

The Roles of the Rod End and the Tail in Vimentin IF Assembly and IF Network Formation

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Abstract. Using mutagenesis, we investigated the importance of two vimentin domains: (a) a highly conserved segment near the carboxy end of the α -helical rod, and (b) the tail, with which the rod end is known to interact. As judged by *in vitro* filament assembly and expression in transiently transfected cells lacking an endogenous vimentin network, the rod-tail interaction is not essential for 10 nm filament structure in

in vitro or for formation of fibrous arrays in culture. However, when mutated, amino acid residues within the rod and the tail segments can cause perturbations in IF assembly and in IF network formation. Finally, our studies show that the vimentin tail seems to play a role both in thermodynamically stabilizing IF structure *in vitro* and in establishing proper IF networks *in vivo*.

INTERMEDIATE filament (IF)¹ proteins can be classified into six types which are expressed in a tissue specific manner (for reviews, see Parry, 1990; Albers and Fuchs, 1992). IF proteins share common structural features, consisting of an α -helical rod domain of conserved secondary structure and size, flanked by nonhelical end domains of variable length, sequence and chemical characteristics (Hanukoglu and Fuchs, 1982; Geisler and Weber, 1982). For a given IF type, sequences within the rod are highly conserved. Throughout the rod are heptad-repeats of hydrophobic residues, indicative of proteins that form coiled-coils (Pauling and Corey, 1953; Crick, 1953; McLachlan and Stewart, 1975). The coiled-coil dimer subunit has been demonstrated for all IF proteins (Woods, 1983; Quinlan et al., 1984; Ip et al., 1985; Aebi et al., 1986). Whereas most IF proteins form homodimers, keratins associate as heterodimers (Coulombe and Fuchs, 1990; Steinert, 1990; Hatzfeld and Weber, 1990). The polypeptide chains of the dimer are parallel and in register (Woods and Gruen, 1981; Quinlan et al., 1984; Woods and Inglis, 1984; Parry et al., 1985). The α -helical rod domain is responsible for the initial association of IF chains into dimers, from which higher oligomers arise.

The building block unit of IFs is a tetramer of two coiled-coil dimers arranged in antiparallel fashion (Ahmadi and Speakman, 1978; Quinlan et al., 1984; Ip et al., 1985; Parry et al., 1985; Geisler et al., 1985; Coulombe and Fuchs, 1990). In most studies, the dimers are partially staggered, with overlapping amino ends and exposed carboxy ends of

the rod domains (Woods and Inglis, 1984; Stewart et al., 1989; Potschka et al., 1990; Geisler et al., 1992).

The nonhelical head (amino) and tail (carboxy) domains of IFs are variable in size and sequence (for review, see Albers and Fuchs, 1992). They are sites of postsynthetic modifications, including phosphorylation (Chou et al., 1990; Ando et al., 1989; Evans, 1989; Geisler and Weber, 1988; Kitamura et al., 1989; Lamb et al., 1989; Peter et al., 1990) and proteolysis (Traub and Vorgias, 1983; Weber et al., 1989). This diversity may render the ends responsible for the variability in IF assembly properties *in vivo* and *in vitro*. Because their roles most likely vary among and even within IF types, elucidating the functional significance of the end domains has been difficult.

Among the more extensively studied IFs are the type III subclass. While the importance of the type III head domain has been underscored by many studies (Traub and Vorgias, 1983; Kaufmann et al., 1985; Georgatos and Blobel, 1987; Herrmann et al., 1992, and references therein), the role of their tails remains obscure. *In vivo* transfection and *in vitro* reconstitution studies have shown that removal of large segments of IF tails often does not prevent filament formation (Kaufmann and Weber, 1985; Shoeman et al., 1990; Raats et al., 1991; Eckelt et al., 1992). However, tailless or partially tailless type III IFs have an increased tendency to aggregate (Kaufmann and Weber, 1985; Perides et al., 1987), and peptides corresponding to a tail segment conserved among type III IFs cause IF aggregation and other perturbations when added to IF assembly reactions (Birkenberger and Ip, 1990; Kouklis et al., 1991). Thus, it seems likely that type III IF tails may play some role in IF network formation. Recently, we discovered that a 15 amino acid residue tag placed onto the carboxy end of vimentin, but not epidermal keratin K14, caused disruption of preexisting IF networks,

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1. *Abbreviations used in this paper:* FPLC, fast performance liquid chromatography; IF, intermediate filament; rt, room temperature.

and blocked de novo IF formation in vivo and in vitro (McCormick et al., 1991). This finding revealed a structural difference between type III and type I tails, and further indicated that while the type III tail domain may not be required for forming a 10-nm filament structure, perturbations in it may nevertheless influence filament network formation and filament assembly. Additional studies suggest that type III IF tails may be involved in higher ordered organization of IF networks via interacting with the nuclear membrane (Georgatos and Blobel, 1987; Djabali et al., 1991).

Precisely how, if at all, the native type III tail is involved in filament assembly and/or filament network formation is presently unknown. However, it is interesting that a subdomain near the carboxy end of the α -helical rod of vimentin has been found to interact directly with a peptide corresponding to the last 30 amino acid residues of a related type III IF protein, peripherin, leading to speculation that formation of an intramolecular loop structure at the carboxy end of vimentin may sterically prevent inappropriate filament-filament aggregation occurring in tailless mixtures (Kouklis et al., 1991). The putative association is intriguing in light of recent chemical crosslinking studies that provide major insights into the structure of type III tetramers and their alignments in protofilaments and protofibrils (Geisler et al., 1992). From these studies, it seems that (a) the type III IF tetramer is staggered, exposing carboxy ends for protofilament elongation and (b) the carboxy end of the rod of tetramers in one protofilament may be in close proximity to the tails of tetramers in an adjacent protofilament. Consequently, elucidating the possible functional significance of the tail domain of vimentin, and specifically of this putative interaction, could be of major importance in understanding the molecular nature of higher ordered interactions in type III IFs.

To this end, we have created a tailless human vimentin, missing the last 54 amino acid residues at the carboxy terminus. In addition, we constructed a series of mutants containing single point substitutions within the rod and tail domains of vimentin. Each mutant was analyzed for its ability to (a) maintain a rod-tail association in vitro, (b) form 10-nm filaments in vitro, and (c) form a de novo vimentin cytoskeletal network in vivo. Our results have enabled us to explore in detail the possible functional significance of the carboxy end of vimentin in IF assembly in vitro and in IF network formation in vivo.

Materials and Methods

Clone Construction

Human vimentin cDNA was cloned into (a) pJay1, containing the SV40 major early promoter and enhancer, to form pJVimwt, and (b) pET-8c, to form pET-8cJVimwt (McCormick et al., 1991). Tailless (Δ T) vimentin cDNA was made by introducing a TAA stop codon at residue 411, immediately after sequences encoding helix 2B. To facilitate sub-cloning, BamHI sites were added 3' to (a) the wild-type TAA stop codon at position 465 (McCormick et al., 1991) and (b) the newly introduced TAA stop codon. All point mutations were made in Bluescript, KS+ (Stratagene Corp., La Jolla, CA) using site-directed mutagenesis (Kunkel, 1985), and mutations were then sub-cloned as Xho I/Bam HI segments into the parent wild-type and tailless vimentin vectors. Each mutant was sequenced, at least through the site of mutagenesis and, where indicated, in its entirety (Chen and Seeburg, 1985).

Transient Transfections and Immunoblots

MCF-7 cells (American Type Culture Collection, Bethesda, MD) were

transiently transfected with wild-type and mutant pJVim constructs according to the calcium precipitate method (McCormick et al., 1991). Tailed transgene products were detected with a 1:50 dilution of mouse monoclonal anti-cVim, specific for an epitope in the vimentin tail (V9; Accurate Chemical Corp., Westbury, NY.) (1:500 dilution for immunoblot studies). Tailless (Δ T) products were detected with a 1:100 dilution of a rabbit polyclonal antiserum, anti-nVim, specific for an epitope located in the head domain (a gift from Dr. Wallace Ip, University of Cincinnati, Cincinnati, OH) (immunofluorescence), or with a 1:3 dilution of a hybridoma supernatant of anti-rVim, a mouse mAb specific for an epitope in the rod (PK15; gift from Dr. Peter Traub, Max-Planck Institute for Cell Biology, Ladenburg, FRG) (immunofluorescence). To detect tailless vimentins on immunoblots, a mouse mAb 3B4 (Boehringer Mannheim Corp., Indianapolis, IN) was used at 1:400 dilution. Secondary antibodies were a 1:50 dilution of either goat anti-mouse IgG Texas red or goat anti-rabbit-FITC (Cappel Biochemicals, Durham, NC). All antisera used for immunofluorescent staining were diluted in 20% normal goat serum. Immunoblots were as described (Letai et al., 1992), using an ECL kit (Amersham Corp., Arlington Heights, IL) according to the manufacturer's instructions.

Fast Performance Liquid Chromatography Purification of Proteins

Vimentin mutants were obtained by subjecting bacterially expressed proteins to anion exchange fast performance liquid chromatography (FPLC) (Coulombe et al., 1990). Protein purity was judged by 8% SDS-PAGE and staining with Coomassie brilliant blue. Each preparation was spun through a centricon-30 column (Amicon, Beverly, MA) at 6,000 rpm for 30–60 min to further ensure that samples did not contain degradation products too small to be detected by SDS-PAGE. Protein concentrations were determined using the Bradford assay kit from Bio-Rad Laboratories (Richmond, CA).

In Vitro Reconstitution of Vimentin Filaments

Vimentins (150 μ g/ml) were dialyzed at room temperature (rt) against (a) 8 M urea, 5 mM Tris-HCl, 1 mM DTT, pH 8.4; (b) 6 M urea, 5 mM Tris-HCl, 1 mM DTT, pH 8.4; and finally (c) 4 M urea, 5 mM Tris-HCl, 1 mM DTT, pH 8.4 (2 h each buffer). Filaments were then dialyzed against 5 mM Tris-HCl, 1 mM EDTA, 0.1 mM EGTA overnight at 4°C followed by rt dialysis against 5 mM Tris-HCl, 1 mM DTT, pH 8.4 for 2 h, and 25 mM Tris-HCl, 160 mM NaCl, pH 7.4 for 3–4 h. Resulting filaments were transferred to 400–500 mesh carbon coated copper grids and stained with 1% uranyl formate, before examination under a Philips CM10 electron microscope at 80 kV. Because slight variations in assembly conditions can lead to differences in overall quality of resulting filaments and/or their degree of aggregation, control reactions containing wild-type vimentin were always run in parallel, and reactions were always repeated in triplicate and with at least two different preparations of purified protein.

Quantitation of Filaments

Assembly efficiency was assessed by visual inspection of electron micrographs for unpolymerized material. Width and length were determined from a random series of 10 micrograph negatives from each experiment as described (Coulombe et al., 1990). Widths were measured from 40 randomly selected filaments. Width measurements reported in tables represent relative, not absolute values, due to variations in negative staining and degree of filament flattening during fixation on grids. Lengths were estimated from selecting at random 25 different filaments and measuring their length and number of visible free ends, and then dividing the total length by the total number of free ends. To assess the percentage of filament unraveling per unit length, 40 randomly selected filaments were examined.

Results

Identifying Point Mutations in the End of Helix 2B that Ablate the Ability of the Rod to Bind to the Tail

Previously, it was shown that a region extending from amino acids 364 to 416 in helix 2B of human vimentin interacted with a 30 amino acid peptide corresponding to a conserved type III tail segment (Kouklis et al., 1991). To examine the importance of these two domains in IF structure, and to ex-

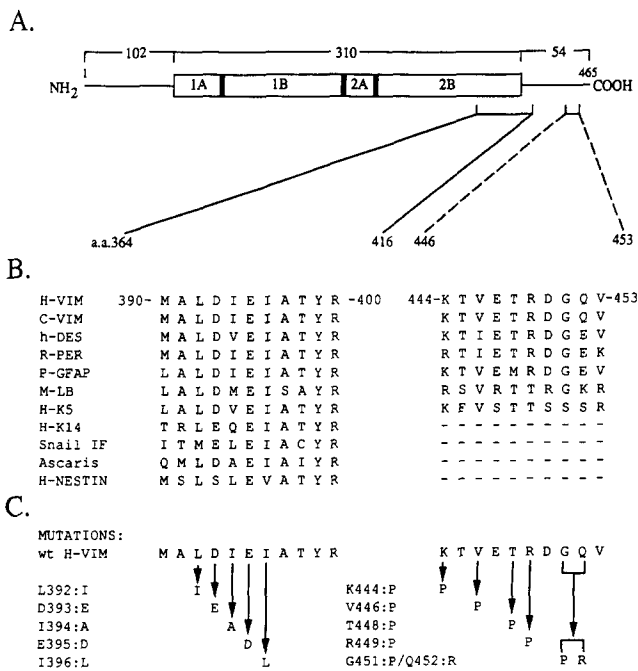
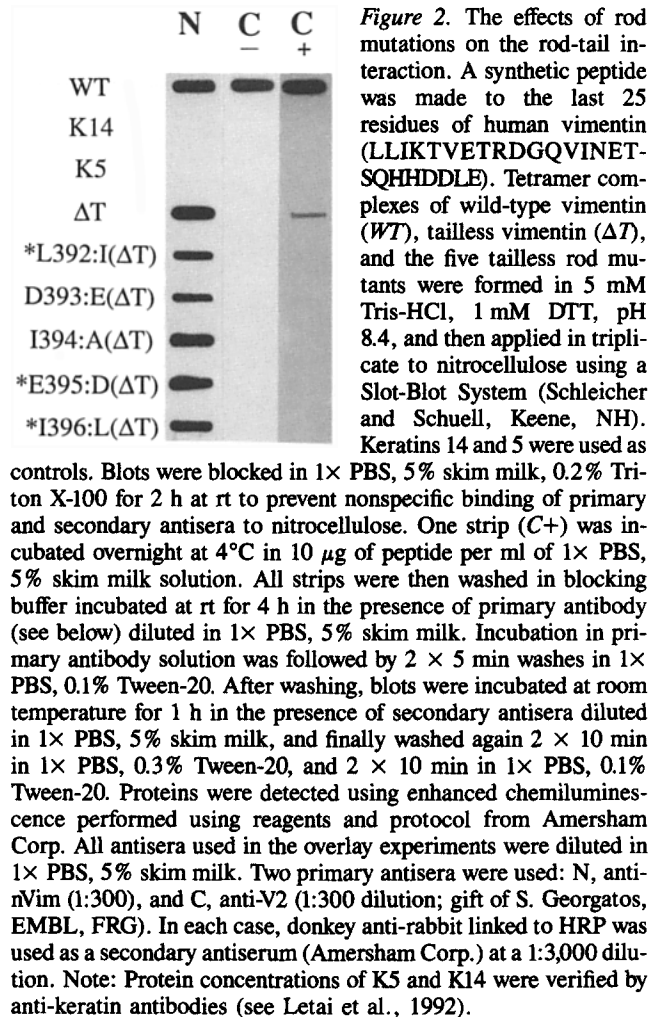


Figure 1. Location of vimentin point mutations. (A) Diagram of human vimentin, denoting the four α -helical domains of the rod (310 a.a.) and flanking nonhelical head (102 a.a.) and tail (54 a.a.) domains. (B) Rod and tail domains selected for mutagenesis. The sequence near the end of helix 2B is given for human vimentin (H VIM) and other IF proteins. The tail segment conserved among type III IF proteins is also given. Abbreviations: C, chicken; h, hamster; R, rat; P, porcine; M, mouse; DES, desmin; PER, peripherin; GFAP, glial fibrillary acidic protein; LB, lamin B. Sequences are reviewed in Albers and Fuchs (1992). (C) Mutations. Arrows denote point substitutions within the rod and tail domains.

to explore the possible functional significance of this interaction, we engineered mutations within the carboxy end of human vimentin (Fig. 1). Rod residues L392, D393, I394, E395, and I396 were chosen for mutagenesis because they are sequential, enabling analysis of amino acid substitutions encompassing 360° about the coiled-coil. L392, E395, and I396 are conserved among nearly all IF types extending to the most distantly related IF proteins (Dodemont et al., 1990). D393 and I394 are more variable, being conserved among most type III IF proteins but divergent among many other IF types (see Fig. 1 B). The five rod mutations were engineered in both wild-type and tailless (ΔT) forms of vimentin, and expressed in bacteria. After purification, tailless mutants were filter-immobilized and tested for their ability to bind a peptide corresponding to the last 25 residues of the tail. Complexes were visualized by anti-V2, an antiserum against the peptide (Fig. 2). Anti-nVim (N) staining, enabling visualization of wild-type and mutant vimentin proteins, verified that similar concentrations of IF proteins had been blotted (first strip). Anti-V2 (C) staining in the absence of competing peptide (-) demonstrated that the antibody was specific for IF proteins containing the vimentin tail domain, and did not cross-react with any of the tailless mutants (middle strip). When the strip was pre-incubated with tail peptide (+) and then stained with anti-V2 (C), binding of peptide was only observed with tailless vimentin, and not with any of the five tailless rod mutants (last strip). These findings suggested that the interaction between the tail and



the rod was dramatically weakened, or lost, as a consequence of these mutations.

Two Mutations Naturally Occurring in Other IF Proteins Are Deleterious to IF Structure when Introduced In Vimentin

When analyzed by gel electrophoresis, L392:I, D393:E, I394:A, E395:D, I396:L and their tailless counterparts migrated as a single species of the expected size (Fig. 3). When subjected to in vitro filament assembly, bacterially expressed human vimentin assembled into 10 nm filaments that were long and uniform (Fig. 4 a). None of the five rod mutants formed wild-type vimentin filaments in vitro (Fig. 4, b-f). Filaments formed from L392:I, D393:E and I394:A were not as long or as uniform as wild-type, and many were unraveled. Unpolymerized material in the background was indicative that assembly was not as efficient as wild-type. In contrast, filaments formed from E395:D were long, but loose and "ribbonlike," suggestive that protofibrillar associations might be affected. The most aberrant filaments were those formed from I396:L, yielding short fibrous stubs of atypical diameter, indicative of an elongation defect.

Table I summarizes the characteristics of filaments assembled from helix 2B rod mutants. The importance of L392, E395, and I396 to IF structure was not surprising, given their high degree of conservation among IF proteins. However for D393 and I394, this was quite remarkable, given that the two

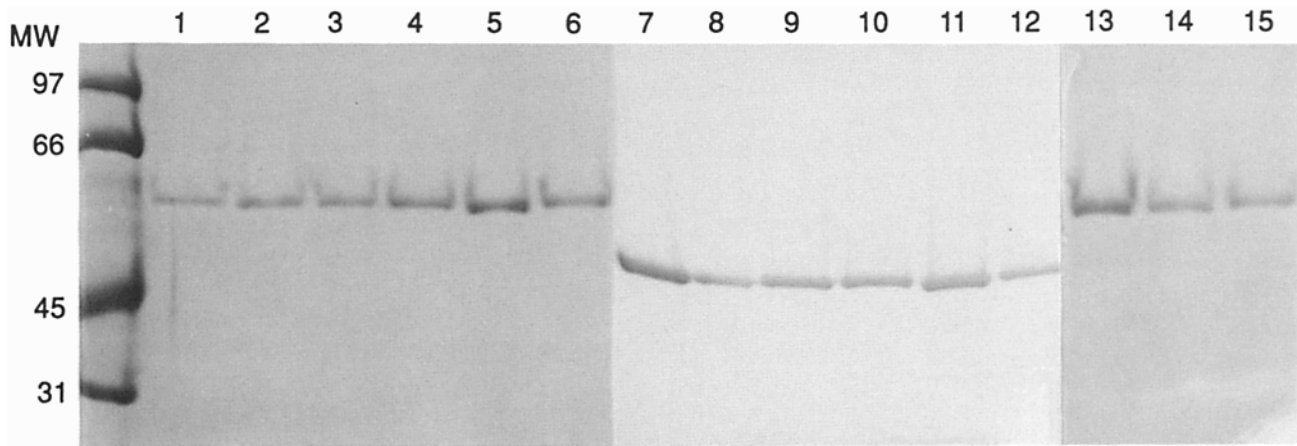


Figure 3. Assessment of the purity of mutant vimentins expressed *in vitro*. Wild-type and mutant vimentin proteins were expressed in bacteria using the pET-8c expression vector. After isolation and purification, proteins were resolved by SDS-PAGE and Coomassie blue staining to examine purity. While this analysis was always performed on all IF preparations, only one IF preparation for each mutant is shown here. Lanes: 1, wild-type vimentin; 2, L392:I; 3, D393:E; 4, I394:A; 5, E395:D; 6, I396:L; 7, tailless (Δ T) vimentin; 8, L392:I Δ T; 9, D393:E Δ T; 10, I394:A Δ T; 11, E395:D Δ T; 12, I396:L Δ T; 13, K444:P; 14, T448:P; 15, R449:P.

substitutions, D393:E and I394:A, are ones which occur naturally in other nontype III IF proteins (Fig. 1 B). These results imply that the environment surrounding these mutations, i.e., their context within the vimentin polypeptide, is critical in generating their deleterious effects.

The Rod-Tail Interaction Is Not Essential for 10 nm Filament Assembly

A priori, it was possible that the inability of our five rod mutants to assemble into proper IFs was due to their inability to interact with the tail. If the rod-tail association is essential for 10 nm filament assembly *in vitro*, then tailless vimentin, Δ T, should not assemble into IFs. However, as shown in Fig. 4 a', tailless vimentin assembled into 10-nm filaments which were only distinguished from wild-type by a slightly increased propensity to unravel (unwinding denoted by *arrowhead* in Fig. 4 a'). This was in agreement with Eckelt et al. (1992), who showed that a tailless *Xenopus* vimentin formed smooth, loosely packed 10-nm filaments *in vitro* (see also Kaufmann et al., 1985; Shoeman et al., 1990). While we did not observe a propensity of tailless vimentin IFs to laterally aggregate, in contrast to that reported by Shoeman et al. (1990), this could be due to differences in assembly conditions.

The Tail Plays a Role in Thermodynamically Stabilizing IF Structure

To test whether our rod mutations could sense the presence or absence of the tail, we constructed these mutations in the context of the tailless (Δ T) vimentin cDNA, and tested their ability to assemble into 10-nm filaments. Filaments assembled from the rod point mutants engineered in Δ T vimentin (Fig. 4, b'-f') were more aberrant than those assembled from tailless vimentin (Fig. 4 a') and often the tailed counterpart (Fig. 4, b-e). Thus, while the tail did not appear essential for IF structure, the tail seemed to thermodynamically stabilize IF structure, particularly when the rod domain had been destabilized by mutation. This enhancing ability of the tail did not involve a rod-tail association, at least of the sort detectable through our ligand-binding assays.

Curiously, one mutant, I396:L, appeared to form filaments which were more uniform in diameter than those produced when the tail was present (Fig. 4 f', compare with Fig. 4 f). One explanation for this surprising result is that the mutation might have caused a downstream alteration in the conformation of the tail such that the tail interfered with filament assembly. This said, I396:L(Δ T) filaments were still not as long as tailless filaments, indicating that the mutation also had an effect on IF structure that went beyond its effects on the tail.

In summary, our IF assembly studies have revealed (a) the importance of five sequential rod residues on IF structure; (b) a role for IF sequence specificity in determining the extent to which certain conservative amino acid substitutions within the rod might perturb IF structure; and (c) the importance of the vimentin tail in enhancing lateral and end-to-end interactions in vimentin IFs. These studies failed to establish a structural importance for the rod-tail association first described by Kouklis et al. (1991).

The Rod-Tail Interaction Is Not Required for Formation of Fibrous Arrays *In Vivo*, but Most Rod End Mutations Alter IF Network Formation

To ascertain the behavior of our vimentin rod mutants on IF network formation, we used the SV40 major early promoter and enhancer to transiently express wild-type and mutant vimentins in the vimentin-free adenocarcinoma cell line MCF-7 (K8⁺, K18⁺, K19⁺, VIM⁻). With only one exception, D393:E(Δ T), cell populations transfected with each transgene produced a single species of vimentin of the expected size and at roughly comparable levels (Fig. 5). Thus, with this exception, any differences we might subsequently observe in the ability of our mutants to form vimentin networks *in vivo* could not be attributed to different levels of protein expression, modification and/or processing. Moreover, since the relative levels of mutants were comparable, it seems most likely that the aberrant behavior of mutants was due to local structural perturbations rather than global defects in the folding of their polypeptide chains.

Wild-type vimentin formed an extensive *de novo* network

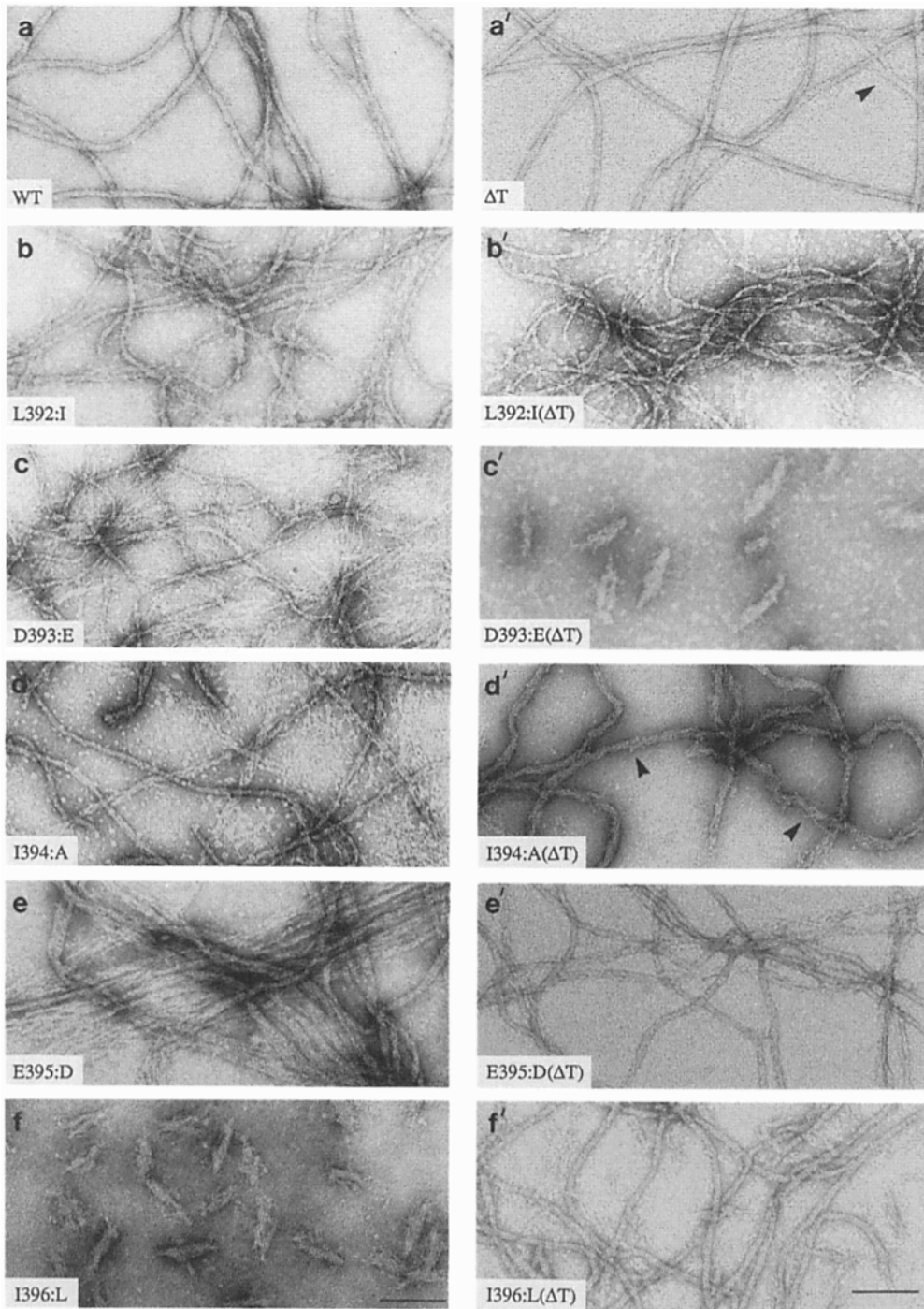


Figure 4. In vitro filament reconstitution of wild-type and rod mutants in the presence and absence of the tail. Wild-type vimentin (*WT*), tailless vimentin (ΔT), and mutants L392:I, D393:E, I394:A, D395:E, and I396:L and their tailless counterparts were expressed in bacteria, purified, and subjected to filament assembly. Shown are representative examples of in vitro reconstituted filaments. Bars, 100 nm.

of densely packed filaments, as judged by anti-cVim staining (Fig. 6 *a*). The filament networks were distinct from keratin filaments (double immunofluorescence performed, but not shown; see McCormick et al., 1991) and were indistinguishable from vimentin IFs seen in other cells that contain endogenous vimentin networks. Similar staining patterns were seen in cells stained with a polyclonal antiserum, anti-V2, against the COOH terminus of the vimentin tail (not shown), and with anti-rVim, a mAb against the rod segment of vimentin (Fig. 6 *b*).

In contrast to wild-type vimentin, most rod mutants formed aberrant filament networks in vivo. Cells transfected

with D393:E, E395:D and I396:L failed to give a discernable IF network, but rather displayed punctate staining at the cell periphery (Fig. 6, *d*, *f*, and *g*). L392:I was less deleterious, often forming cytoskeletal fibers, albeit ones which frequently collapsed around the nucleus (Fig. 6 *c*). The most surprising rod mutant was I394:A, which produced an extensive cytoskeletal network remarkably similar to wild-type (Fig. 6 *e*), even though the networks in these cells collapsed occasionally (not shown). Subtle differences aside, the fact that I394:A and L392:I could assemble into extensive fibrous arrays in vivo, coupled with their inability to bind the tail domain peptide in vitro, argued against a major

Table I. Summary of Characteristics of IFs Assembled from Vimentin Rod Mutants

Mutant	Diameter	Length	Degree unraveling
	(n = 40)	(n = 25)	(n = 40)
WT	10 ± 1 nm	>10,000 nm	<2%
L392:I	9 ± 2 nm	740 ± 420 nm	50%
D393:E	9 ± 1 nm	680 ± 310 nm	30%
I394:A	9 ± 1 nm	590 ± 370 nm	40%
E395:D	(**)	>10,000 nm	>90%
I396:L	5–20 nm	<200 nm	na
ΔT	11 ± 1 nm	>10,000 nm	10%
L392:I(ΔT)	10 ± 2 nm	1000 ± 500 nm	40%
D393:E(ΔT)	5–20 nm	<200 nm	na
I394:A(ΔT)	14 ± 1 nm	1400 ± 700 nm	50%
E395:D(ΔT)	8 ± 2 nm	>10,000 nm	10%
I396:L(ΔT)	8 ± 1 nm	640 ± 320 nm	10%

Note: In cases where unraveling was appreciable, diameter of filaments could not be accurately determined and is denoted by (**). *nd*, not determined; *na*, not applicable; *n*, number of filaments used in measurements.

importance of the rod-tail association in vimentin IF network formation.

Certain Effects of the Tail In Vivo Are Dependent upon Mutations Within the Rod

Irrespective of its association with the rod, the tail might still be important for IF network formation in vivo. To test this possibility, we tested our ΔT versions of these mutants in transiently transfected MCF-7 cells (Fig. 6, *c'–g'*). Significantly, for L392:I and I394:A, removal of the tail did indeed have a deleterious effect on IF network formation (Fig. 6, *c'* and *e'*). Thus, at least for these mutants, the tail appeared to have a positive effect on fibrous network formation

in vivo. Curiously, although clearly aberrant, E395:D(ΔT) assembled a more extensive network than its tailed counterpart (compare Fig. 6, *f'* with *f*). This finding suggested that in the context of the E395:D mutation, the tail was in a conformation that interfered with network formation. Thus, the effects of the tail on filament network formation was dependent upon the specific mutation within helix 2B of the rod.

Mutations Within a Motif Conserved among Type III IF Proteins Perturb IF Network Formation and IF Assembly

The realization that a number of our rod mutations were sensitive to the presence of the tail prompted us to explore the importance of specific residues in the nonhelical tail domain. Our initial interest was on the extreme carboxy terminus, because we had previously demonstrated that replacing the four carboxy terminal amino acids of vimentin with 15 amino acids of substance P compromised the ability of this modified vimentin to form an intermediate filament network in transfected MCF-7 cells in vivo (McCormick et al., 1991). We later showed that this mutant, VIMP, was unable to assemble into 10-nm filaments in vitro (data not shown). Extensive mutagenesis of the COOH terminus revealed that it was the addition of the P-tag and not the removal of the COOH-terminal residues from the COOH terminus of vimentin that was responsible for compromising the function of the modified vimentin. These results are summarized in Table II.

Our interest in the vimentin tail broadened as a consequence of studies by Kouklis et al. (1991), demonstrating that a peptide corresponding to the last 30 residues of a related type III IF protein, peripherin, associated with residues 370–420 of human vimentin (Kouklis et al., 1991). Within this tail segment, a sequence (K-T-X-E-T-R-D-G), where X is variable, is found in all type III IF proteins (see Fig. 1 B),

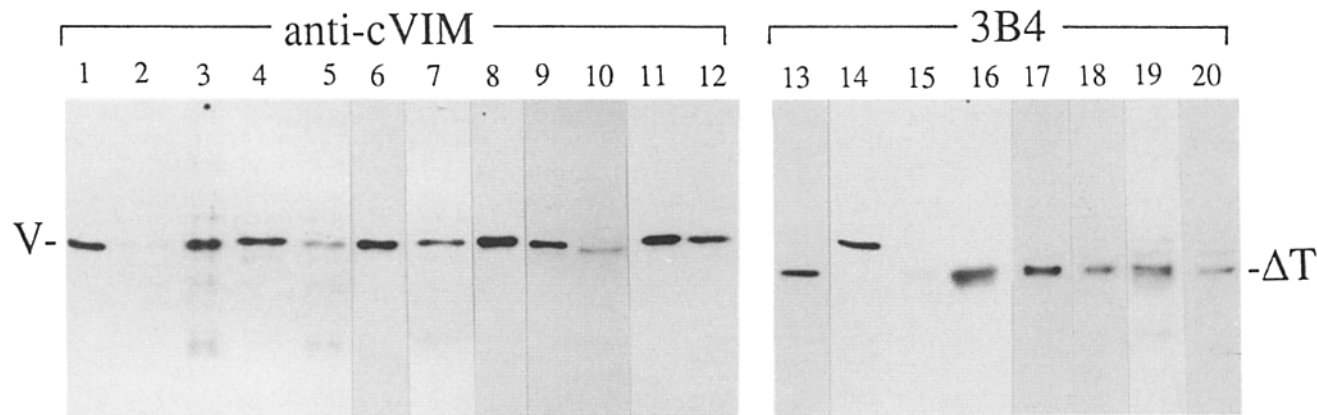


Figure 5. Analysis of vimentin mutant protein expressed by transiently transfected MCF-7 cells. 60-mm dishes and glass chamber slides of cells were transiently transfected in duplicate with wild-type or mutant vimentin expression vectors. 65 h post transfection, the glass chamber slide cultures were subjected to immunofluorescence microscopy using anti-cVim antisera (tailed constructs) or anti-rVim antisera (tailless constructs). From these slides, the relative efficiency of each transfection was estimated by determining the ratio of positively stained to unstained cells. From culture dishes, IF proteins were extracted, resolved by electrophoresis through 8.5% polyacrylamide SDS gels, and transferred by electroblotting to nitrocellulose paper (McCormick et al., 1991). Blots were hybridized with the antibodies indicated, followed by chemiluminescence development. Blots were exposed to x-ray film for times that were adjusted for slight variations in transfection efficiencies. Lanes 1 and 13, bacterially expressed wild-type vimentin and ΔT vimentin, respectively. IF extracts were from cells transfected with: lane 2, untransfected; lane 3, pJVim; lane 4, L392:I; lane 5, D393:E; lane 6, I394:A; lane 7, E395:D; lane 8, I396:L; lane 9, K444:P; lane 10, V446:P; lane 11, R449:P; lane 12, G451:P/Q452:R; lane 14, same as 1; lane 15, same as 2; lane 16, ΔT; lane 17, L392:I(ΔT); lane 18, I394:A(ΔT); lane 19, E395:D(ΔT); lane 20, I396:L(ΔT).

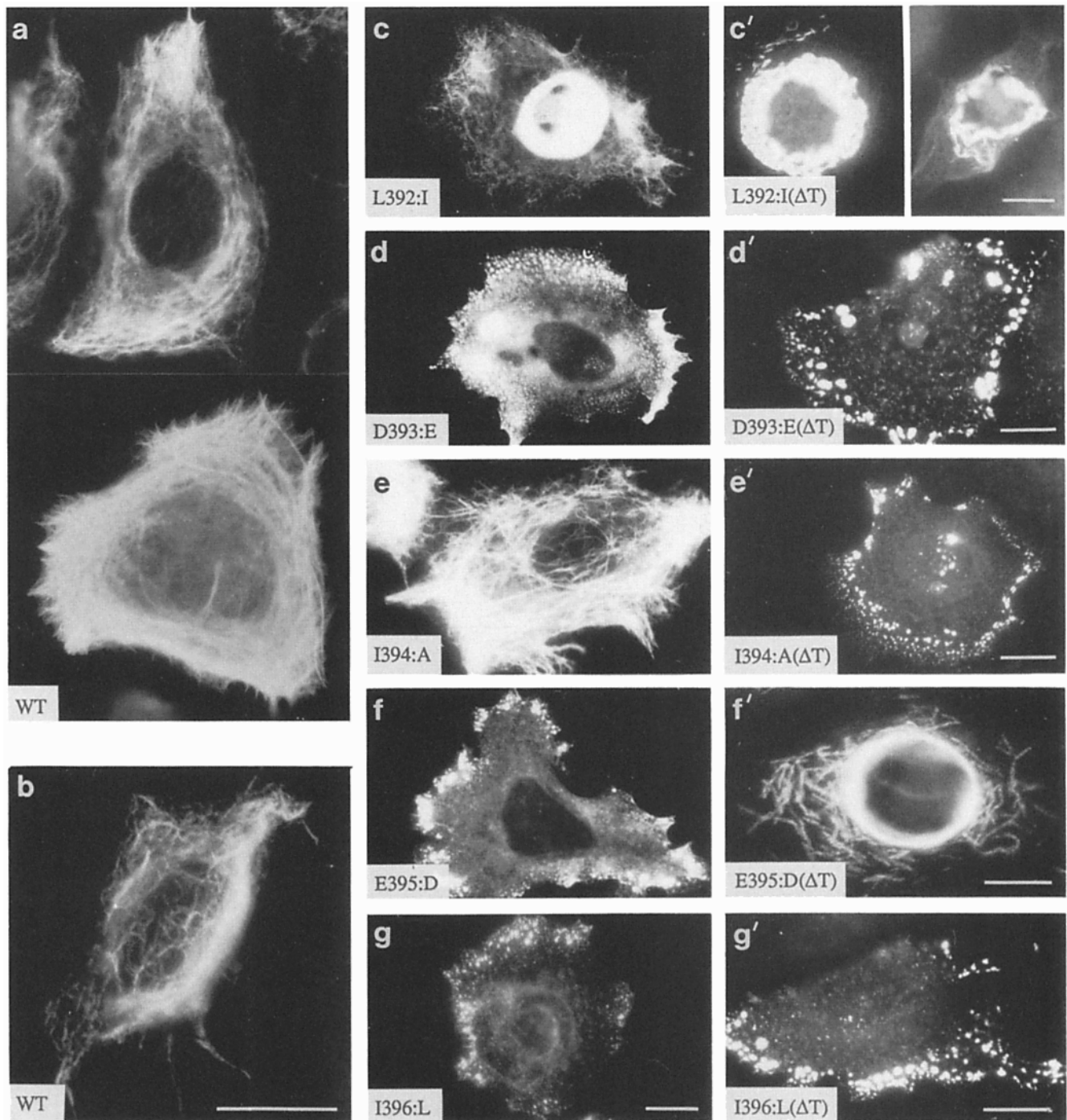


Figure 6. Transient expression of wild-type and rod mutants in epithelial cells. Wild-type vimentin (WT), and mutants L392:I, D393:E, I394:A, D395:E, and I396:L and their tailless counterparts were transiently transfected into MCF-7 cells, and 65 h later, cells were stained with anti-cVim (a, c–g) or anti-rVim/anti-nVim (b, c'–g'). Bars, 30 μ m.

suggesting a possible role for this domain in type III IF assembly or function.

To examine more closely the importance of this sequence in de novo vimentin network formation in vivo, we created a series of proline mutations in the consensus sequence of the vimentin tail (Fig. 1 C and Table II). As judged by indirect immunofluorescence microscopy of transiently transfected cells, V446:P and V453:P, had no apparent effect on de novo vimentin filament formation, and generated filamentous networks indistinguishable from wild-type controls

(Fig. 7 a; shown is V446:P). It may be relevant that these two residues vary among type III IF sequences, and our findings suggest that they either play specialized roles unique to each type III IF protein, or perhaps no role at all. Other tail mutations which had no effect on IF assembly are given in Table II.

In 25% of cells transfected with the double mutant, G451:P/Q452:R, vimentin networks were indistinguishable from wild-type. However, in more than 75% of cells, fibers made de novo from this mutant appeared somewhat thicker

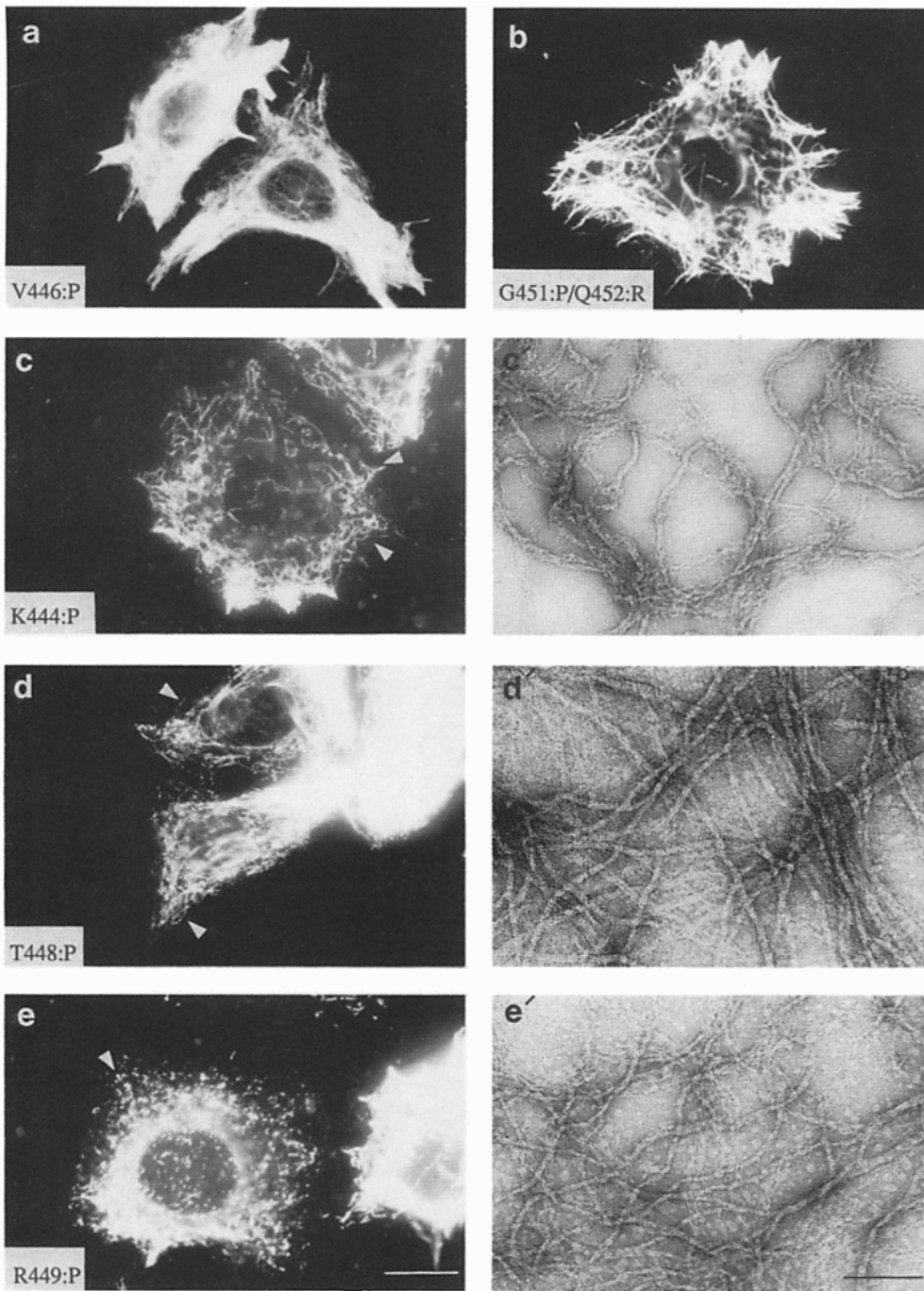


Figure 7. Transient transfection and in vitro filament reconstitution of tail mutants. (a-e) The tail mutants, V446:P, G451:P/Q452:R, K444:P, T448:P, and R449:P, were transiently transfected into MCF-7 cells, and 65 h later, cells were stained with anti-cVim. (c'-e') The same mutants were expressed in bacteria, purified, and subjected to IF assembly in vitro. Shown are IFs from mutants in (c-e). Arrows: (c-e) beaded fiber structures. Bars: (a-e) 30 μ m; (c'-e'), 100 nm.

than normal, suggesting that the mutant might have had a subtle effect on vimentin filament network formation (Fig. 7 b). K444:P, T448:P, and R449:P had significantly greater deleterious effects, generating networks of fibers with a kinked and/or beaded appearance (Fig. 7, c-e, respectively; see also Table II). K444:P, T448:P and R449:P also formed aberrant filaments in vitro (Fig. 7, c'-e', respectively). Of the other mutations in the tail consensus region, only G451:P/Q452:R generated filaments in vitro that were distinguishable from wild-type (not shown). While the in vivo and in vitro effects of these tail mutants were appreciable, none were as deleterious as three of the subtle rod mutations, D393:E, E395:D, and I396:L (compare with Fig. 6).

In summary, substantial changes in the tail could be made without compromising IF network formation and IF assembly. However a few changes, particularly those within the conserved tail sequence, seemed to alter tail conformation in a fashion deleterious to these processes. Whether these mutations perturbed the rod-tail association described by Kouklis et al. (1991) seems likely, but was not addressed.

The Tail of Vimentin Plays a Role in IF Network Formation In Vivo

A priori, it could be that the three tail mutations acted by steric hindrance to interfere with IF network formation in

Table II. Summary of Vimentin Tail Mutants

Mutant	In vitro	In vivo	Diameter*	Length*	Degree unraveling*
			(n = 40)	(n = 25)	(n = 40)
WT	+++‡	+++§	10 ± 1 nm	>10,000 nm	<2%
K444:R	nd	+++	nd	nd	nd
K444:P	+	++	8 ± 1 nm	1,000 ± 400 nm	75%
V446:L	nd	+++	nd	nd	nd
V446:P	nd	+++	nd	nd	nd
T448:S	nd	+++	nd	nd	nd
T448:P	++	+	9 ± 1 nm	1,400 ± 500 nm	20%
R449:P	+	+	8 ± 1 nm	950 ± 700 nm	50%
G451:P/Q452:R	++	++	10 ± 1 nm	>10,000 nm	10%
V453:P	nd	+++	nd	nd	nd
S458:T	nd	+++	nd	nd	nd
Q459:N	nd	+++	nd	nd	nd
H460:G	nd	+++	nd	nd	nd
H461:G	+++	+++	10 ± 1 nm	>10,000 nm	<2%
D462:E/D463:E	+++	+++	10 ± 1 nm	>10,000 nm	<2%
E465:P	nd	+++	nd	nd	nd
E465:Y	++	+++	11 ± 1 nm	>10,000 nm	<2%
VIMP			na	na	na

* Measurements from in vitro assembled filaments; n, number of filaments used in measurements.

‡ + + +, Wild-type in appearance, i.e., smooth, long, and uniform in diameter; ++, IFs described by two of the three wild-type characteristics; +, IFs described by one of the three characteristics.

§ Networks not readily distinguishable from wild-type in + + +, >95%; ++, 20–50% and +, <20% of transfected cells.

|| Complete block in IF assembly in vitro or no wild-type networks in transfected cells.

na, Not applicable; nd, not determined.

vivo. If so, removing the vimentin tail altogether might be expected to be tolerated in vivo. In striking contrast, however, cells transfected with tailless vimentin formed a fibrous network which was atypically concentrated at the protruding edges of the cells (Fig. 8). The peculiar staining pattern could not be attributed to the antibody, since anti-rVim and anti-nVim gave this pattern (Fig. 8, a and b), which was distinctly different from that produced when these antisera were used to stain cells transfected with wild-type vimentin (for example, see Fig. 6 b). The peripheral location of the tailless network was intriguing in light of studies by Georgatos and Blobel (1987), suggesting that the vimentin tail is involved in interactions between IFs and the nuclear envelope.

The peculiar IF networks formed with tailless vimentin showed a tendency to collapse, as evidenced by the appear-

ance of thick cables of IFs in >50% of cells examined at late times (≥65 h) after transfection (Fig. 8 c). Collapsed networks were not seen in cells transfected with wild-type vimentin, providing further evidence that the tail of vimentin plays an important role in establishing proper IF networks within cells.

Networks Formed with Mutant Vimentin Do Not Disrupt Microtubule Networks

Treatment of cultured cells with microtubule-disrupting drugs is known to cause a collapse of vimentin networks into perinuclear whorls (Franke et al., 1979). However, injection of anti-vimentin antibodies into fibroblasts does not conversely cause perturbations in the microtubule network (Lin

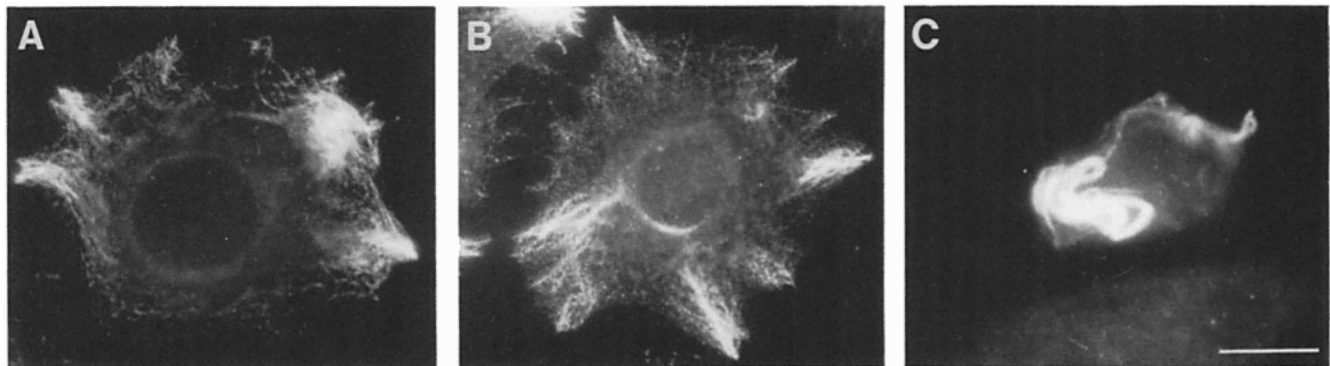


Figure 8. Tailless vimentin in vivo. Tailless (ΔT) vimentin was transiently expressed in MCF-7 cells and 65 h later, cells were stained with anti-rVim or anti-nVim. (a) ΔT , anti-rVim; (b) ΔT , anti-nVim; (c) ΔT , anti-nVim, example of collapsed tailless vimentin network. Bar, 30 μ m.

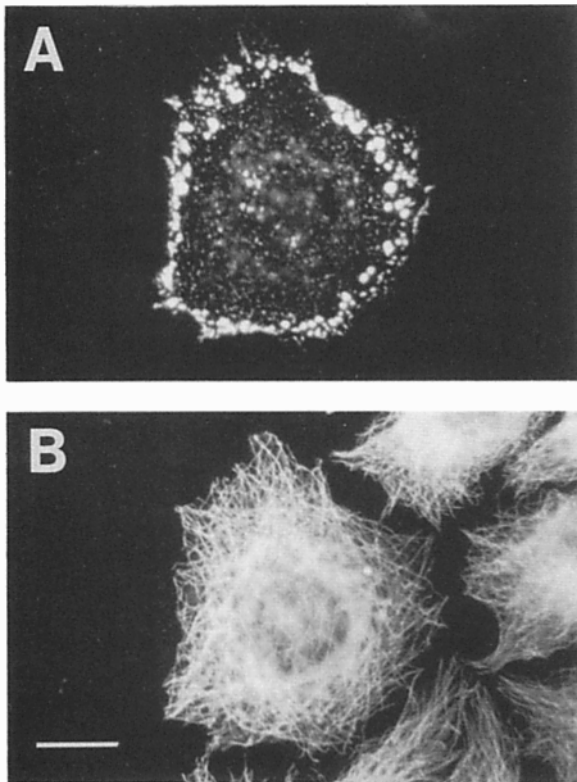


Figure 9. Vimentin mutants not competent to form an IF network do not interfere with formation of a microtubule network. MCF-7 cells transiently transfected with the vimentin mutant I396:L(Δ T) were stained with anti-rVim (A) and anti- α tubulin (B). Bar represents 30 μ m.

and Feramisco, 1981; Gawlitta et al., 1981). In agreement with this finding, aberrant vimentin aggregates had no apparent effect on the microtubule network (Fig. 9). This was true for all of our mutants, irrespective of whether they were tailless. Thus, the microtubule network seems to be significantly more resistant to perturbations in the vimentin network than the vimentin network is to the microtubule network. Whether the differences in behavior of various mutants *in vivo* might be due to changes in their interactions with microtubules could not be addressed by the assays described here.

Discussion

The Importance of Residues Near the Carboxy End of Helix 2B

We have identified five residues (L392–I396) just upstream from the highly conserved TYRKLLLEGEE sequence, that are critical to proper filament assembly *in vitro* and *in vivo*. Our findings are consistent with and extend a previous report illustrating that nonconservative mutations of three residues in this region of desmin (one corresponding to residue E395 of vimentin, also mutated in our study), blocked *de novo* desmin network formation in MCF-7 cells (Raats et al., 1991). What becomes apparent from our studies is that even conservative substitutions within these residues can have profound deleterious effects on filament network formation and 10-nm filament assembly.

Another important finding was that some conservative changes, e.g., D393:E and I394:A, caused major perturbations in vimentin filament formation, and yet these precise substitutions are found naturally in other IF proteins. This implies that the surrounding residues can have a profound influence as to whether a specific amino acid residue change can be tolerated in an IF protein. Moreover, it is a signal that caution should be exerted in comparing mutations from one IF protein to another, particularly when the residue in question is not strictly conserved among all IF proteins.

Finally, because IF assembly and network formation were compromised by five successive residues in the α -helical rod segment, its importance must extend throughout a complete turn of the α -helix, including not only the residues involved in forming the hydrophobic, coiled-coil seal (L392 and I396) but also those involved in higher ordered interactions. This finding is further supported by the variety of higher ordered interactions affected by our mutations. These included (a) aberrancies in protofibrillar associations (e.g., E395:D), (b) defects in efficient incorporation of tetramers into a filament (e.g., L392:I and D393:E), and (c) defects in efficient longitudinal packing at the protofilament/protofibril level (e.g., I396:L).

All five point mutations also prevented or greatly weakened the association between the tail and rod. However, the effects of these rod mutations on IF structure went beyond mere disruption of this interaction, because deleterious effects were still detected when the tail domain was removed entirely from these mutants. Moreover, because tailless vimentin assembled into 10 nm filaments *in vitro*, the tail (and consequently the rod-tail association) appeared to be largely dispensable for IF structure *in vitro*.

Possible Functional Significance of the Rod-Tail Interaction

We cannot say that the rod-tail association first noted by Kouklis et al. (1991) is irrelevant to IF network formation and IF assembly. However, as judged by our *in vitro* studies, removing the tail altogether had only very subtle effects on 10-nm filament assembly. Moreover, while helix 2B rod mutations disrupted the rod-tail association and also caused aberrations in IF assembly, these mutations were deleterious to IF assembly even after the tail was removed. Finally, because filamentous arrays can develop *in vivo* with the mutant I394:A, in which the rod-tail association is ablated, it seems unlikely that the association is critical for forming any of the major architectural features of an IF network, at least as the network exists in cultured cells flattened on the dish surface. One possibility is that the rod-tail interaction occurs inconsequentially when the tail folds into a conformation that (a) does not interfere with IF assembly, and/or (b) allows it, i.e., the tail, to perform its roles in IF assembly and network formation (see below). If the interaction plays a more substantial role, it must be one that is not readily observed by the methods of detection used here.

The Importance of the Vimentin Tail in IF Network Formation and in IF Structure

Our transfection studies provided graphic illustration that the tail is critical for IF network formation *in vivo*. The fibrous arrays of tailless vimentin at the cell periphery were initially surprising, given that Raats et al. (1991) did not observe

filamentous networks *in vivo* from tailless vimentin or desmin. Moreover, while Eckelt et al. (1992) detected IFs in bovine mammary cells transfected with *Xenopus* tailless vimentin, they also observed accumulation of IFs in the nuclei, a phenomenon not observed in our studies. We do not yet know the underlying reasons for these various differences. However, the peripheral localization of these networks is consistent with studies by Georgatos and Blobel (1987), who found that a chemically generated tailless vimentin could not associate with the nuclear lamina.

The role of the tail in IF structure has been more difficult to assess. The slight unraveling of tailless vimentin IFs most likely reflects a relative weakening of interprotofibrillar interactions in tailless filaments, perhaps an indication that such associations may be thermodynamically less favorable in tailless versus wild-type filaments. This notion is generally consistent with reports implicating the nonhelical tails of IFs in maintaining proper distances between IFs and in thermodynamically favoring the optimal packing of subunits, leading to seemingly more stable IFs (Kaufmann et al., 1985; Lu and Lane, 1990; Hatzfeld and Weber, 1990; Shoeman et al., 1990; Wilson et al., 1992; Eckelt et al., 1992). However, our data showing that most rod mutants formed better IFs when the tail was present, than in its absence, underscore more clearly than before that the tail plays a role in thermodynamically stabilizing IF structure.

While removal of the tail has provided some insights into the role of the tail in IF structure, little is known about the structure of the tail itself, or how the tail might influence tetramer or higher ordered packing. Our extensive mutagenesis revealed that many changes to the tail were tolerated without major consequence, and that most of the deleterious effects of the tail seemed specific for a few residues within a segment conserved among type III IFs. Thus, point mutations in a number of residues downstream from this conserved sequence did not affect filament formation *in vivo* or *in vitro* (see Table II), and moreover, a peptide to an upstream tail sequence did not interfere with 10-nm filament assembly (Kouklis et al., 1992).

Secondary structure predictions suggest that residues in the tail might form a beta turn or hairpin structure (Kouklis et al., 1991). However, our knowledge of tail structure is not sufficient to understand why certain residues but not others within the conserved tail segment could tolerate proline substitutions without major consequence to IF structure or network formation. Future studies will be necessary to ascertain whether proline mutations such as R449:P are deleterious because they (a) create a tail structure that sterically interferes with 10-nm filament assembly or (b) perturb a certain tail structure that in its wild-type form is critical in controlling the assembly process.

Taken together, our data agree best with a model where the tail is not essential for IF structure, but is involved in enhancing protein interactions within the 10-nm filament. Coupled with recent structural studies of desmin (Geisler et al., 1992), indicating that the carboxy ends of two antiparallel dimers are in the nonoverlapping ends of the tetramer, our findings point to the notion that the tail might influence the association-dissociation kinetics involved in the packing of tetramers into protofilaments. This could explain why a number of mutations within helix 2B and the tail domain of vimentin affected filament length.

A priori, it is possible that the tail may function to alter the rate or efficiency of assembly, without perturbing internal alignment of tetramers. Through, e.g., intermolecular associations, proper alignment of subunits could be abetted and the critical concentration required for assembling tetramers or higher ordered structures could be reduced. Experimental indications that the vimentin tail may function to enhance intertetrameric interactions are (a) the enhanced protofibrillar unraveling of tailless vimentin, and (b) the enhanced protofilament/protofibril shortening, protofibrillar unraveling and inefficiency of subunit incorporation caused by removal of the tail from helix 2B mutants.

An alternative, if perhaps less likely, model is that alignment of at least some tetramers in tailless vimentin filaments is aberrant, giving rise to perturbations in filament structure too subtle to detect in our *in vitro* assays. In this scenario, the tail could function to sterically prevent an otherwise thermodynamically favored, but structurally incorrect, molecular interaction in the self assembly process. If intramolecular, this could occur at the level of aligning the anti-parallel dimers, perhaps favoring the staggered tetramer conformation at the expense of the unstaggered conformation (see, e.g., Aebi et al., 1988; Coulombe and Fuchs, 1990; Steinert, 1991). If intermolecular, the steric hindrance could occur at the level of tetrameric or higher oligomeric packing. This mechanism has recently been proposed to account for the tail interactions found in nonmuscle myosin, a protein structurally related to vimentin (Hodge et al., 1992).

Finally, the notion that the tail may temporarily prevent formation of inappropriate intra- and/or interchain interactions is intriguing, because this is an important function of a recently described class of proteins called chaperones (for review, see Gething, 1991). The mitochondrial chaperonin hsp60, for instance, requires itself to fold into a functional protein (Cheng et al., 1990). Because IF assembly *in vitro* occurs under conditions that are significantly more stringent than physiological, it seems reasonable to expect that the process of IF assembly is controlled *in vivo*. A self-chaperone role for the tail provides an attractive model for the regulated macromolecular assembly of vimentin and other type III IF proteins.

At the moment, our data are not sufficient to distinguish between these models. Irrespective of the precise assembly step regulated by the tail, the mechanism must be consistent with our findings that (a) addition of the 15 residue P tag interferes with all tail functions, (b) specific sequence features of vimentin's tail appear to be important for tail function, (c) some subtle mutations in the rod domain appeared to have more deleterious effects on IF structure when the tail domain was missing, and (d) the IF networks produced by tailless vimentin were clearly aberrant. As additional studies are conducted, it should be possible to further elucidate the molecular pathways that are controlled by the tail of vimentin.

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