

# Modulation of Desmin Intermediate Filament Assembly by a Monoclonal Antibody

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**Abstract.** We have used a monoclonal antibody against desmin to examine the assembly of intermediate filaments (IF) from their building blocks, the tetrameric protofilaments. The antibody, designated D76, does not cross react with any other IF proteins (Danto, S. I., and D. A. Fischman. 1984. *J. Cell Biol.* 98:2179-2191). It binds to a region amino-terminal to cys-324 of avian desmin that is resistant to chymotrypsin and trypsin digestion, and in the electron microscope appears to bind to the ends of tetrameric protofilaments. In combination, these findings suggest that the epitope of the antibody resides at the amino-terminal end of the  $\alpha$ -helical rod domain. Preincubation of desmin protofilaments with an excess of D76

antibodies blocks their subsequent assembly into IF. In the presence of sub-stoichiometric amounts of antibodies, IF are assembled from protofilaments but they are morphologically aberrant in that (a) they are capped by IgG molecules at one or both ends; (b) they are unraveled to varying degree, revealing a characteristic right-handed helical arrangement of sub-filamentous strands of different diameters. The antibody binds only to the ends but not along the length of desmin IF. The most straightforward explanation for this is that the epitope resides in a part of the desmin molecule that becomes buried within the core of the filament upon polymerization and is therefore inaccessible to the antibody.

INTERMEDIATE filaments (IF)<sup>1</sup> have long been thought to be stable and static components of the cytoskeleton, in part because of their well-recognized insolubility in physiological solvents. The functions attributed to IF likewise have been ones that are passive and structural, such as keeping other subcellular organelles in their proper places and maintaining cell form. Thus, IF are thought to be at least in part responsible for the centering of the nucleus in some cell types (Woodcock, 1980; Granger and Lazarides, 1982; Goldman et al., 1985; Wang, 1985), and for the positioning of desmosomal components at the plasma membrane during the morphogenesis of desmosomes in some epithelial cell lines (Jones and Goldman, 1985). Neurofilaments, the IF of neurons, are similarly thought to provide a skeletal framework through which components of axoplasmic transport traverse (Lasek et al., 1985). In the earlier literature it was considered likely that, in striated muscle, IF coordinated the contractile activities of myofibrils by keeping their Z lines in lateral register (Lazarides, 1980, 1982). More recent immunolocalization studies at the electron microscope level (Tokuyasu et al., 1985) do not support this notion, but point to the alternative that the IF cytoskeleton as a unit provides a scaffold upon which myofibrils are assembled.

Their apparent static nature notwithstanding, the assembly and disassembly characteristics of IF are of importance because they may reveal information relating to the subunit

composition of the filament and how the subunits are fitted together. Furthermore, in skeletal and cardiac muscle, the IF, which are made of desmin, undergo extensive organizational rearrangement during development. In cultured embryonic muscle cells, before the appearance of well-defined striated myofibrils, desmin is found by immunofluorescence to be localized as a filamentous network similar to those seen in other cell types, but upon myofibril assembly the desmin becomes condensed at the Z disk and in the interfibrillar space (Bennett et al., 1979; Gard and Lazarides, 1980; Tokuyasu et al., 1984). Although there is evidence that the desmin is in the filamentous form both before and after the reorganization (Tokuyasu et al., 1985), how the rearrangement takes place is unknown. On one hand, it may involve filament disassembly, relocation of subunits and reassembly of IF at a new location. On the other hand, whole filaments may be translocated as such, although other examples of this type of translocation are few. More recently, studies on the proteins of the nuclear lamina, lamins A and C, have revealed that they are highly homologous to IF polypeptides (McKeon et al., 1986) and that the nuclear lamina is in fact populated with filaments morphologically similar to cytoplasmic IF (Aebi et al., 1986). Since the nuclear envelope assembles and disassembles cyclically during each cell cycle, one is led to wonder if IF are indeed as static as they are commonly thought to be.

Recently, Danto and Fischman (1984) reported the generation of a monoclonal antibody, designated D76, which binds

1. *Abbreviations used in this paper:* IF, intermediate filament(s); NTCB, 2-nitro-5-thiocyanobenzoic acid.

to desmin in mature muscle cells but not in embryonic heart, cultured cardiac myocytes, or early myotubes. This study suggests that the immunoreactivity of desmin may change during development such that the epitope of D76, which is not available for antibody binding during embryonic stages, becomes exposed upon maturation. Such a change could occur through a rearrangement of the subunits of the IF as discussed above, or via modification of the desmin polypeptide during development. In this paper, we explore these possibilities by further defining the epitope of this monoclonal antibody and by examining its ability to modulate the assembly of desmin into IF *in vitro*. We demonstrate that the D76 epitope most probably resides at the amino-terminal end of the  $\alpha$ -helical rod domain. Filaments assembled in the presence of nonsaturating amounts of this antibody often have aberrant morphology, being unraveled along their length and splayed at their ends as a result of binding of the antibody molecules. Under saturating conditions this antibody blocks the assembly of desmin into IF, possibly by physically preventing aggregation of protofilaments, the functional building blocks of IF.

## Materials and Methods

### Purification of Desmin

Desmin was purified from fresh chicken gizzard according to the method of Huiatt et al. (1980). In more recent experiments the final ion exchange chromatography was performed using a Mono Q HR 5/5 column (Pharmacia Inc., Piscataway, NJ). Fractions containing crude desmin eluted from a hydroxyapatite column were loaded onto the Mono Q column in 6 M urea, 14.3 mM  $\beta$ -mercaptoethanol, 10 mM Tris-HCl, pH 7.5, and eluted with a linear 0–250 mM NaCl gradient in 20 ml of the same buffer at a flow rate of 1 ml/min. At the end of the gradient the salt concentration was held at 250 mM for an additional 5 ml. Under these conditions the desmin eluted at  $\sim$ 200 mM salt.

### Chemical and Proteolytic Modification of Desmin

Desmin was cleaved at its single cysteine (residue 324) by using 2-nitro-5-thiocyanobenzoic acid (NTCB; Sigma Chemical Co., St. Louis, MO) (Jacobsen et al., 1973). A 38  $\mu$ M solution of desmin, in 0.1% SDS or 6 M urea, 50 mM sodium borate, pH 9.0, was mixed with a 10-fold molar excess of NTCB over total sulfhydryl and, after the pH was adjusted to 9.5 by the addition of NaOH, incubated for 2–24 h at 37°C. The reaction was terminated by the addition of an equal volume of 2 $\times$  SDS-PAGE sample buffer (Laemmli, 1970) and heating at 95°C for 3 min.

Chymotryptic cleavage of desmin was carried out according to the method of Geisler et al. (1982). Desmin was used in the form of protofilaments, in 10 mM Tris-HCl, pH 8.5. The protease ( $\alpha$ -chymotrypsin; Sigma Chemical Co.) was added at a concentration equal to 1:400 of that of desmin and the digestion was allowed to proceed for 5–60 min at 23°C. It was then terminated by the addition of phenyl methyl sulfonyl fluoride (PMSF) to 1 mM and an equal volume of 2 $\times$  SDS-PAGE sample buffer, followed by heating at 95°C for 3 min.

CT-desmin, a derivative consisting of residues 97–415, was generated by sequential treatment with chymotrypsin and trypsin as described by Kaufmann et al. (1985). Protofilaments were first digested with chymotrypsin for 5–20 min as described above. TPCK (*L*-1-tosylamide-2-phenylethyl-chloromethyl ketone; Sigma Chemical Co.) was then added to 1 mM to inactivate the chymotrypsin, followed by TPCK-treated trypsin (Sigma Chemical Co.) at a concentration equal to 1:200 of that of desmin. The digestion was stopped by the addition of an equal volume of 2 $\times$  SDS-PAGE sample buffer and heating at 95°C for 3 min.

The removal of residues 1–67 of desmin using thrombin (Sigma Chemical Co.) was performed as described by Lu and Johnson (1983) and the reaction was terminated by the addition of PMSF to 0.2 mM.

### Antibody Preparation

The preparation of the monoclonal antibody, D76, has been reported (Danto and Fischman, 1984). Hybridoma cultures were reestablished from previously frozen stock. Ascites tumors were induced in Pristane-primed nude mice by intraperitoneal injection of  $10^6$ – $10^7$  hybridoma cells. Either the hybridoma culture supernatant or the ascites fluid was used as a source of antibodies. For Western blot analyses the ascites fluid was diluted in Tris-buffered saline containing 1% bovine serum albumin or 3% Carnation brand powdered milk. Purified IgG preparations were used for electron microscopy. IgG was purified from the ascites fluid either by chromatography on a CNBr-Sepharose goat anti-mouse IgG column or by anion exchange chromatography on a Mono Q column after ammonium sulfate fractionation at 50% saturation. In the latter case the crude preparation was loaded onto the Mono Q column in 20 mM Tris-HCl, pH 7.5, and eluted with a linear 0–225 mM NaCl gradient in 35 ml. The IgG eluted at 120 mM NaCl under these conditions.

Anti-IFA, a monoclonal antibody that reacts with all IF proteins (Pruss et al., 1981), was a generous gift of Dr. Rebecca Pruss, Merrill Dow Research Institute, Cincinnati, OH.

### Electrophoresis and Immunoblotting

Gel electrophoresis was performed using 15% separating gels in miniature apparatuses (Hofer Scientific Instruments, Inc., San Francisco, CA) according to the method of Laemmli (1970). Western blot analyses were carried out as described by Towbin et al. (1979). The secondary antibody used was affinity-purified goat anti-mouse IgG coupled to horseradish peroxidase (Boehringer-Mannheim Diagnostics, Indianapolis, IN), and color development was done using diaminobenzidine and H<sub>2</sub>O<sub>2</sub>. Alternatively, goat anti-mouse IgG coupled to alkaline phosphatase (Promega Biotech, Madison, WI) was used, and the color development was carried out using the substrates supplied by the manufacturer.

### Electron Microscopy

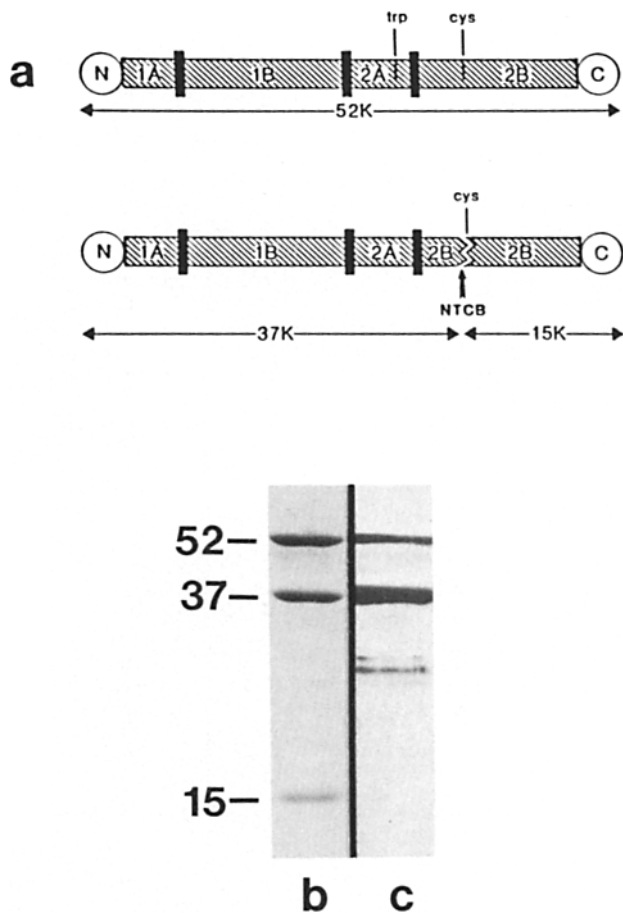
The quick-freeze, deep-etch technique, as described by Heuser (1983), and the glycerol-spray technique of Tyler and Branton (1980), were used to visualize the binding of D76 IgG to desmin IF and subunits. In the former, antibody-treated protofilaments or assembly mixtures were allowed to adsorb to freshly prepared microscopic mica flakes for 10–15 s and then immediately quick frozen by using a liquid helium-cooled Cryopress (Med-Vac, Inc., St. Louis, MO). The frozen samples were freeze fractured, deep etched, and replicated exactly as described by Heuser (1983). In the latter approach, antibody-desmin mixtures were thoroughly mixed with an equal volume of glycerol and immediately sprayed onto freshly cleaved mica sheets by using a glass nebulizer. The samples were then dried *in vacuo* in a freeze-etch machine (model 301; Balzers, Hudson, NH) and rotary replicated with platinum-carbon at 5°C. The extent of IF formation in the presence of antibodies was monitored by negative staining using 1% aqueous uranyl acetate.

## Results

### Localizing the D76 Epitope

We first sought to determine the location of the D76 epitope along the desmin molecule by protein chemical experiments. The molecule contains a single cysteinyl residue at position 324, which is amenable to specific cleavage by the reagent NTCB. The sizes and locations of the cleavage fragments are illustrated schematically in Fig. 1 *a*. The 52,000-*M<sub>r</sub>* molecule is cleaved into an amino-terminal piece that has a mass of  $\sim$ 37,000 daltons and a carboxy-terminal piece of mass  $\sim$ 15,000 daltons. The identity of these fragments has been established previously by amino acid sequencing (Geisler and Weber, 1981).

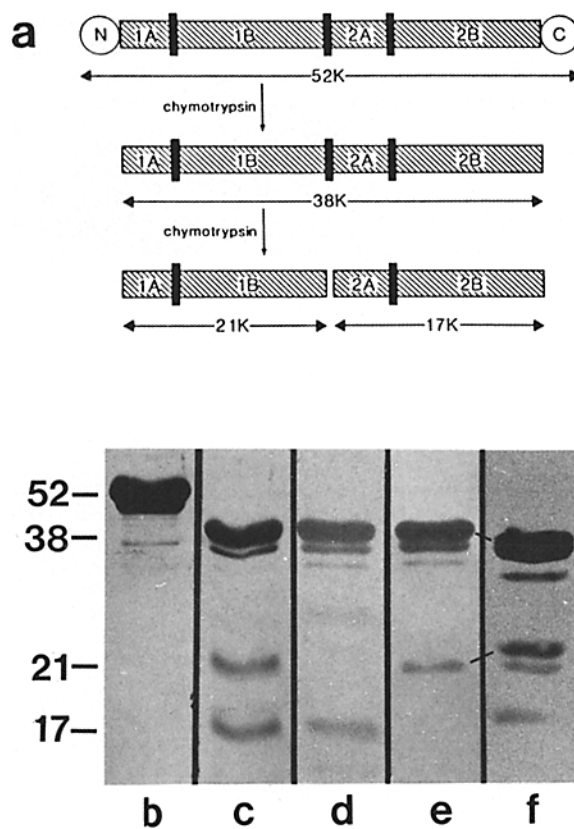
Immunoblotting experiments using these fragments produced results shown in Fig. 1 *b* and *c*. The D76 antibody showed reactivity toward the 52,000-*M<sub>r</sub>* intact desmin molecule and the 37,000-*M<sub>r</sub>* fragment, indicating that its epitope resides within a region amino-terminal to cysteine 324. The



**Figure 1.** (a) Diagrammatic illustration of the cleavage of desmin at cys-324 by NTCB. The shaded region denotes the  $\alpha$ -helical rod domain of the molecule while the circles at both ends represent the headpiece and tailpiece domains. The black vertical bars within the rod domain denote the non- $\alpha$ -helical spacers which subdivide helices 1 and 2 (after Steinert et al., 1985b). The parent desmin molecule, with a relative molecular mass of 52,000, is cleaved into an amino-terminal fragment of 37,000 and a carboxy-terminal fragment of 15,000  $M_r$ . (b) Coomassie Blue-stained 15% SDS-polyacrylamide gel of desmin and its NTCB-cleavage products. (c) Immunoblot of the gel shown in (b) reacted with monoclonal antibody D76. Only the amino-terminal fragment is reactive. We do not know the identity of the minor reactive band at  $\sim 30,000 M_r$ , but think that it is an amino terminal NTCB-fragment of the desmin rod domain (see Results).

minor reactive band below the amino-terminal fragment in Fig. 1 c is probably an amino-terminal NTCB fragment of the desmin rod domain: we noted that in overloaded SDS gels of our desmin preparation (e.g., Fig. 2 b) we could often detect a small contamination of the 38,000- $M_r$  rod domain, which probably arose due to endogenous proteolysis during the early stages of purification.

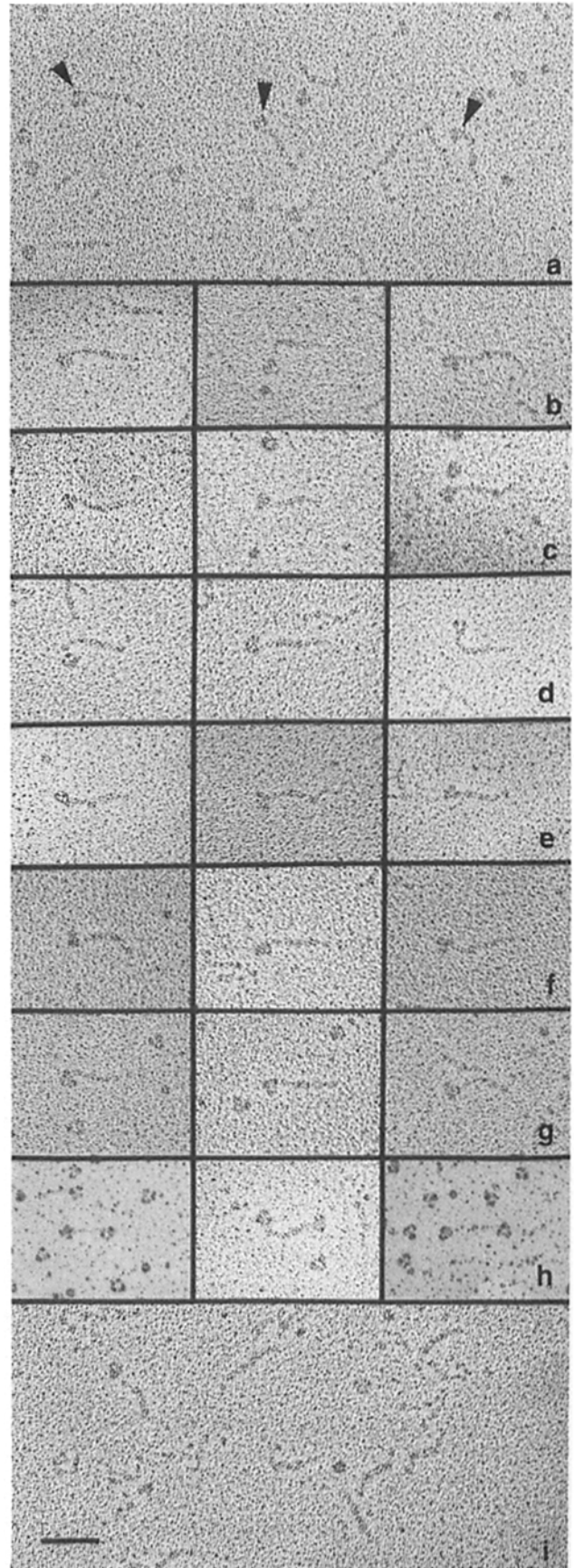
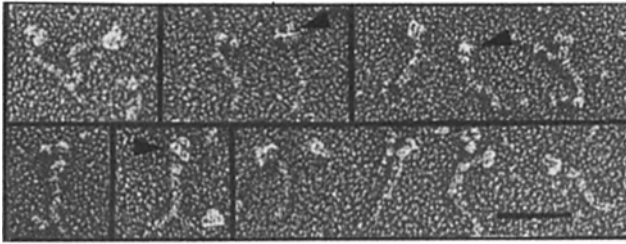
To further define the D76 epitope we carried out limited digestion of desmin using  $\alpha$ -chymotrypsin and trypsin. As noted by others (Traub and Nelson, 1981; Geisler et al., 1982), desmin is extremely sensitive to proteolytic attack, particularly at the non- $\alpha$ -helical regions of the molecule. In the presence of chymotrypsin, the desmin molecule first loses its non- $\alpha$ -helical head and tail domains, resulting in a



**Figure 2.** (a) Diagrammatic illustration of the limited proteolysis of desmin by  $\alpha$ -chymotrypsin. First to be removed from the 52,000- $M_r$  parent molecule are the non- $\alpha$ -helical headpiece and tailpiece, leaving a 38,000- $M_r$  rod domain. The latter subsequently gives rise to a 21,000- $M_r$  amino-terminal fragment and a 17,000- $M_r$  carboxy-terminal fragment. (b) SDS gel of intact desmin. This overloaded gel shows a minor contamination of the 38,000- $M_r$  rod domain. (c) SDS gel of desmin after 15 min of chymotryptic digestion. (d) Immunoblot of a 15-min chymotryptic digest probed with anti-IFA, a monoclonal antibody whose epitope has been mapped to within the last 20 amino acids of the rod domain of all IF proteins (Geisler et al., 1983). The antibody labels the 38,000- $M_r$  rod domain and the 17,000- $M_r$  carboxy-terminal fragment. (e) Immunoblot of a 15-min chymotryptic digest of desmin probed with monoclonal antibody D76. The rod domain and the 21,000- $M_r$  amino-terminal fragment are labeled. (f) Immunoblot of CT-desmin probed with D76. Desmin protofilaments were sequentially digested with chymotrypsin and trypsin before being transferred to nitrocellulose. The upper band indicated by a solid triangle is CT-desmin, the lower one is the CT-derivative of helix 1. Both are recognized by D76.

38,000- $M_r$   $\alpha$ -helical rod (Fig. 2 a). Further chymotryptic digestion cleaves the rod domain at its middle non- $\alpha$ -helical spacer, generating an amino-terminal half (helix 1,  $M_r = 21,000$ ) and a carboxy-terminal half (helix 2,  $M_r = 17,000$ ) (Geisler et al., 1982; Kaufmann et al., 1985). The addition of trypsin to the  $\alpha$ -helical rod removes 23 amino acid residues from its amino-terminal end (Kaufmann et al., 1985), resulting in a species known as CT-desmin (Fig. 2 f).

Under the conditions used in our experiments (chymotrypsin/desmin molar ratio = 1:400, 23°C), the intact desmin molecule was no longer detectable in Coomassie Blue-stained



**Figure 3.** (Left) Quick-freeze, deep-etch images of desmin protofilaments incubated with D76 IgG at a ratio of one IgG per protofilament. Each protofilament, a rod-shaped structure 5 nm wide and 48 nm long, appears to have a single IgG molecule attached to it. Some IgG molecules are indicated by arrowheads. (Right) Desmin tetramer-D76 IgG complexes visualized by glycerol-spraying followed by rotary replication. (a) A representative field of such complexes made from a 1:2 mixture of thrombin-treated protofilaments and D76 IgG. Arrowheads indicate complexes. Rows b and c are one-end-labeled immune complexes. These complexes were, of necessity, sprayed onto mica under low ionic strength, high pH conditions; few adhered to the mica. Rows d-g are complexes of thrombin-treated protofilaments and D76 which were sprayed at 50% glycerol, 50 mM NaCl, 10 mM Tris-HCl, pH 7.5. Row h shows three thrombin-treated protofilaments that appear to be labeled at both ends by antibodies. These were made from a mixture containing 2.5 IgG molecules per protofilament; hence the relatively high background of IgG. (i) A control preparation of protofilaments incubated with an unrelated mouse IgG. Bars, 50 nm.

gels after 5 min. Within a short time thereafter, the 38,000-*M<sub>r</sub>* rod domain gave rise to helices 1 and 2 (Fig. 2 *c*). When the two fragments were blotted and incubated with anti-IFA, a monoclonal antibody whose epitope had been mapped to within the last 20 amino acid residues of the rod domain of all IF proteins (Pruss et al., 1981; Geisler et al., 1983), only helix 2 showed reactivity (Fig. 2 *d*), further ascertaining the identity of the two fragments. In contrast, the monoclonal antibody D76 recognized helix 1 specifically (Fig. 2 *e*). In addition, D76 recognized CT-desmin (residues 97–415) and its smaller chymotryptic Helix 1 derivative (Fig. 2 *f*). Thus, in corroboration with the results obtained from cysteinyl cleavage, these results place D76 epitope conclusively within the amino-terminal half of the  $\alpha$ -helical rod.

The functional building blocks of IF are tetrameric structures referred to as protofilaments (Geisler and Weber, 1982; Pang et al., 1983; Ip et al., 1985*b*). During IF assembly in vitro monomeric polypeptides initially associate into the tetrameric species, which in turn assemble in a stereo-specific fashion, first to attain the 10-nm diam characteristic of all IF, then to elongate into filaments micrometers long (Crewther et al., 1983; Geisler et al., 1985; Ip et al., 1985*a*; Quinlan et al., 1986). It was therefore important to determine the position of the D76 epitope along the tetrameric desmin protofilament.

Affinity-purified D76 IgG was incubated with desmin protofilaments in ratios ranging from 0.25 to 2.5 IgG molecules per protofilament in 10 mM Tris-HCl, pH 8.5, for 1–3 h at 4°C. (A particle mass of  $4 \times 53,000 = 212,000$  daltons was assumed for the protofilament). The mixtures were adsorbed onto mica flakes, quick frozen, and freeze etched for electron microscopy (Fig. 3 *left*). In these preparations, the vast majority of the protofilaments appeared to have an IgG molecule attached to one end.

Under low ionic strength, high pH conditions, protein molecules adsorb relatively poorly to mica (Heuser et al., 1987). To ensure that poor adsorption did not lead to nonrandom sampling of immune complexes, the same experiment was also performed in two alternative ways as follows: (*a*) the glycerol spray method of Tyler and Branton (1980) was used in place of the quick-freeze, deep-etch technique, and (*b*) the thrombin derivative of desmin (Lu and Johnson, 1983) was substituted for intact desmin. This derivative of desmin, which lacks residues 1–67, forms tetramers that do not assemble into IF under physiological conditions (Kaufmann et al., 1985), thus permitting the experiment to be done in neutral, salt-containing solutions. In some experiments, the antibody–protofilament complexes were chromatographed on a Sephacryl S-300 column before processing for electron microscopy. Results from these variations did not differ significantly from those obtained by freeze etching on mica, and are illustrated in Fig. 3 *right*. When the antibody/protofila-

ment ratio was increased to 2.5, and the total protein concentration adjusted to give a reasonable distribution of particles on the mica surface, the number of free IgG in the background rose markedly and immune complexes were more difficult to find. About 10% of the proto-filaments appeared to have been labeled at both ends (Fig. 3 *right, h*, and Table I), in contrast to the study of Geisler et al. (1985) in which two-ended labeling of protofilaments was predominant, if not exclusive, under similar conditions of antibody excess. The binding of antibody molecules elsewhere along the protofilament was not observed.

### Effects of D76 on Desmin Assembly

Several lines of evidence have pointed to the assembly of IF as being a multistep event involving, at a minimum, the side-to-side and end-to-end accretion of subunits (Geisler et al., 1982; Aebi et al., 1983; Sauk et al., 1984; Ip et al., 1985*a*). Thus, the location of the epitope of D76 at the end of the protofilament strongly suggested that the presence of the antibody might interfere with the association of subunits and therefore the assembly of desmin IF from its building blocks. This possibility was also explored using an ultrastructural approach.

A solution of desmin protofilaments was cleared of aggregates by a 20-min centrifugation at 143,000 *g* in an Airfuge and diluted to 10–20  $\mu$ g/ml in 170 mM NaCl, 1 mM MgCl<sub>2</sub>, 10 mM imidazole, pH 7.0 at 0°C. Under these conditions desmin does not polymerize into IF because its concentration is subcritical for polymerization (unpublished observation). D76 IgG in the same buffer was then added to the protofilaments at ratios of 1–2.5 IgG molecules per protofilament and the mixtures incubated at 0°C for 1 h. Polymerization was then induced by transferring the desmin–antibody mixtures to 37°C for 2 h, at the end of which samples were withdrawn for electron microscopy.

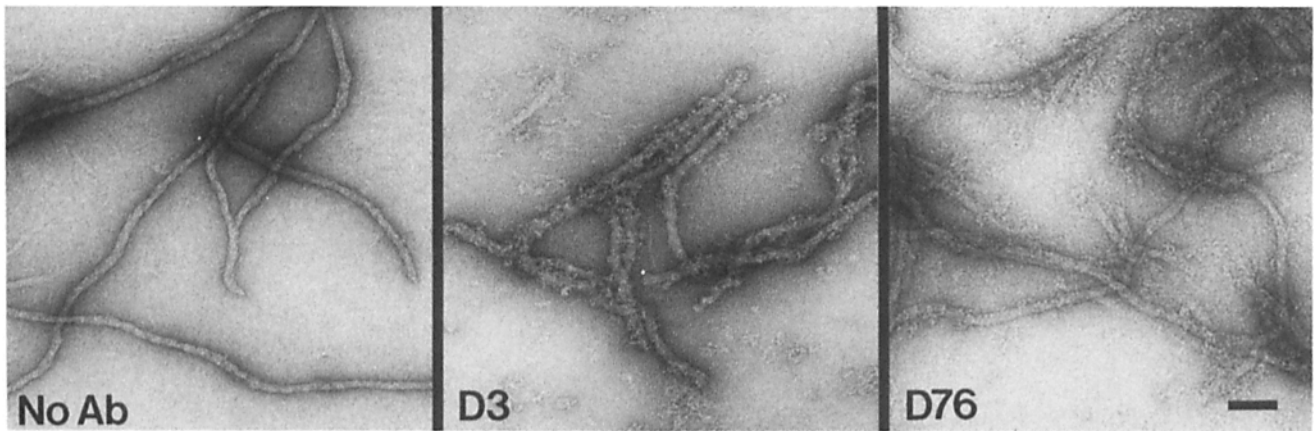
In samples taken from a reaction mixture that contained 2.5 antibody molecules per protofilament, we were unable to detect by negative stain electron microscopy any IF of normal morphology after 2 or more hours of incubation. In their place were numerous large dark-staining amorphous clumps of aggregated protofilaments and a few extremely short (ca. 100 nm) filaments (not shown). The normal assembly of desmin protofilaments into IF, or at least filament elongation, was apparently inhibited by the presence of D76 antibodies under these conditions.

In reaction mixtures containing <2.5 antibody molecules per protofilament we could not detect, by electron microscope observation, complete inhibition of filament assembly of the kind described above. There were fewer filaments, and they tended to be shorter than those assembled under normal conditions. In negatively stained preparations the assembled filaments did not appear to be labeled with antibodies to an appreciable degree, being normally calibrated for the most part. This point is illustrated in Fig. 4, in which the image of IF assembled in the presence of D76 is contrasted to that of those treated with another monoclonal antibody, D3, which does label the filament along its length. No attempts were made to quantify the extent of assembly inhibition in these experiments because we did not feel that filament length and number under the conditions used were sufficiently meaningful criteria for such purposes.

**Table I. Extent of Labeling of Thrombin-treated Desmin Protofilaments by D76 at 2.5 Times Antibody Excess**

| One end labeled | Both end labeled | Unlabeled* | Number counted |
|-----------------|------------------|------------|----------------|
| %               | %                | %          |                |
| 64.0            | 9.7              | 26.3       | 175            |

\* Includes protofilaments that could not be scored with confidence.



**Figure 4.** Negatively stained images of desmin IF illustrating the lack of labeling along the filament shaft by D76. (*Left*) Untreated desmin IF. (*Center*) Desmin IF treated with monoclonal antibody D3. (*Right*) IF assembled in the presence of a sub-stoichiometric amount of D76. Note the fuzzy appearance and enlarged diameter of the IF in the presence of D3 but not D76. Bar, 50 nm.

To better understand the interaction of the antibodies with the desmin protofilaments from a structural standpoint, we withdrew samples for quick-freeze, deep-etch electron microscopy. We wished to know in particular how the antibodies brought about inhibition of filament assembly, in view of the finding that they did not label fully assembled filaments to an appreciable degree. The images of desmin filaments assembled in the presence of nonsaturating concentrations of D76 IgG (Fig. 5) provide at least a partial answer to this question. Upon examination of deep-etch replicas of these filaments, we observed that most of the short filaments present (presumably equivalent to those seen by negative staining) were labeled by a cluster of IgG molecules at their tips (Fig. 5, *a-f*). This suggests to us that the presence of the antibody molecules at those locations blocked further addition of protofilaments to effect elongation. Unfortunately, it is technically unfeasible to trace a sufficiently large number of filaments from one end to the other to determine whether both ends are always labeled by antibodies. This is because during sample preparation the fracture plane jumps frequently and full-length filaments are rarely contained in any single plane unless they are extremely short, such as those shown in Fig. 5, *e* and *f*.

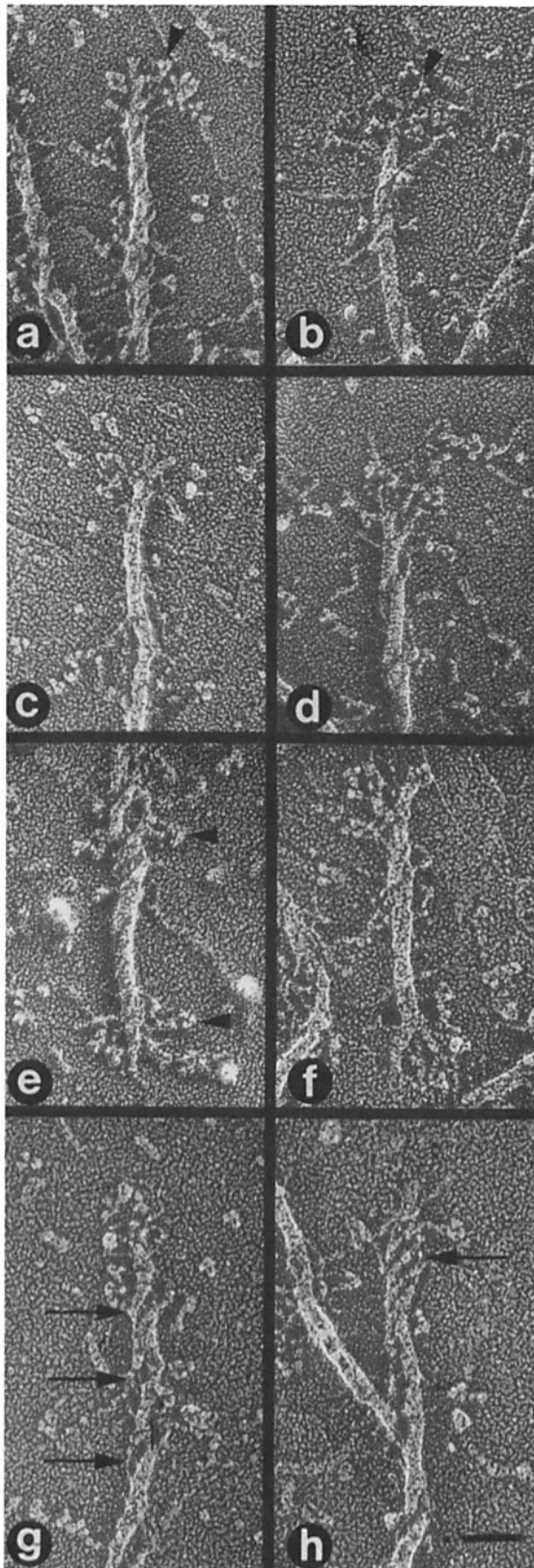
The number of protofilament ends per cross section of IF is generally considered to be eight (Aebi et al., 1983; Steven et al., 1983; Ip et al., 1985*a*), yet we rarely found more than four IgG molecules capping the end of an IF (Fig. 6). Even in cases where a greater number of IgG molecules were seen at the end of a filament (e.g., Fig. 5 *b*), viewing the images in stereo suggested that the more peripherally located ones may be in actuality not bound to a protofilament. Apparently, then, not all the protofilaments at the end of an IF are accessible to antibody molecules in solution. It is also noteworthy that even in these capped filaments, few antibody molecules are seen along their length, in agreement with our observation by negative staining that D76 does not label the shafts of IF assembled in its presence (Fig. 4) or those of preformed IF (data not shown).

Also of considerable interest is that in virtually all of the IF assembled in the presence of antibodies, the filament shaft appears unraveled at multiple points along their length, re-

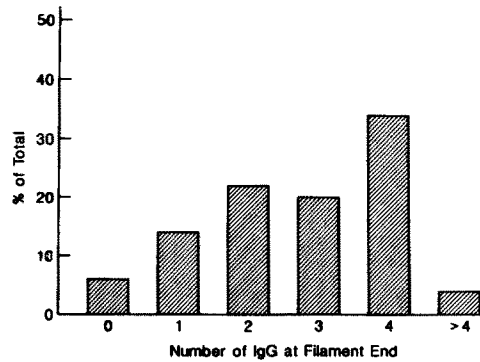
vealing a characteristic right-handed helical substructure (Fig. 5, *d, g*, and *h*). This substructure is similar to that seen by Heuser (1983) and by Aebi et al. (1983) in keratin filaments although the cause of the unraveling is different in all three cases.

Further details of the unraveling brought about by assembling desmin IF in the presence of D76 IgG are illustrated in Fig. 7. Fig. 7 *a* shows a desmin IF which exhibits a variety of structural anomalies that are attributable to the presence of antibodies. Unraveling to varying extent is seen at a number of locations along the filament and, at two such locations, assembly is so extensively disrupted that the filament bifurcates into branches. The diameter of these branches varies considerably, although it is substantially >2–3 nm, the diameter of a single protofilament. In fact, in many instances the branches themselves further unravel into 2–3-nm wide strands. The different levels of antibody-induced unraveling are further illustrated in the stereo micrographs presented in Fig. 7, *b-e*. Fig. 7 *b* depicts a short IF whose middle one-third is unraveled into at least three 6-nm strands distinguishable when viewed in stereo. The possible presence of an IgG molecule below the uppermost strand is also indicated. In contrast, Fig. 7 *c* depicts an IF of similar length but whose integrity is much more disrupted, exhibiting in its middle region a number of strands of the 2–3 nm variety. Again, amidst the unraveled strands are globular entities suggestive of IgG molecules. Further evidence that the disruption is due to the presence of D76 is found in Fig. 7 *d*. A segment of an IF is shown, the middle one-third of which is completely unraveled to 2–3-nm substrands. Two of these substrands are clearly capped by IgG molecules (Fig. 7 *d*, *arrows*), which evidently terminated their elongation prematurely. The remaining substrands that are unaffected by antibodies continue to elongate and emerge as a normal-appearing IF past the unraveled region. Finally, as illustrated in Fig. 7 *e*, the antibody-induced unraveling of IF provides a direct demonstration that these filaments are organized in several levels of structural hierarchy. At least two types of subfilamentous strands are visible in this image; those of the 6 and the 3-nm varieties. Similar images have been presented by Aebi et al. (1983) in previous studies of keratin IF assembly.





**Figure 5.** Monoclonal antibody D76 blocks desmin IF assembly by binding to filament ends. Desmin IF are assembled in the presence of one IgG molecule per protofilament. This concentration of antibody is insufficient to block filament assembly completely. Many of the IF that are formed have either one end (*a-d*) or both ends (*e* and *f*) capped by IgG molecules. Some of the IgG molecules,



**Figure 6.** Histogram illustrating that the number of antibody molecules labeling the end of desmin IF rarely exceeds four. 50 filament ends were scored directly from paired negatives viewed in stereo, enlarged ten times from the electron microscope magnification using a zoom stereo viewer (Bausch and Lomb, Inc., Rochester, NY).

## Discussion

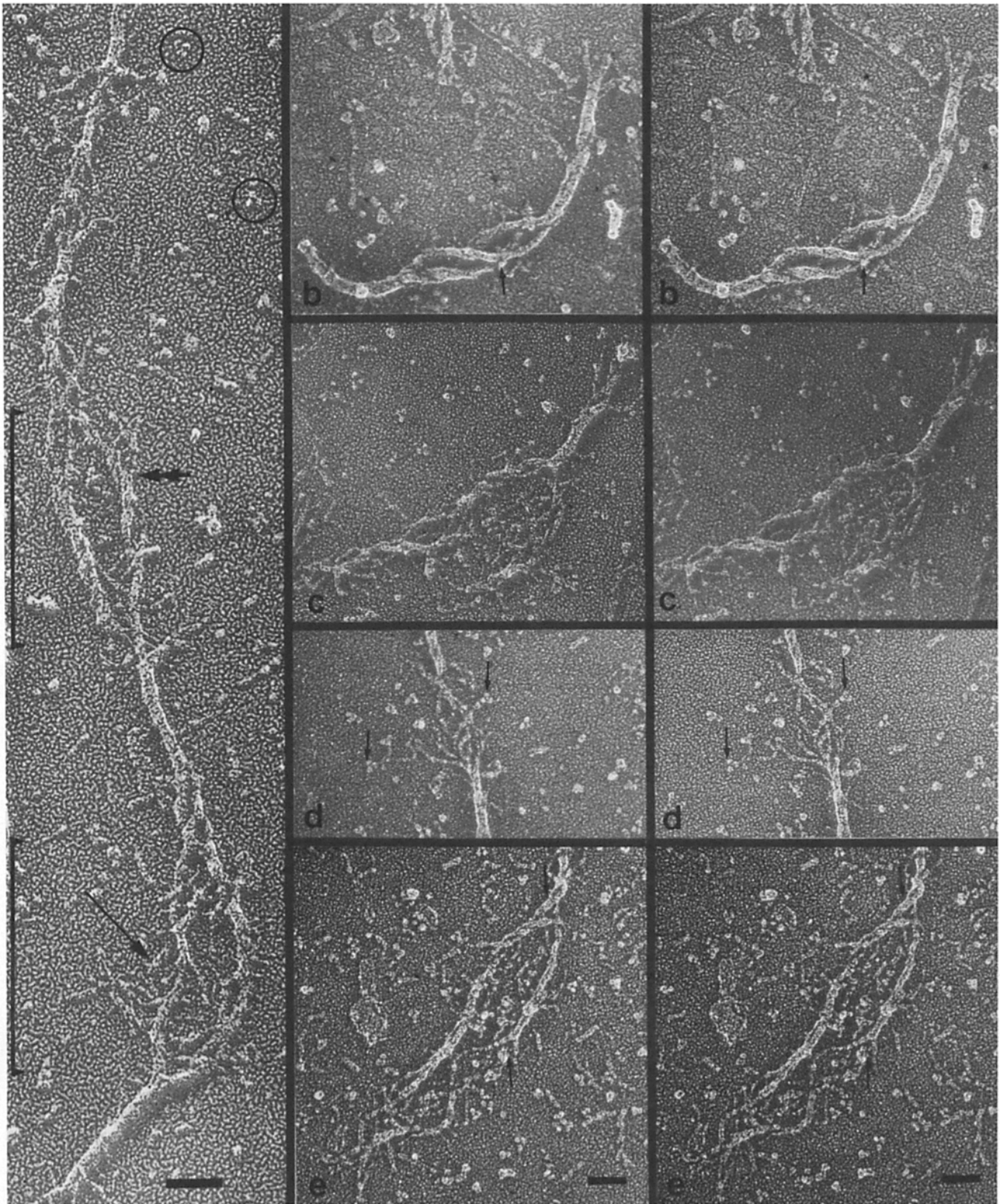
### The Epitope of D76

Despite their considerable size differences, all members within the IF family of proteins share a common structural theme (reviewed by Steinert et al., 1985*b*; Weber and Geisler, 1985, Osborn and Weber, 1986). The typical IF molecule consists of a rod domain composed of amino acid residues capable of forming  $\alpha$ -helical coiled-coils, punctuated by non- $\alpha$ -helical regions at the amino- and carboxy-terminal ends and at least two breaks conservatively located among the IF polypeptides. The break that occurs approximately half way along the rod domain divides it into the so-called helix 1 and helix 2; the one that occurs within helix 1 further divides this portion into helix 1A and helix 1B. A third break occurs within helix 2 of certain IF polypeptides. This general structural principle provides a convenient framework for defining the location of the epitope of monoclonal antibody D76.

Western blot analyses using cysteine cleavage fragments show that D76 recognizes a site that is amino-terminal to cysteine 324. This means that the epitope could be within one of the following four regions: the headpiece (residues 1-73), which consists of  $\beta$ -sheet and several  $\beta$ -turns (Geisler and Weber, 1982); the amino-terminal half of the  $\alpha$ -helical rod domain including helix 1 (residues 74-241); the non- $\alpha$ -helical middle spacer (residues 242-262), or residues 263 to 324 of helix 2. That this site remains with the 21,000-*M<sub>r</sub>* rod subdomain after chymotrypsin digestion puts the epitope between residues 74 and 255 (Kaufmann et al., 1985), and that D76 binds to CT-desmin further eliminates residues 74-96 as candidates for its epitope, leaving residues 97-255.

From the labeling of unpolymerized protofilaments (Fig. 3) and from the capping of the tips of assembled IF (Figs.

which have a typical trilobular appearance, are indicated by arrowheads. (*g* and *h*) Unraveling in the presence of D76 antibodies, showing a characteristic right-handed helical substructure (*arrows*). Note again that antibody labeling is concentrated at the tips of the IF and very few antibody molecules are seen along the shafts of the filaments. Bar, 50 nm.



**Figure 7.** Morphologically aberrant desmin IF assembled in the presence of monoclonal antibody D76. (a) Survey view of IF unraveling. This filament exhibits unraveling at various stages all along its length, but particularly at two regions where it bifurcates into two strands (indicated by *brackets*). At the location marked by the long arrow the unraveling of the filament has resulted in further untwisting into finer substrands with diameter similar to that of protofilaments. Two typically trilobular IgG molecules are circled; similarly profiled structures are seen within the upper unraveled region, between the separated branches, at the level indicated by a horizontal double arrow. (b) Stereo view of a short filament that has unraveled into three 6-nm branches which clearly participate in a right-handed helix. An IgG molecule may be present underneath the uppermost branch (*arrow*). (c) Stereo view of an extensively disrupted filament exhibiting a number of 2–3-nm strands. (d) In this segment of IF the capping of two protofilaments by IgG molecules (*arrows*) has terminated their elongation,



4, 5, and 7), we have concluded that D76 IgG molecules bind to a site very close to the physical end of the protofilament. This would tend to argue that its epitope is very near the amino-terminal end of the rod, possibly in helix 1A. On the other hand, this antibody does not cross react with vimentin (Danto and Fischman, 1984), whose sequence homology with desmin from residue 97 to the end of Helix 1A is 90% (4 of 38 residues differ significantly in charge and hydrophilicity). A more precise localization of this epitope must await higher resolution analyses.

It is by now widely recognized that the functional building block of IF is the tetrameric protofilament. There is strong evidence that this structure consists of two dimeric  $\alpha$ -helical coiled-coils (Geisler and Weber, 1982; Pang et al., 1983; Quinlan et al., 1984) and that the polypeptides within each coiled-coil are aligned in parallel and in register (Parry et al., 1985), but it is less clear how the dimers themselves are arranged within the tetramer. Protein chemical and ultrastructural observation (Geisler et al., 1982; Ip et al., 1985a; Quinlan et al., 1986) suggest that the two coiled-coils cannot be staggered by much, if at all, since the lengths of the dimeric coiled-coil and the tetrameric protofilament are virtually identical at 48–50 nm. However, polymorphism at this level of organization has been reported by Potschka (1986), who has detected tetrameric species of vimentin which are some 64 nm long. Recently, Geisler et al. (1985) reported that a monoclonal antibody to desmin, DE-U-10, whose epitope is located at the carboxy-terminal end of Helix II, bound to both ends of protofilaments, implying an antiparallel arrangement of the dimeric coiled-coils. We have attempted to confirm this finding with our antibody, D76, but despite repeated trials have only been able to achieve two-ended labeling of protofilaments (or their thrombin derivatives) in ~10% of the complexes encountered, even when the mixtures contain a 2.5-fold excess of antibodies over protofilaments. At present we can offer several possible explanations for this discrepancy. (a) There may be subtle structural differences between the two ends of the protofilament, possibly arising from a slight stagger of the coiled-coils, that are recognized by D76 but not by DE-U-10. (b) There may be polymorphism in the assembly of IF polypeptides, similar to those reported by Potschka (1986), 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). This point is further underscored by the recent studies of Georgatos and Blobel (1987) in which two distinct populations of vimentin IF attachment sites were detected in avian erythrocytes, one at the nuclear envelope and the other at the cytoplasmic face of the plasma membrane, suggesting that the two ends of a filament may not be structurally identical. (c) Finally, one reviewer of this manuscript suggested that it is possible that the binding of D76 antibodies to the protofilaments might destabilize their tetrameric organization, resulting in parallel, unstaggered dimers labeled by D76 at one

end. At the moment we do not have sufficient data to discriminate among these possibilities. More definitive experiments to address the question of IF polarity are in progress.

### *D76 Interferes with Desmin Assembly: Implications on IF Structure*

We have presented ultrastructural evidence that desmin protofilaments preincubated with D76 IgG fail to polymerize into IF when induced to do so. Because the size of the IgG molecule is large compared to that of the protofilament, a priori it is possible that D76 blocks either the end-to-end or lateral aspects of protofilament association during filament assembly. Indeed, the images shown in Figs. 5 and 7 suggest that these possibilities may not be mutually exclusive. That most of the IF formed in the presence of substoichiometric amounts of antibodies are short and capped by antibody molecules strongly argues that the antibodies interfere with filament elongation. However, the lateral packing of protofilaments must also have been compromised by the presence of the antibody, as evidenced by the unraveled appearance of many of the formed filaments. It is nevertheless conceivable that unraveling may be a secondary effect; the incorporation of an IgG-labeled protofilament onto the end of an existing IF may preclude further axial addition of protofilaments in a particular substrand, while elongation along other substrands remains unaffected. This may eventually result in an IF containing an unraveled region, much like the ones shown in Fig. 7.

We previously proposed a low resolution model of IF subunit arrangement (Ip et al., 1985a), the main feature of which is that adjacent protofilaments within the IF are staggered by half the length of a protofilament (~22–24 nm). This model was invoked to explain the discovery of an assembly intermediate of vimentin measuring 1.5 times the length and twice the width of a protofilament (Ip et al., 1985a), a species that has subsequently been detected also in assembly mixtures of thrombin-treated desmin (Kaufmann et al., 1985). The present observation that the number of D76 antibody molecules labeling the end of a given IF never reached a full complement of eight (the presumed number of protofilament ends per IF cross section) lends support to the proposed staggered arrangement of protofilaments. Indeed, according to the model four of the eight protofilaments making up the IF would be recessed from the filament end by 22–24 nm, so that one would not expect them to be accessible to antibody molecules.

D76 does not label desmin IF that are already assembled (Figs. 4, 5, and 7), suggesting that the D76 epitope, although accessible in protofilaments before polymerization, is apparently not available for antibody binding after they are incorporated into the IF. One obvious possibility is that the epitope becomes buried within the IF backbone upon polymerization. In this context, in a recent model of IF structure Steinert and co-workers have put forth the view that the rod domains of IF polypeptides associate side-to-side to form the core structure of the filament, while the end domains protrude

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leaving the remaining protofilaments in a loosely wound right-handed helical pattern. (e) Multiple levels of subfilamentous organization are evident in this stereo pair of an unraveled IF. At the top of the field, the 10-nm filament unwinds into two 6-nm branches (*upper arrow*) and the one on the right further branches into smaller caliber strands (*lower arrow*). Bar, 50 nm.

radially away from the filament backbone (Steinert et al., 1985a). Such a model would predict that an epitope located within the end domain of an IF polypeptide would probably remain accessible to antibodies even after the incorporation of the protofilament into an IF, since it would still be on the surface of the filament. However, we do not consider this contradictory to our results because the epitope of D76 may be far enough into the molecule that even if it is on the filament surface it may still be inaccessible to the antibody. The resolution of this issue will have to await further structural studies and the availability of more suitable probes. Parenthetically, it is of interest to note that another monoclonal antibody against desmin, designated D3 (Danto and Fischman, 1984), whose epitope is also within the  $\alpha$ -helical rod domain but is more centrally located, does not interfere with desmin IF assembly but does label previously formed desmin IF (Fig. 4). In this case the epitope is evidently exposed regardless of whether the protofilament is within an IF or not.

Our interest in monoclonal antibody D76 stemmed from its reactivity toward desmin that appears to be related to the developmental program of muscle cells. In embryonic skeletal muscle cell cultures D76 binds to desmin only after it has made the transition from a filamentous network to a Z disk location; by immunofluorescence it does not recognize desmin in presumptive myoblasts and in IF longitudinally disposed within early myotubes, although the presence of desmin can be detected biochemically by immunoblotting with other antibodies of more conventional binding properties (Lazarides et al., 1982; Fischman and Danto, 1985). Similarly the antibody does not recognize desmin in parts of embryonic chick heart or cultured cardiac myocytes derived therefrom, but does bind to desmin in adult cardiac Z disks. Two possible explanations that have been offered are that during development, either the desmin becomes biochemically altered, or a specifically associated protein becomes removed; in either case a new antigenic site would be exposed (Lazarides et al., 1982). Whereas the results of the present study do not directly address the first possibility, they do not support the second either. The experiments were conducted using desmin purified from adult tissue and thus should be devoid of any associated protein present during development. In any case the removal of any putative associated protein during filament assembly would be expected to produce the opposite result, i.e., increased antibody binding to assembled filaments compared with protofilaments. The results from this study would therefore tend to be more compatible with the idea that a subtle structural modification of desmin takes place during its transition from cytoplasmic filaments to the Z disk.

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