Romano et al., 1986; Franke 1987; Krauss and Franke, 1990), suggesting that this region may have a cytoplasmic orientation and may serve an as yet unidentified function. This, together with the recent observation by Quinlan et al. (1989) that a recombinant glial fibrillary acidic protein fusion protein lacking a carboxy terminus failed to form filaments in vitro, motivated us to examine if the tailpiece might play any role in IF structure and/or function. We used a two-step approach. First, we generated an antibody against a synthetic peptide modeled after the conserved region to probe if the carboxy terminus is exposed on the filament surface. Second, the synthetic peptide itself was used as a probe to evaluate the involvement and/or contribution of the carboxy terminus in the assembly of desmin filaments.

Materials and Methods

Peptide Synthesis

Two synthetic peptides were used in this study. The sequences for both were taken from the sequence of avian desmin published by Geisler and Weber (1982). The first one, which is referred to as D442-450, covers residues 442–450 and has the sequence K-T-I-E-T-R-D-G-E. The second peptide, which we used as a control for these studies, covers residues 26-36 and is named D26-36. Its sequence is P-R-A-S-F-G-S-R-G-S-G. An additional cysteine was added at the carboxy termini of peptides which were to be used as immunogens. Both peptides were synthesized at the Macromolecular Structure Analysis Facility, University of Kentucky at Lexington.

Generation of Antibodies

D442-450 was conjugated to keyhole limpet hemocyanin (KLH) via three separate cross-linkers: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), glutaraldehyde, and *m*-maleimidobenzoyl-*N*-hydroxysucciminide ester (MBS) (Pierce Chemical Co., Rockford, IL). The three conjugates were then mixed and the "cocktail" was emulsified with CFA before using for immunization. Rabbits were initially injected subcutaneously with 200 μg of peptide and intraperitoneal boost injections were given monthly with peptide emulsified with Freund's incomplete adjuvant. Serum was collected 10 d after injection. This procedure was continued until the rabbit produced antibodies of a sufficiently high titer. Preimmune sera were screened by Western blot analyses.

Purification and Characterization of Anti-D442-450

For Western blotting and immunofluorescence antisera were used. For EM, anti-D442-450 was further purified by chromatography on a desmin affinity column, which was constructed by coupling monomeric desmin to Affigel 15 (Bio-Rad Laboratories, Richmond, CA). Monospecific antibodies were eluted with 0.2 M glycine, pH 2.3. Fractions were identified by SDS-PAGE and stained with Gel-Code silver stain (Pierce Chemical Co.). Some experiments were done with Fab′ derived from affinity-purified IgG by the method of Brackenbury et al. (1977).

Immunofluorescence studies were performed with glycerinated myofibrils and with unfixed and −20°C methanol-fixed frozen sections of chicken gizzard smooth muscle. Myofibrils were released from stretched, glycerinated rabbit psoas by homogenization in 150 mM NaCl, 2 mM $MgCl_2$, 1 mM β-mercaptoethanol, 4 mM EGTA, and 15 mM Tris-HCl, pH 7.5. After antibody incubations, samples were fixed in 4% formaldehyde in TBS followed by antiquench (CitiFluor, London, England) before observation. Several commercial antibodies to desmin were used as controls, including mAbs from Amersham Corp. (Arlington Heights, IL) and Dako Corp. (Santa Barbara, CA), and a polyclonal preparation from Sigma Chemical Co. (St. Louis, MO). Anti-IFA, an mAb that recognizes all IF proteins (Pruss et al., 1981) was also used.

SDS-PAGE was performed using 10% separating gels according to the method of Laemmli (1970). Western blots were performed as described by Towbin et al. (1979) except that Immobilon P (Millipore Corp., Bedford, MA) was used in place of nitrocellulose. TBS containing 5% Carnation dried milk was used as the blocking agent. Affinity-purified goat-anti-rabbit Ig conjugated to alkaline phosphatase (Sigma Chemical Co.) was used as the secondary antibody and the colorimetric substrate was nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP).

Biotin Labeling of Synthetic Peptides

Peptides were labeled with *N*-hydroxy succinimide LC biotin (Pierce Chemical Co.) at a fivefold molar excess under a nitrogen atmosphere for a minimum of 3 h at 23°C. Tris was added to a final concentration of 20 mM to quench any excess reactive *N*-hydroxy succinimide groups. To ascertain that the peptides were biotinylated, they were cross-linked to rabbit serum albumin (RSA) using EDC, run on an SDS polyacrylamide gel and probed by Western blot analysis with alkaline phosphatase–conjugated antibiotin antibodies (Sigma Chemical Co.).

Purification and Proteolytic Modification of Desmin

Desmin was purified according to the method of Geisler and Weber (1980) with minor modifications. In place of Sephadex G-25 gel filtration, the crude desmin was concentrated with an Amicon concentrator fitted with a YM30 membrane to remove low molecular weight contaminants. It was then loaded onto a DEAE-Sepharose-CL6B column (Pharmacia Fine Chemicals, Piscataway, N.J.) and eluted with a 0–500-mM NaCl gradient. Desmin-containing fractions were identified by SDS-PAGE.

The removal of desmin residues 1–67 with thrombin (Sigma Chemical Co.) to produce T-desmin was done according to Lu and Johnson (1983). Chymotrypic cleavage of tetramers to produce rod domains was carried out in 10 mM Tris pH 8.5 [Geisler et al., 1982] at 22°C, at a final chymotrypsin:desmin molar ratio of 1:250. L-desmin (desmin lacking its carboxy-terminal 27 residues) was produced according to Kaufmann et al. (1985). Endoproteinase lys-C (Boehringer-Mannheim Biochemicals, Indianapolis, IN) digestion was carried out on either IFs in 50 mM NaCl, 0.1 mM DTT, 10 mM Tris-HCl, pH 7.5, or tetramers in 10 mM Tris, 0.1 mM DTT, pH 8.5. The final concentration of lys-C was 1% protease/mg of IF, 2.5%/mg of tetramers. The reaction was terminated with aprotinin (Sigma Chemical Co.) and digestion products were analyzed on 10–22.5% SDS polyacrylamide gels.

Binding Studies

Desmin at different oligomeric states was incubated with biotin-labeled synthetic peptide at 200× molar excess for 30 min at room temperature. To assay binding during assembly, desmin was initially dissolved in 8 M urea, 5 mM EGTA, 0.1% β-mercaptoethanol, 10 mM phosphate buffer, pH 7.5. It was then mixed with 3 vol of the same buffer without urea but containing the synthetic peptide, such that the urea concentration is reduced to 2 M in the presence of the peptide. To assay binding to tetramers, desmin and peptide were, respectively, dialyzed against and dissolved in 10 mM Tris, 0.1% β-mercaptoethanol, pH 8.5, before mixing. To assay for binding to IFs, desmin and peptide were mixed in 170 mM NaCl, 10 mM Tris, 0.1% β-mercaptoethanol, pH 7.0. After incubation, samples were cross-linked for 30 min with 30 mM glutaraldehyde in the same buffer in which the binding reaction was carried out, with continuous stirring. The reaction was then stopped by the addition of SDS-PAGE sample buffer, followed by heating to 95°C for 3 min. Samples were analyzed using either 10% or 10-22.5% gradient gels, as indicated in the figure legends. The blots were probed in one of two ways: (*a*) using anti-biotin antibodies conjugated to alkaline phosphatase (Sigma Chemical Co.) or (*b*) using antidesmin antibodies (Dako Corp.) followed by anti-rabbit IgG conjugated to alkaline phosphatase.

Electron Microscope Procedures

The position of the epitope of anti-D442-450 along the tetramer was determined by rotary shadowing of T-desmin-antibody complexes. Affinity-purified anti-D442-450 IgG or Fab′ were incubated with T-desmin in molar ratios up to 2:1, diluted with 50% glycerol and sprayed onto freshly cleaved mica (Tyler and Branton, 1980). Using a freeze-etch apparatus (model 301 or 400D; Balzers Corp., Hudson, NH) the samples were rotary replicated at 5° with platinum-carbon followed by a thin carbon coat. Replicas were floated off on water then picked up onto uncoated grids.

For immunoelectron microscopy of IFs, purified desmin filaments were adsorbed onto carbon-coated, poly-lysine-treated grids, and then blocked with cytochrome *c*. Grids were then incubated by flotation on successive drops of affinity-purified primary antibody, washing solution, 5-nm gold-conjugated, affinity-purified, secondary antibody and washing solution. The grids were then negatively stained using 1% uranyl acetate. The conjugation of secondary antibodies to 5 nm colloidal gold was carried out in the presence of 2% fish gelatin to reduce nonspecific binding (Birrell et al., 1987).

Fresh carbon films without formvar support were used for negative staining of filaments formed in the presence of synthetic peptide D442-450. Glow discharge, a procedure commonly used to render carbon films hydrophilic, was specifically avoided, because normal desmin and vimentin IFs often unravel when deposited on glow-discharged carbon supports (Birkenberger, L., and W. Ip, unpublished). Untreated carbon films, on the other hand, often retain the minute oil droplets that are generated in a rotary pump- and oil diffusion pump-based vacuum evaporator, and these oil droplets appear as numerous round particles in the background of a negatively stained specimen, e.g., Fig. 7.

Results

Characterization of Anti-D442-450

Anti-D442-450 is highly specific for desmin (Fig. 2), in addition to being highly reactive with the immunogenic synthetic peptide (data not shown). Not only does it recognize desmin specifically in a crude whole muscle cell homogenate (Fig. 2 *A*); it also distinguishes among the IF family of proteins in a manner commensurate with the degree of homology. The type III IF proteins—desmin, vimentin, GFAP and peripherin—share extensive homology in the region covered by D442-450, there being only two conserved (ile → val, glu → gln) amino acid substitutions in vimentin, one conserved (ile → val) and one unconserved (thr → met) change in GFAP and a single lys → arg substitution in peripherin (Fig. 1). Anti-D442-450 recognizes neither vimentin nor GFAP, but does cross-react with peripherin, as judged by Western blot analyses (Fig. 2 *A*). Anti-D442-450 does not recognize NF-L, NF-M, or NF-H, which do not share sequence homology with desmin in this region.

The specificity of anti-D442-450 for the carboxy-terminal tailpiece domain of desmin was further demonstrated by Western blot analyses using well-defined products of limited proteolysis. Three proteases—chymotrypsin, thrombin, and endoprotease lys-C—were used because of their previously demonstrated specificities. Chymotrypsin removes the headpiece and tailpiece domains rapidly under the conditions used (chymotrypsin:desmin molar ratio of 1:250, at 23°C); longer incubations result in cleavage of the surviving rod domain into a 21,000-M_r, amino-terminal Helix 1 and an

DESMIN	K	T	I	E	T	R	D	G	E
VIMENTIN	-	-	V	-	-	-	-	-	Q
GFAP	-	-	V	-	M	-	-	-	-
NIFP	R	-	-	-	-	-	-	-	-

Figure 1. Amino acid sequences of the type III IF proteins in the region covered by residues 442–450 of avian desmin (D442–450). The data for chicken desmin, hamster vimentin, and mouse glial fibrillary acidic protein are taken from Geisler and Weber (1986). The sequence for peripherin is taken from Thompson and Ziff (1989).

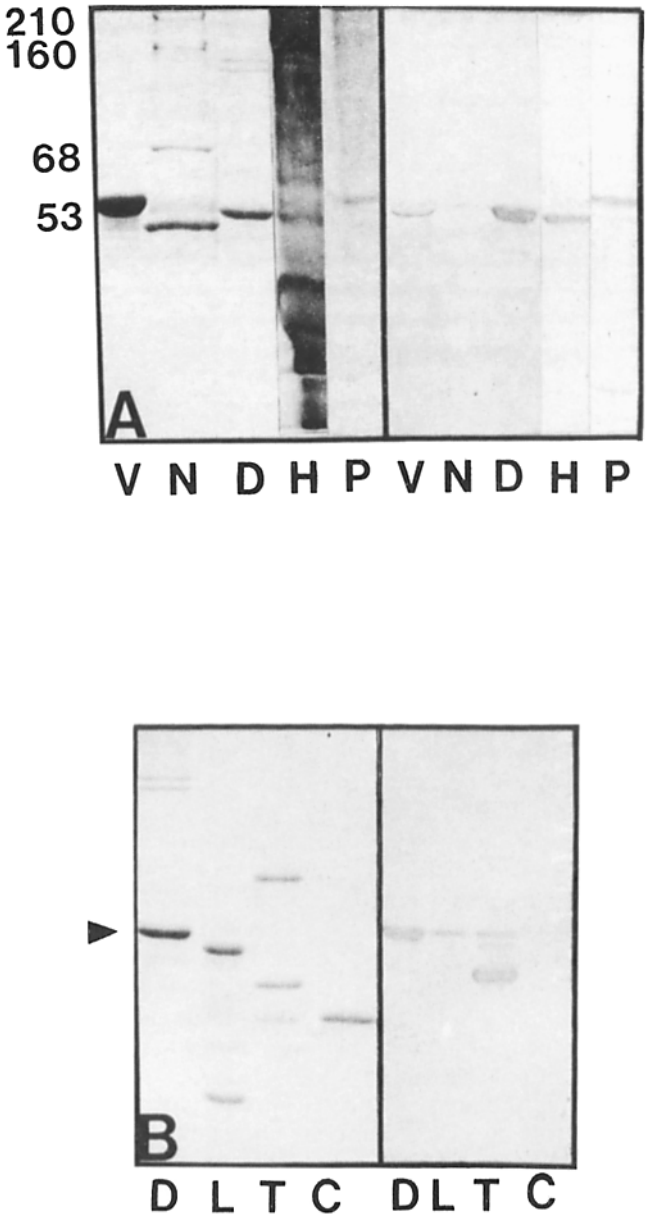

Figure 2. Western blot analysis of anti-D442-450 reactivity. (*A*) Purified porcine aortic vimentin (lanes *V*), a crude bovine neurofilament preparation (lanes *N*) containing NF-H, NF-M, NF-L, and GFAP, purified gizzard desmin (lanes *D*), crude chicken gizzard homogenate (lanes *H*) and a cytoskeletal preparation of PC12 cells containing peripherin (lanes *P*) were run on SDS gel (*left*) and the blot probed with anti-D442-450 (*right*). The antibody recognized only the avian desmin, the trace amount of desmin that copurified with the porcine vimentin, and peripherin. It identified a single, desmin band in the crude gizzard homogenate. Lanes *H* and *P* in the left panel were actually from gels stained after electrotransfer, which removed over 80% of the total proteins. The numbers on the right denote relative molecular mass in thousands. (*B*) The domain-specificity of anti-D442-450 was demonstrated by Western blot analysis of proteolytic derivatives of desmin. (*Left*) SDS-PAGE; *right*, immunoblot. *D*, intact chicken gizzard desmin. *L*, L-desmin, which lacks residues 437–463 of the tailpiece. *T*, T-desmin, which lacks residues 1–67 of the headpiece. *C*, the desmin rod, which spans residues 94–415. The antibody reacted only with T-desmin and the remaining intact desmin in each preparation. The band indicated by an arrowhead is desmin. The high molecular weight band in lane *T* is thrombin, used to generate *T*-desmin.

18,000-M_r, carboxy-terminal Helix 2 (Geisler et al., 1982). Thrombin digestion of desmin removes residues 1-67, producing T-desmin, a molecule with a truncated headpiece, while endoprotease lys-C cleaves at lys-436 and lys-437, resulting in L-desmin, a species with a truncated tailpiece (Kaufmann et al., 1985). Western blot analyses (Fig. 2 *B*) showed that anti-D442-450 recognizes only those desmin species that contain residues 442-450, i.e., intact desmin and T-desmin; the rod and L-desmin are not recognized. Thus, the epitope for anti-D442-450 lies carboxy-terminal to lys-437, in good agreement with the location of the immunogenic peptide along the molecule.

Anti-D442-450 Labels Free, Unassembled Desmin Tetramers

The tetramer represents a level of subunit organization of considerable importance because it is a stable intermediate of the assembly of IFs from monomeric polypeptides (Crewther et al., 1983; Geisler et al., 1985; Ip et al., 1985*a*,*b*; Quinlan et al., 1986). This species has been detected in the cytoplasm of fibroblasts in the form of a small but distinct IF subunit pool (Soellner et al., 1985) and is therefore considered to be a fundamental building block of IFs. It is of interest to localize the epitope of anti-D442-450 along the desmin tetramer for two reasons. First, the arrangement and orientation of the dimeric coiled-coils within the tetramer is not known with certainty (see Geisler et al. [1985] and Ip [1988] for different possibilities) and the localization of a tailpiece-specific epitope along it might provide information in this regard. Second, it is commonplace to find antipeptide antibodies reacting with proteins from which their immunogenic sequences are derived, only under denaturing conditions such as those encountered during Western blotting but not under native conditions (Dyson et al., 1988). Thus, it was important to verify that anti-D442-450 is indeed capable of recognizing desmin in a native conformation, i.e., in the absence of a denaturant.

T-desmin tetramers were used in this experiment. This desmin derivative lacks the amino-terminal 67 residues and therefore does not polymerize into IFs under the ionic conditions used for antibody–antigen binding and rotary replication (Kaufmann et al., 1985). Anti-D442-450 IgG purified on a protein A-agarose column were incubated with T-desmin tetramers in 100 mM NaCl, 10 mM Tris HCl, pH 7.0, for 2 h at room temperature, at ratios of up to 2 mol of antibodies per mol of desmin monomer. The mixture was then thoroughly mixed with an equal volume of glycerol, sprayed onto freshly cleaved mica and replicated with platinum-carbon. Fig. 3 is a montage of electron microscope images of the immune complexes, showing that the antibodies bind predominantly to one end of T-desmin tetramers. In one instance we did find what appeared to be binding of IgG molecules to both ends of the tetramers (Fig. 3, *bottom right*), although the length of the desmin–antibody complex raises doubt as to whether it consists of one or two tetramers. We did not observe labeling elsewhere along the tetramers. This further argues for the specificity of the antibodies and, more importantly, demonstrates that they do recognize desmin in an oligomeric form.

Anti-D442-450 Does Not Label Assembled Filaments

Several investigators have postulated that the terminal domains of IF proteins are located outside of the filament backbone, which consists of α-helical rod domains (Steinert et al., 1985*a*,*c*; Geisler and Weber, 1986). Two lines of evidence support this hypothesis. First, several proteolysis studies have shown that substantial amounts of end domain peptides could be released from keratin IFs (Steinert et al., 1983, 1985*b*) without apparent disruption of the morphologic integrity of the filaments. Likewise, desmin from which the carboxy-terminal 27 amino acids have been excised retains its ability to polymerize into IFs of normal appearance (Kaufmann et al., 1985). Although these studies do not directly address the issue of where the tailpiece domain of a given polypeptide is physically located in relation to the entire IF, the inference from these observations is that it probably resides outside of the IF backbone (Steinert et al., 1985*c*). Second, similar results have also been reported in proteolysis studies on neurofilaments (Chin and Eagles, 1983; Eagles and Maggs, 1985), and Hisanaga and Hirokawa (1988) have demonstrated by electron microscopy that NF-M and NF-H, which have anomalously large carboxy-terminal extensions, reassemble into filaments bearing peripheral sidearms.

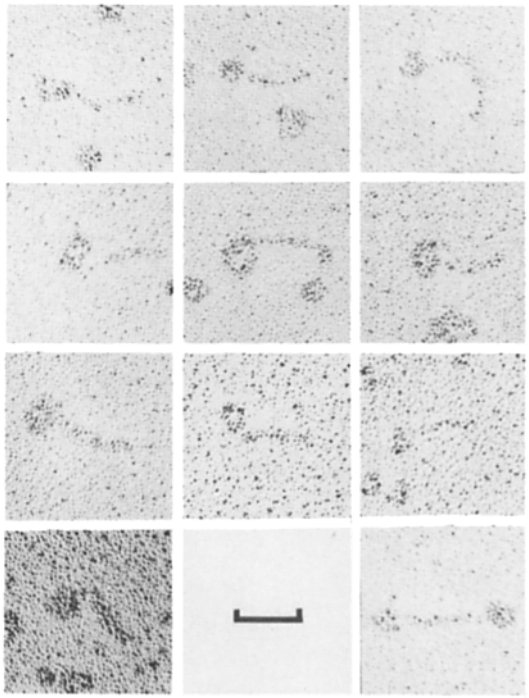

Figure 3. Gallery of T-desmin tetramers treated with an excess of anti-D442-450 IgG. Note predominant one-ended labeling. The immune complex shown on the lower right is the only clear case of two-ended labeling, although it is considerably longer than other complexes. Bar, 50 nm.

By virtue of its specificity, anti-D442-450 would seem to be an excellent probe for examining this question. According to the hypothesis, one would expect residues 442–450 of chicken desmin to be peripheral to the filament backbone because this segment of the chain is situated at the carboxy terminal end of the tailpiece domain and also because its hydrophilic nature suggests a cytoplasmic orientation. It follows that anti-D442-450 should label intact IFs because its epitope should be accessible. This was tested by immunofluorescence microscopy of isolated skeletal muscle myofibrils and frozen sections of chicken gizzard smooth muscle, which

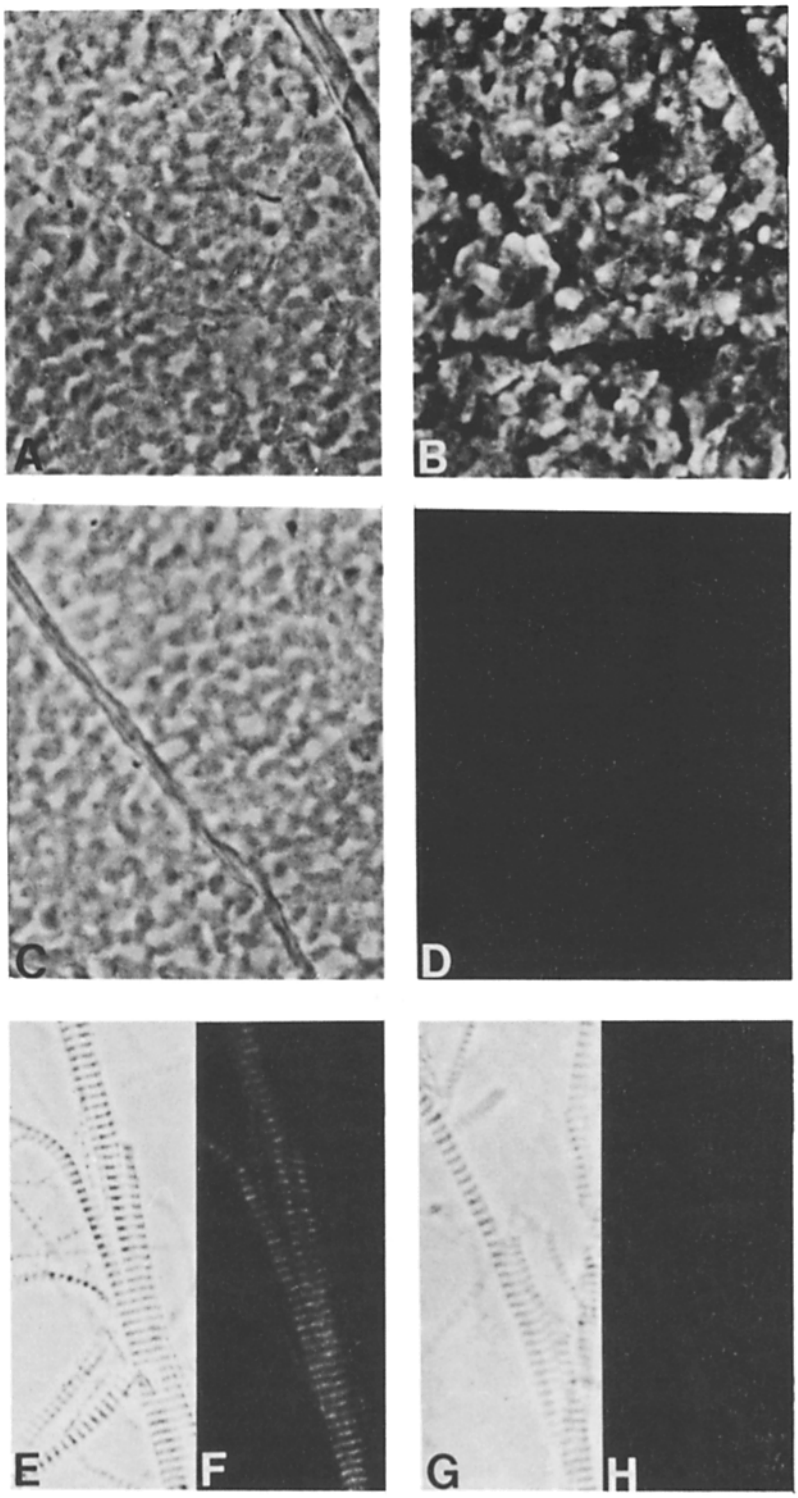

Figure 4. Immunofluorescence of muscle tissue demonstrating the lack of staining of anti-D442-450. (*A* and *B*) Frozen sections of chicken gizzard smooth muscle stained with a polyclonal antidesmin antibody (Dako Corp.). (*C* and *D*) Chicken gizzard smooth muscle stained with anti-D442-450. (*E* and *F*) Skeletal myofibrils stained with D3, a monoclonal anti-desmin (Danto and Fischman, 1984). (*G* and *H*) Myofibrils stained with anti-D442-450. The first of each pair is a phase contrast micrograph, the second a fluorescence micrograph. Note complete absence of immunoreactivity in the case of anti-D442-450. 872×.

are both rich in desmin. Surprisingly, anti-D442-450 did not recognize Z bands or any other structure in myofibrils, and failed to stain any cells in fixed or unfixed frozen sections of gizzard smooth muscle, either in the form of IgG or Fab′ fragments (Fig. 4). Under identical conditions, commercial polyclonal antidesmin antibodies (Amersham Corp.) and a monoclonal anti-desmin (D3; Danto and Fischman, 1984) stained the same tissues strongly at locations known to contain desmin, the Z-bands of myofibrils, the cytoplasm of individual smooth muscle cells within the muscular tissue stroma of gizzard, and the tunica media of surrounding arteries.

To examine the possibility that the epitope in muscle cells might be masked by the presence of associated protein(s), we attempted to label desmin IFs in vitro. Filaments reconstituted from purified desmin were adsorbed to carbon-coated electron microscope grids, and incubated via flotation on sequential drops of purified anti-D442-450 IgG and

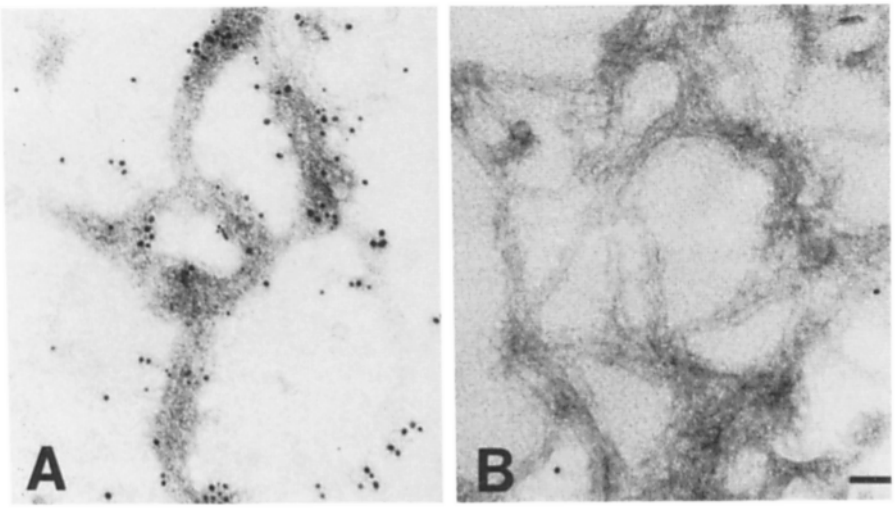

Figure 5. Negative-stain immunoelectron microscopy of reconstituted desmin IFs. (*A*) Desmin IFs sequentially incubated with a biotinylated polyclonal antidesmin (Sigma), goat antibiotin IgG and rabbit-anti-goat IgG conjugated to 5 nm colloidal gold. (*B*) Desmin IF incubated with affinity-purified anti-D442-450 followed by goat-anti-rabbit IgG conjugated to 5 nm gold. Note extensive labeling in *A* but the complete lack of gold label in *B*. Bar, 50 nm.

affinity-purified anti-rabbit IgG conjugated to 5 nm colloidal gold. The grids were then negatively stained with 1% aqueous uranyl acetate for EM. Under these conditions we could not detect significant gold labeling even when the incubation was carried out with a fivefold excess of affinity-purified anti-D442-450 IgG over desmin (Fig. 5), whereas the commercial polyclonal antibodies (Sigma Chemical Co.) gave heavy gold labeling under identical conditions. We therefore conclude that anti-D442-450 labels desmin tetramers but does not label assembled desmin IFs.

Binding of Synthetic Peptide D442-450 to Desmin

The two polypeptides in an IF protein dimer are parallel and in-register (Crewther et al., 1983; Steinert et al., 1984; Parry et al., 1985), such that homologous stretches of the two strands are very closely apposed. Consistent with this is our recent observation that two cyanogen bromide–derived fragments of desmin self-associate in a homologous fashion under filament assembly conditions (Saeed and Ip, 1989), suggesting that like regions of an IF protein may interact to stabilize the filament. We therefore sought to determine if synthetic peptides modeled after portions of the desmin sequence would bind desmin.

A binding experiment was first carried out using biotinylated peptides. To ascertain that the peptides were biotinylated, they were each cross-linked to RSA using EDC and analyzed by Western blotting using alkaline phosphatase–conjugated antibiotin antibodies. This experiment verified that both RSA–peptide conjugates were recognized by the antibiotin antibodies whereas RSA alone was not. Western blots were also probed with the corresponding antipeptide antibodies to verify that the peptides were bound to the RSA (results not shown).

Monomeric desmin in 8 M urea, 5 mM EGTA, 0.1% β-mercaptoethanol, 10 mM phosphate buffer, pH 7.5, was used initially to maximize the accessibility of binding sites. It was mixed at different molar ratios with synthetic peptide (either D442-450 or D26-36) dissolved in buffer without urea, such that the urea concentration was simultaneously reduced to 2 M. Samples were then cross-linked with glutaraldehyde and analyzed by SDS-PAGE and Western blotting (Fig. 6). In agreement with Quinlan et al. (1986), chemical cross-linking of desmin in 2 M urea produced an SDS-insensitive species that migrated just ahead of the myosin heavy chain molecular weight marker and therefore has an apparent M_r of ~200,000 (Fig. 6, *second lane from left*). In keeping with the assignment of Geisler and Weber (1982) we interpret this species as a tetramer of desmin. When the formation, and subsequent cross-linking, of desmin tetramers were done in the presence of biotinylated D442-450, a species whose mass was indistinguishable from that of the desmin tetramer alone was obtained (Fig. 6, *fourth lane from left*). Western blot analysis of this cross-linked product with antibiotin antibodies (Fig. 6, *right, fourth lane from left*) revealed that it contained biotin, indicating that D442-450 must be present. In a control experiment wherein the synthetic peptide D26-36 was substituted for D442-450, the corresponding 200,000-M_r species was found not to be biotinylated. This indicates that, in the desmin dimer, the binding of homologous stretches of neighboring monomers to one another is not a generalized, nonspecific phenomenon, but one that may be specified by the actual sequence involved.

The same experiment was then carried out, starting with desmin tetramers (in 10 mM Tris, 0.1% β-mercaptoethanol, pH 8.5) to assess peptide binding. The results from this experiment (Fig. 6) closely parallel those of the previous one, indicating that D442-450 does bind tetrameric desmin. Again, synthetic peptide D26-36 did not bind desmin tetramers in a parallel experiment. Neither peptide bound when added to preassembled desmin IFs. This could not be assayed using the cross-linking strategy because cross-linked IFs would not migrate into the polyacrylamide gel, but was instead assessed by immunolabeling with anti-D442-450 (see Fig. 10, below).

To address the question of whether the synthetic peptide binds to a specific region of desmin, proteolytically modified products were used. Endoproteinase lys-C cleaves desmin into two fragments, L-desmin (which includes the amino terminus, the rod and a small portion of the carboxy-terminus) and a fragment consisting of the carboxy-terminal 27 residues of the tailpiece (Kaufmann et al., 1985). Digested desmin tetramers in 10 mM Tris, 0.1 mM DTT, pH 8.5, were incubated with a 200-fold molar excess of biotinylated D442-

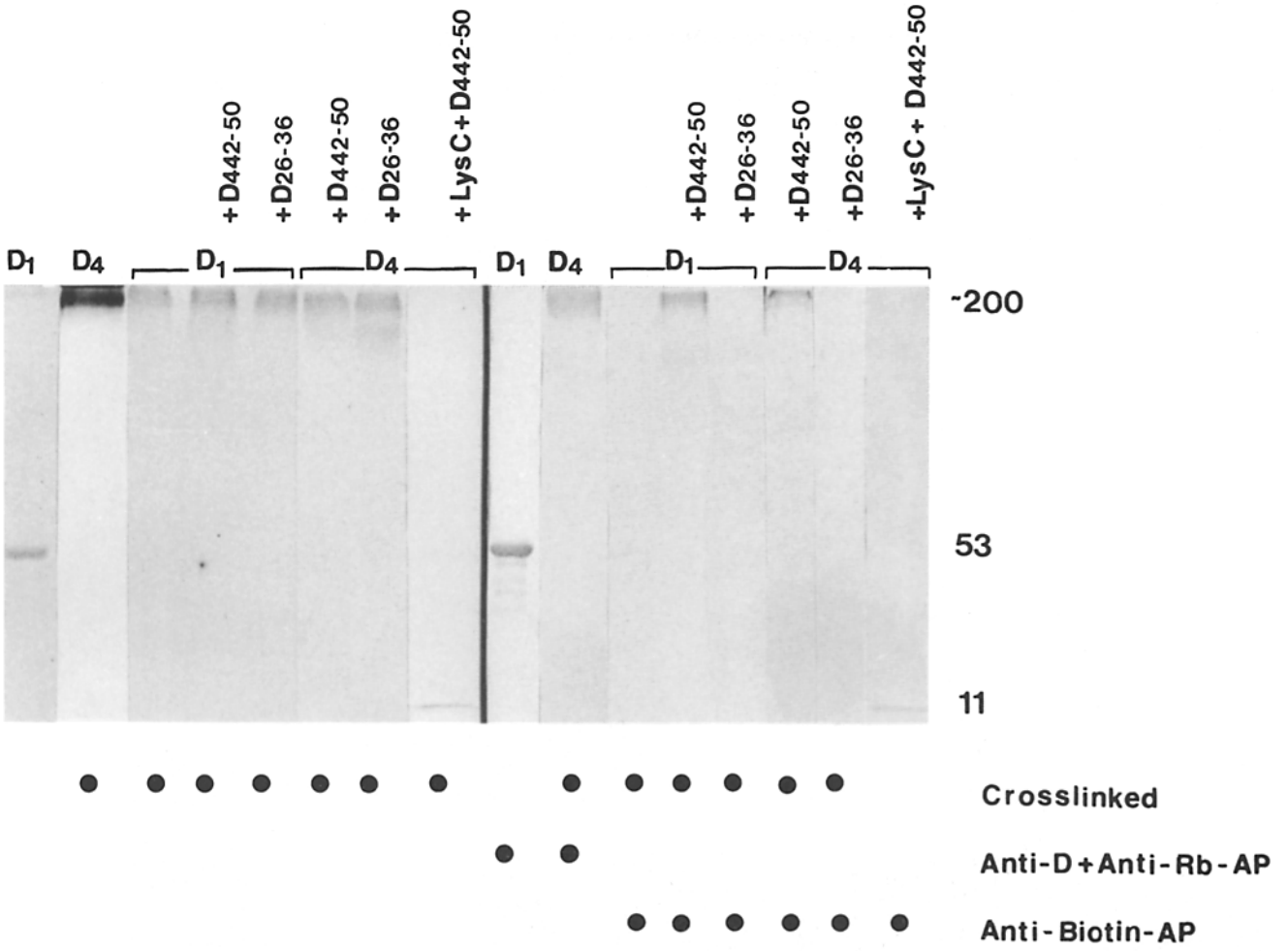

Figure 6. Immunoblot analysis of the binding of synthetic peptides D442-450 and D26-36 to desmin. (*Left*) SDS-PAGE. (*Right*) Immunoblot. The presence or absence of black dots across the bottom of the panels denote whether or not the protein or protein–peptide adduct was cross-linked, and in the case of the right panel, the antibody with which the blot was probed (antidesmin polyclonal followed by alkaline phosphatase-conjugated goat-anti-rabbit antibodies, or directly with alkaline phosphatase-conjugated anti-biotin antibodies). D_1 denotes desmin monomers, D_4 denotes desmin tetramers. The first and second lanes on the far left of each panel show the migration positions of desmin without and with cross-linking, respectively. The next three lanes demonstrate that D442-450, but not D26-36, incorporates into tetramers, as indicated by the presence of biotin in only the D+D442-450 cross-linked product. The next two lanes demonstrate that D442-450, but not D26-36, bind to desmin tetramers, as indicated by the presence of biotin in only the D+D442-450 cross-linked product. The lane on the far right shows endoproteinase lys-C digestion products incubated with D442-450 and subsequently cross-linked. The biotin is found only in the 11,000-M_r fragment which contains the carboxy-terminal 27 residues cleaved by lys-C. This establishes that D442-450 binds to a site within this region. (The lane at the far right was from a 10-22.5% gradient gel; all others were 10%.)

450, cross-linked with glutaraldehyde and then analyzed by SDS-PAGE and Western blotting (Fig. 6). The cross-linking yielded two SDS-insensitive products, a 200,000-M_r species corresponding to L-desmin tetramers and an 11,000-M_r species containing the carboxy-terminal 27 residues cleaved during proteolysis. Western blot analysis of these cross-linked products with anti-biotin showed that only the 11,000-M_r species contained biotin. This led us to conclude that the synthetic peptide D442-450 binds intact desmin at a location between lys-436/lys-437, the lys-C-cleavage site, and the carboxy terminus at leu-463.

Filaments Assembled in the Presence of D442-450 Have Altered Morphology

In view of the specificity of peptide binding, it was important to determine whether filaments could be assembled in the presence of the peptide, and if so, whether their structure was detectably altered. Desmin monomers were incubated with non-biotinylated D442-450 at molar ratios ranging from 1- to 300-fold, diluted as described above to form tetramers. These were then further dialyzed into assembly buffer (170 mM NaCl, 10 mM Tris, 2 mM β-mercaptoethanol, pH 7.0) overnight at 4°C, negatively stained and examined by EM. Fig. 7 compares representative fields of desmin IFs assembled in the presence (Fig. 7 *A*) and absence (Fig. 7 *B*) of D442-450. While many of the filaments formed in the presence of peptide were normal in morphology, a significant population appeared to have larger apparent diameter and lighter staining (Fig. 7 *B*). Closer examination of these aberrant filaments (Fig. 7 *C*) revealed that the larger

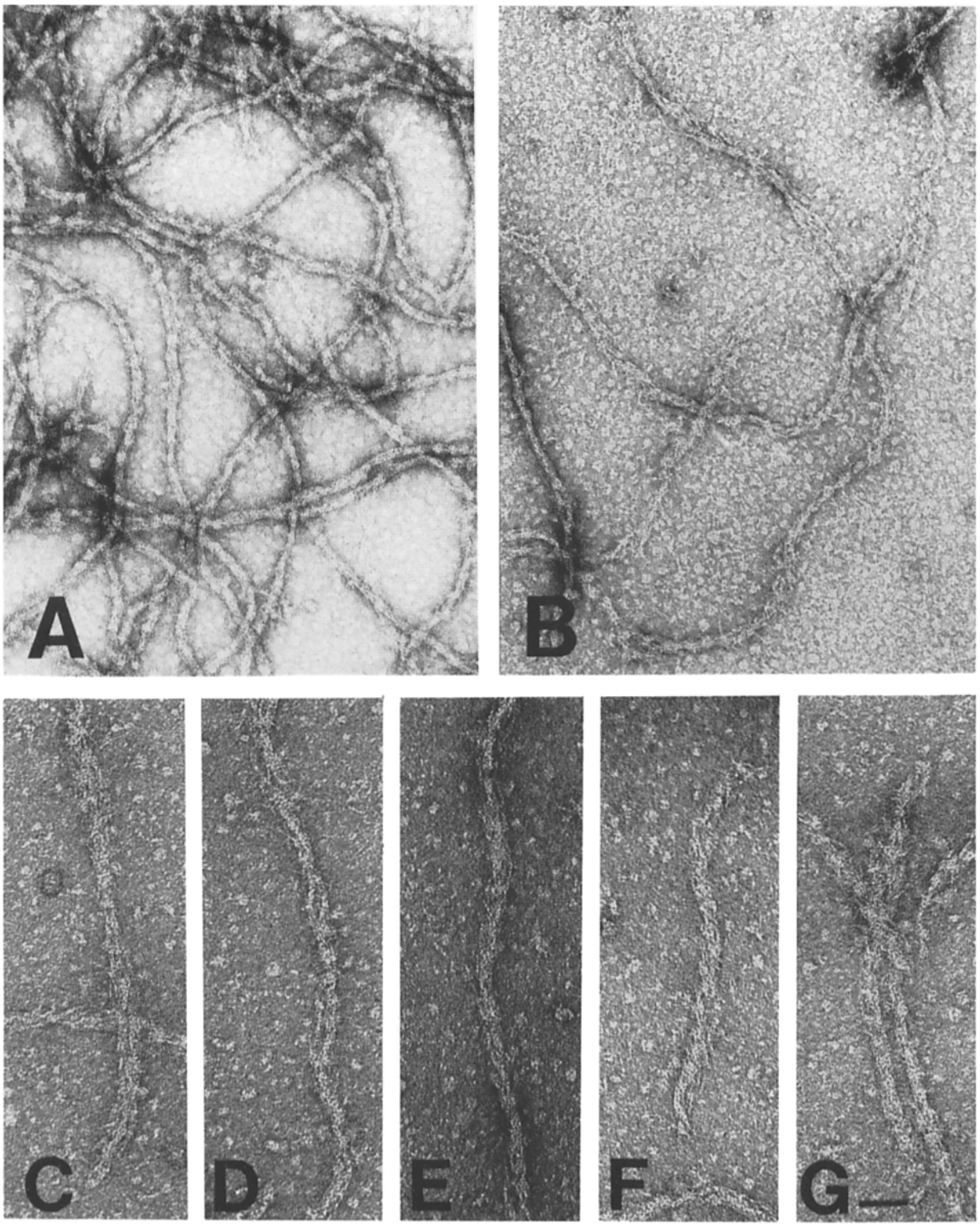

Figure 7. Modification of desmin IF structure by D442-450. (*A*) IFs formed by dialysis against assembly buffer; (*B*) desmin was mixed with D442-450 in 8 M urea, 10 mM Tris, pH 8.5, diluted with 3 vol of 10 mM Tris to lower the urea concentration to 2 M to allow tetramerization, and then dialyzed against assembly buffer. Note uniform caliber of IFs in *A*, but lightly stained, loosely packed IFs in *B*. Not all filaments were unraveled. (*C–G*) Higher magnification details of peptide-modified IFs. Note the larger apparent diameters of the modified filaments, the loosely woven appearance and the tetramers/protofibrils that have become more evident as a result of the unraveling. Bars: (*A* and *B*) 31.25 nm; (*C–G*) 50 nm.

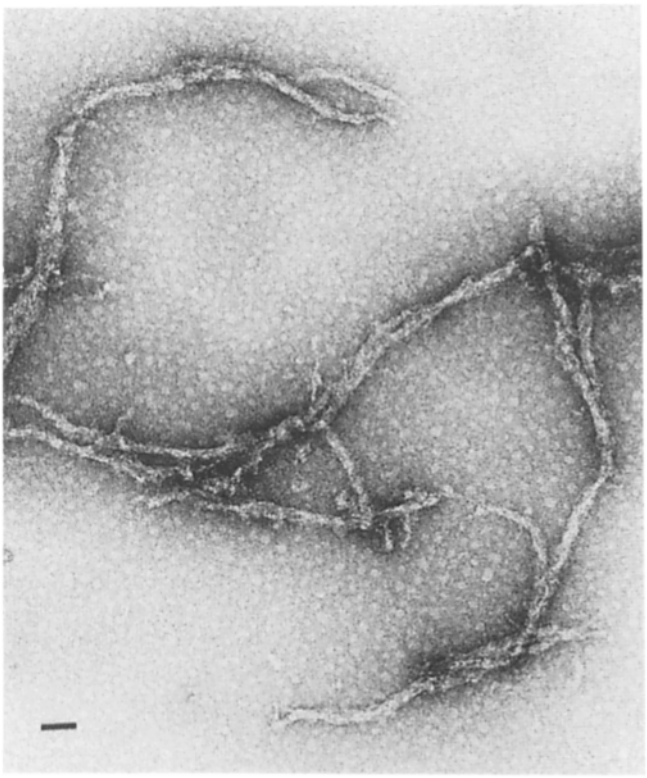

Figure 8. Desmin filaments assembled from tetramers to which D442-450 was added. These filaments are irregular in caliber and are ribbon like. In many cases they give the appearance of bifurcating, with the split ends eventually recombining again. Bar, 50 nm.

apparent diameter was a result of looser packing of subfilaments, reminiscent of the phosphate-induced unraveling of IFs previously described by Aebi et al. (1983). Very fine fibrils, suggestive of protofibrils, were frequently recognized. These fine fibrils appeared to be considerably longer than the length of a tetrameric subunit (48–60 nm; Geisler et al., 1982; Ip et al., 1985*a*; Potschka, 1986; Quinlan et al., 1986, 1989), suggesting that elongation of the protofibrils was less affected than lateral association. These structural alterations of IFs assembled from peptide-bound desmin was observed when peptide was present at molar ratio as low as 1:1. Increasing molar excess of peptide brought about more numerous altered IFs but no attempts were made to quantify these results.

Assembly of tetramers incubated with D442-450 also yielded loosely packed filaments, but to a lesser degree (Fig. 8). In contrast to those formed from peptide-bound monomers, filaments observed in this experiment appeared to be more ribbon-like, with varying diameter along their length.

Anti-D442-450 Antibodies Label Filaments Formed in the Presence of Synthetic Peptide D442-450 But Not Those Formed in its Absence

The failure of anti-D442-450 to label reconstituted IFs suggest that, in a filament, the portion of the tailpiece covered by residues 442–450 is partially or not at all exposed. However, another plausible explanation may be that the affinity of the antibodies was not sufficiently high to withstand the vigors of multiple washings. One way to test this would be to perform immunolabeling of the IFs assembled in the presence, and hence have an overabundance, of synthetic peptide. Since these filaments are loosely packed (Fig. 7), one might expect the epitope covered by D442-450 to be more accessible, and whether or not anti-D442-450 would decorate these modified filaments under the same incubation and washing protocol would provide an indication as to how tightly the antibodies bind during immunoelectron microscope studies. Filaments were assembled as described previously, starting with desmin monomers, using a 200-fold molar excess of peptide. They were then placed onto carbon coated grids, successively incubated via flotation over drops of affinity-purified anti-D442-450 and anti-rabbit IgG conjugated to 5 nm colloidal gold and stained with 1% uranyl acetate. Control filaments, i.e., IFs assembled in the absence of peptide, were not labeled by anti-D442-450 (Fig. 9 *A*). In contrast, filaments formed in the presence of the synthetic peptide D442-450 were extensively labeled (Fig. 9 *B*). A further control was carried out to rule out nonspecific adhesion of synthetic peptide to IFs. Pre-assembled filaments were sequentially incubated with peptide, buffer wash, anti-D442-450, and finally gold-labeled secondary antibodies. No labeling was observed (data not shown). As a rule, the filament backbone of labeled IFs was less sharply delineated in the electron microscope than that of unlabeled one due to the accumulation of antibody molecules on the surface. Consequently, no periodicity of labeling was discernable.

Discussion

We have used a synthetic peptide corresponding to residues 442-450 of desmin, and polyclonal antibodies directed against it, to examine the location of the carboxy-terminal tailpiece domain of desmin in the subunit and in the assembled filament. The major findings of this study are (*a*) in the assembled IF, residues 442-450 are inaccessible to our antibody probe, and (*b*) the synthetic peptide D442-450 binds to desmin at a location between lys-436/lys-437 and the carboxy terminus, and (*c*) its presence during filament assembly leads to the formation of filaments with aberrant morphology.

The Disposition of the Desmin Tailpiece Domain in an IF

Our immunolabeling studies suggest that the epitope for anti-D442-450 is accessible to the antibodies when desmin is in the unassembled form but not when desmin is in the filamentous form. This would imply that, upon assembly, the portion of the tailpiece corresponding to residues 442–450 is positioned in such a way that it is only partially or not at all exposed to the cytoplasm. This could be because the tailpiece domain is not entirely peripheral to the IF backbone as previously proposed, or because the interactions that take place between end domains during assembly mask the epitope in question. To the extent that it is a negative result, it is incumbent upon us to rule out other plausible explanations before accepting the implied conclusion of a "hidden" tailpiece.

There are several plausible explanations in addition to the one we favor. First, it is possible that the conformation as-

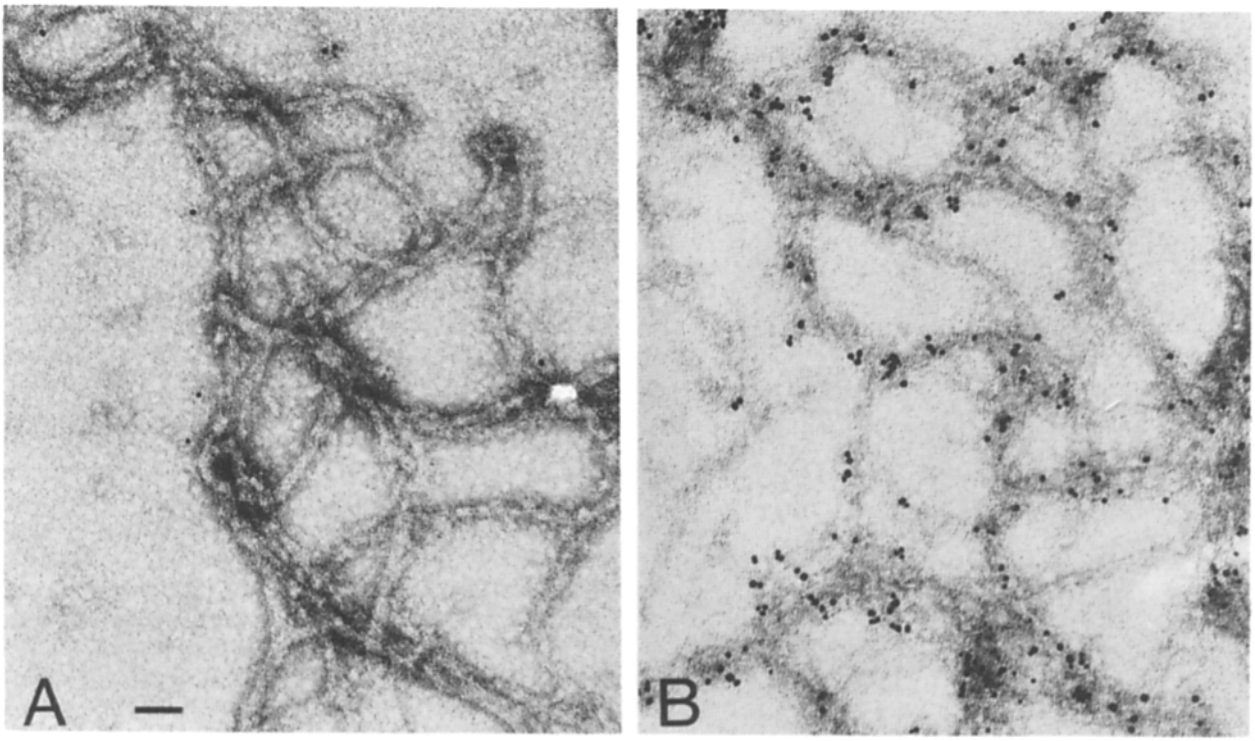

Figure 9. Immunoelectron microscopy of desmin filaments formed in the presence of D442-450. Filaments were assembled as described in Fig. 7 *B* and then labeled with anti-D442-450 followed by 5 nm gold-conjugated goat-anti-rabbit IgGs. (*A*) Control desmin filaments incubated with anti-D442-450. This essentially confirms the image of Fig. 5 *B*. (*B*) filaments formed in the presence of peptide. Note the extensive labeling as a result of incorporated peptide. Only filaments assembled in the presence of peptide are labeled; adding peptide to preassembled desmin IFs does not result in labeling (data not shown). Bar, 50 nm.

sumed by the synthetic peptide in solution differs significantly from that of residues 442-450 of desmin in an IF, so that the antipeptide antibodies were raised against an immunogen essentially unrelated to desmin. Second, our fixation protocol for immunolabeling might have artifactually masked the epitope. Third, in muscle cells a number of associated proteins (IFAPs) have been reported (reviewed by Steinert and Roop, 1988), and one or more of these might have masked the epitope. Fourth, the affinity of anti-D442-450 might not have been sufficiently high to withstand the vigors of repeated incubations and washings during specimen preparation.

We submit that the first of these possibilities is unlikely because the antibodies do label desmin tetramers, which were reassembled from monomers by removal of urea (Fig. 3; further discussed below). While these subunits are stable only in solutions of low ionic strength and high pH (typically 10 mM Tris, pH 8.5), biophysical studies (Geisler et al., 1982; Quinlan et al., 1986) have shown that they possess considerable secondary structure and are, at least to that extent, in a folded conformation. The conditions under which the immunoelectron microscopy was performed also render the second and third explanations unlikely. In this experiment, the labeling was carried out using unfixed, reconstituted IFs deposited on an electron microscope grid. This eliminates the possibility that the fixation protocol for immunofluorescence somehow rendered the epitope of anti-D442-450 inaccessible. Furthermore, since purified desmin contained negligible amounts, if any, of IFAPs, the masking of the epitope by their presence is also unlikely. Finally, anti-D442-450 did decorate filaments which are formed in the presence of excess synthetic peptide (Fig. 9). Formally, we have no direct proof that the synthetic peptide or residues 442-450 of the protein is exposed on the surface of these morphologically modified filaments. However, we do know that the synthetic peptide bound to the tailpiece between residues 436 and 463, and our electron microscope images show that the filaments are extensively unravelled as a result of peptide binding, thus possibly exposing the epitopes for antibody decoration. The fact that the antibodies did label these filaments after having been taken through the identical incubation and washing protocol used to label unmodified IFs argues against the possibility that the affinity of the antibodies was too low for immunolabeling studies.

How then do our results reconcile with the hypothesis that the end-domains of IF proteins are peripheral to the filament backbone? We have noted several possibilities, which we will discuss below.

In their elegant study Kaufmann et al. (1985) showed that the lysine-specific protease, lys-C, cleaves chicken desmin at lys-436 and lys-437, but found no cleavage at lys-442, the beginning of the nine-residue peptide which is the immunogen of our antibody probe. Since the digestion was performed with desmin filaments, lys-436 and lys-437 must be considerably more accessible to the protease than lys-442 when the protein is incorporated into a polymer. This would be in agreement with our present results and would suggest that the tailpiece of desmin may in fact be partially buried within the IF backbone.

Second, the studies that lend direct support to the hypothe-

sis of exposed end domains, i.e., those that show that end domains can be excised from IFs without affecting their apparent morphologic integrity, were done with keratin IF (Steinert et al., 1983, 1985*b*) and neurofilaments (Chin and Eagles, 1983; Eagles and Maggs, 1985). Similar studies with type III IF have not been reported. On the other hand, considerable differences exist among the carboxy-terminal sequences of the different types of IF proteins. Those of the keratins range from unusually glycine-rich to nonexistent, those of the neurofilament triplet vary greatly in size, are extremely lysine- and glutamic acid-rich, contain all of their phosphorylation sites and are thus highly charged, whereas those of the type III IF proteins do not possess any of these characteristics but instead share a homologous region among themselves. It is therefore possible that the tailpieces of the type III IF proteins are located differently within the IF than those of their more distant relatives in the IF protein family. Future studies using genetically engineered IF proteins should shed light on this issue.

Third, whereas domain deletion experiments have shown that the tailpiece domain is not essential for the assembly of IFs, it is not clear that filaments assembled from tailpiece-truncated protein species are identical in every respect to those assembled from intact proteins. Specifically, the stability and other physical properties of the modified filaments have not been studied. Therefore, the possibility cannot be ruled out that the tailpiece, though not directly involved in assembly, may play an as yet unidentified role in IF dynamics. Indeed, our studies using the synthetic peptide D442-450 (discussed below) show that it may destabilize IFs when present during assembly of IFs from intact desmin.

Fourth, we note that there is emerging evidence for a similar masking of small regions of the headpiece of a type III IF protein as a result of filament assembly. Recently, Geisler et al. (1989) mapped the cAMP-dependent (kinase A) phosphorylation sites of vimentin, and reported that certain serine residues in the headpiece were much more highly phosphorylated in the subunit form than in the filamentous form. In fact, some serine residues were not phosphorylated at all in the filament form, supporting the notion that a significant structural change must take place during assembly which renders those serine residues inaccessible to kinase A.

Assembly of IFs May Be Mediated by a "Registration Peptide"

Our binding studies with the synthetic peptide had two purposes. We wished to determine if the synthetic peptide could bind to desmin in a specific manner, and if so, could it be used as a modulator of desmin assembly. Synthetic peptides have been successfully used by other investigators to modify interaction between selected sites on proteins (Pierschbacher and Ruoslahti, 1984; Akiyama and Yamada, 1985; Georgatos et al., 1988; Kelly et al., 1988), to mimic the assembly of transmembrane proteins (Bormann et al., 1989) and to study protein folding (Hall and Frieden, 1989). If successful, it would provide a means for generating IF-specific probes, the lack of which has been a major stumbling block for studying IF structure and function.

Our results demonstrate that the synthetic peptide D442-450 does indeed bind desmin at a site between lys-436 and leu-463, the carboxy terminus. This is consistent with previous work showing that the rod domains within an IF protein dimer are parallel and in-register (Crewther et al., 1983; Steinert et al., 1984; Parry et al., 1985), and together with it, suggests that the peptide binds to residues 442–450 of desmin in a homophilic fashion. Interestingly, we have also observed recently that D88, a cyanogen bromide–derived fragment of desmin that corresponds to the first 88 amino acids of the headpiece domain, self-associates predominantly into dimers under IF assembly conditions (Saeed and Ip, 1989). Collectively, these observations support the view that the mutual recognition of like IF polypeptides, of necessity one of the earliest events of filament formation, may at least in part be mediated by homophilic binding of like domains of two molecules. The concept of recognition peptides is of course not new; however, in this case like-domain recognition may take on additional significance because it may also serve as a mechanism of molecular registration. It is conceivable that the binding of homologous regions facilitates coiled-coil interaction between the α-helical rod domains by bringing them into close proximity. At the same time, the axial registration of the two α-helices is also assured by the homophilic binding, and a zipper-like mechanism may then ensue to complete the coiled-coil dimer.

There are two points concerning this concept that merit discussion. First, a full in-register association of homologous regions of amino acids could expose stretches of like charge to each other, an energetically unfavorable situation. However, this is unlikely because, for the hydrophobic seams of two rod domains (the residues at the *a* and *d* positions) to interact to form a dimer, the two α-helices must be rotated 180° from one another so that all the *a* and *d* residues face inwards, i.e., they must have dyad symmetry. In this arrangement, homologous amino acid stretches in the head and tail domains likewise would also be rotated with respect to each other, so that they are not paired up residue for residue. Moreover, the sequence covered by D442-450 is predicted to be α-helical by both the Chou-Fasman and the Garnier algorithms (Birkenberger, L., and W. Ip, unpublished observation), and as such it has the propensity to be stabilized by interaction with another α-helix, including itself. Clearly, more detailed information on the secondary and higher order structure of the end domains is necessary to draw more concrete conclusion concerning this point. A second point is that the keratins form obligate heteropolymers and are therefore never in a situation where registration peptides are paired. In this connection, it is worthwhile considering the speculation that the length polymorphism reported for IF protein tetramers (Geisler et al., 1982; Ip et al., 1985*a*; Quinlan et al., 1986; Potschka, 1986; Aebi et al., 1988) is due to a degree of permissivity in the hydrophobic interaction between rod domains during dimerization, and that the registration peptide is an additional mechanism, found in homopolymeric IFs, to ensure proper lateral alignment of rod domains during assembly in vivo.

Ultrastructurally, the presence of D442-450 during assembly causes the filaments to be much more loosely packed (Fig. 7), and allows the filaments to be labeled by anti-D442-450 (Fig. 9). We do not know at this time whether the antibodies bind to the "exogenous" synthetic peptide or to the epitope on the desmin tailpiece itself, but that does not affect our conclusions as stated. This effect was observed at as low a molar ratio as 1:1, and hints at the feasibility of using judi-

ciously designed synthetic peptides as site-specific disrupting agents. However, for this approach to be informative it is important that the peptides be fully characterized as to their binding specificity.

Issues Relating to IF Polarity

As the hierarchy of filament assembly progresses, less is known about the actual arrangement of the subunits within a filament. The pair of polypeptides within a dimeric coiled-coil are most probably parallel and in-register (Parry et al., 1985), but the orientation of the dimers within a tetramer is less clear. The more widely accepted view is an antiparallel arrangement, based largely on the report by Geisler et al. (1985) that an mAb directed against the carboxy terminus of the desmin rod domain bound to both ends of the rod, and on the work of Stewart et al. (1989) in which antiparallelism was inferred from analyses of the banding patterns of genetically engineered GFAP paracrystals. In our hands, labeling of tetramers with both amino terminus (Ip, 1988) and carboxy terminus specific (Fig. 3) antibodies have resulted in binding at only one end. These observations seem to favor a parallel arrangement of the dimers and at present we can only explain this discrepancy in a speculative way.

In their 1985 study, Geisler et al. labeled desmin rods with an mAb directed against the carboxy end of the rod domain. In our earlier study (Ip, 1988) we labeled intact tetramers with a monoclonal antibody directed against the amino end of Helix 1 (D76, originally developed by Danto and Fischman [1984]), and in the present study we labeled T-desmin (headpiece-truncated but otherwise intact) with tailpiece-specific antipeptide antibodies. Bearing in mind that like-domains may self-associate, it is conceivable that the binding of our antibodies may have interfered with that association and caused the tetramers to split into dimers. This would have resulted in the one-headed labeling images that we have seen, as one reviewer of an earlier version of this paper pointed out. This would not have happened in the study of Geisler et al. since there were no end domains in their preparation.

Another equally tenable possibility is that there may be polymorphism in the assembly of IF polypeptides (Potschka, 1986; Quinlan et al., 1989), such that both parallel and antiparallel arrangement of the coiled-coils are possible. Indeed, Aebi and co-workers have long suggested that polymorphic forms of IF and their assembly intermediates may exist, particularly in in vitro assembly studies wherein cytoplasmic constraints do not present themselves (Aebi et al., 1983; Aebi, 1986). In this context, it may be pertinent to point out that both polarity arrangements have advantages when considering higher level IF organization. The parallel, polar tetramer is easier to reconcile with the vectorial mechanism of assembly whereby filaments assemble at nucleation sites at the nuclear envelope and elongate toward the plasma membrane (Georgatos and Blobel, 1987; Albers and Fuchs, 1989; Vikstrom et al., 1989). On the other hand, the antiparallel, apolar tetramer is more compatible with a nonvectorial mechanism of assembly whereby subunits insert into IFs along their length (Angelides et al., 1989; Ngai et al., 1990).

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