Diversity of Intermediate Filament Structure

EVIDENCE THAT THE ALIGNMENT OF COILED-COIL MOLECULES IN VIMENTIN IS DIFFERENT FROM THAT IN KERATIN INTERMEDIATE FILAMENTS*

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Although vimentin intermediate filaments (IF) are morphologically similar to all other IF types, cells have evolved different ways of manipulating vimentin and keratin IF. The structural basis for such differences is unknown. We have explored this by use of cross-linking experiments on vimentin oligomers, polymers, and intact IF to determine the axial length of vimentin molecules and the degrees to which neighboring molecules are aligned in IF. Our data reveal that the homodimer vimentin molecule (43.9 nm) is clearly shorter than a keratin heterodimer molecule (48.2 nm). Vimentin assemblies contain three modes of antiparallel molecular alignments: A11 and A22 in two-molecule or larger oligomeric assemblies, in which the two molecules are staggered so as to bring their 1B and 2B rod domain segments, respectively, into register; and A12 in higher order molecular assemblies in which the two neighboring molecules are largely overlapped. Since the repeat axial length of the vimentin assemblies (42.6 nm) is less than the molecular length, this means there is an overlap (designated as alignment A12(n)) of about 1 nm (5–10 residues) between the end of the 2B and beginning of the 1A rod domain segments of similarly directed molecules in the IF. Interestingly, these four modes of nearest neighbor molecular alignments also occur in keratin IF. However, the degree of stagger of alignments in the A11 and A22 modes is different (stagers of −19.5 for vimentin versus −16.6 nm for keratin, and 23.3 and 28.6 nm, respectively). Two-dimensional surface lattice maps of the two IF types are very similar, except for differences in molecule alignments and different axial repeats of 21.4 nm in vimentin and 22.6 nm in keratin IF. Although vimentin-keratin hybrid molecules can be induced to form in vitro, they do not assemble into higher order structures. The data suggest that vimentin and keratin are incapable of assembly into IF in vitro or in vivo simply because their molecules are of different axial lengths and because the exact axial alignments of neighboring molecules are different.

Intermediate filaments (IF) constitute one of the three major components of the cytoskeleton of most eukaryotic cells. The IF family consists of about 40 different protein chains that have clear and well understood patterns of expression during development and differentiation. Based on sequence comparisons and gene structures, six types of IF have now been identified, including about 15 type I and about 15 type II keratins of "hard" and "soft" epithelia; four known type III chains, including vimentin, desmin, peripherin, and the glial fibrillary acidic protein; several type IV chains expressed in neuronal tissues; several type V nuclear lamins; and a single type VI protein (1, 2). Recent data have documented that IF are highly dynamic structures in living cells, continually exchanging protein along their length in response to a variety of stimuli or cellular processes and undergo reversible cycling of disassembly and reassembly such as during mitosis (2). In the latter case, these cycling events are mediated by phosphorylation of the IF chains with specific protein kinases, which promote disassembly, followed by dephosphorylation by phosphatases, which allows spontaneous reassembly (2). IF also undergo rebuilding processes during development and differentiation in which one type of IF chain is replaced by another, as in neuronal tissues, or replacement of one set of keratin chains by another, as is common in epithelial systems. This is thought to occur by dynamic exchange/replacement mechanisms (3) that may also involve phosphorylation.

Analyses of the primary sequence data reveal that all IF chains are built on a common plan (4, 5): a central α-helical rod domain of conserved properties, and amino- and carboxyl-terminal end domains of widely varying properties. The rod domain consists of four distinct segments, 1A, 1B, 2A, and 2B, that possess a 7-residue repeating motif common to proteins capable of forming an α-helical coiled-coil. In the case of the five types of vertebrate cytoplasmic IF, each segment is of identical size and likely secondary structure (4, 5). The segments are joined by the linker sequences L1, L12, and L2 that confer additional flexibility to the rod or allow it to kink at these points. The L1 link varies widely in size and sequence within the family of IF chains, whereas in contrast, the third link, L2, is highly conserved in both length and sequence.

Current models for the structure of the several types of IF are based on the view that the rod domains of the constituent chains pack together in the same way, or at least limited sets of ways, to form a central core. This packing is in large part governed by the conserved periodicities of charged residues along the rod domains (4, 5). The hypervariable end domains...
are thought to protrude from this central core to interact with other cytoplasmic constituents (6,7). Certain of the details of IF structure have been studied in detail. The first step is the formation of a coiled-coil dimer molecule of two compatible chains, formed by alignment of the chains in parallel and close axial registration (8–10). In the special case of KIF, this molecule is a heterodimer consisting of one type I and one type II chain (9–11); in types III and VI IF, this is usually a homodimer (12); and in types IV (13) and V (14) IF, this may be a homodimer or facultative heterodimer (15). There is still some controversy as to the next step. Some studies with type I and keratin (11,16), type III desmin (17,18) or vimentin (19), and type IV neurofilament (13,20) IF have suggested that a pair of molecules form a "four-chain building block," which serves as the basic unit from which IF are assembled. More recent work (21,22) has suggested instead for KIF that such a unit consists of a range of small oligomers, containing one to four molecules. Whereas several of the earlier studies suggested that a pair of molecules is aligned antiparallel and in register (11,16,17,20), more recent studies on both desmin (18,23,24) and keratin (25–27) IF have shown that the molecular axes of the adjacent molecules are antiparallel and partly staggered. Indeed, detailed cross-linking studies of keratin 1/keratin 10 (K1/K10) KIF (26) and K5/K14 KIF (27) identified a total of three alignments involving antiparallel molecules: A14, in which the adjacent molecules are staggered by about -17 nm so that their 1B rod domain segments are aligned next to each other; A22, in which the molecules are staggered by about 29 nm so that their 2B segments are aligned; and A44, in which the molecules are almost perfectly overlapped (stagger < 0.1 nm). A sufficient number of cross-links was obtained in the case of these KIF to deduce axial registrations and to calculate the likely lengths of the rod domain coiled-coil as well as non-coiled-coil linker segments. The A14 alignment mode occurs only in a three- or four-molecule KIF assembly, in which the third and fourth molecules are aligned in close axial registration with respect to their neighbors (25–27). Based on the axial parameters of these alignments in these KIF, a fourth alignment termed A23 was deduced, in which the last 5–10 residues of the 2B segment of one molecule overlap the first 5–10 residues of the 1A segment of a similarly directed molecule (25,27). Thus KIF consists of alternating antiparallel in-register and staggered rows of molecules. However, because of insufficient numbers of cross-links (23,24), it has not been possible yet to ascertain the similar axial parameters for the type III desmin IF. To date, there are only a few clues on how to build a three-dimensional model for IF. The two-dimensional surface lattice drawn from the alignments adduced in the above KIF cross-linking data require a discontinuity or packing irregularity along the axis in a KIF (26,27), and it has been suggested that this may serve as a seam along which KIF exchange molecules during development and differentiation, or disassemble and reassemble during phosphorylation-dependent cycling (26–28). Also, the size of this seam varies with the total numbers of molecules/unit molecule length in the various size polymorphs of IF (26,27).

The question as to whether the axial parameters adduced for K1/K10 and K5/K14 KIF are common to those of other IF types is important in terms of the higher levels of structure. Several different sorts of experiments have documented that cells can manipulate the different types of IF systems in different ways. When keratin or vimentin chains are microinjected singly or in combination into transformed cell lines that possess separate type I/II and type III IF networks, the proteins are sorted out and attached to the appropriate network and do not coassemble. On the other hand, the vimentin IF network of fibroblasts can assimilate transfected type IV neurofilament chains (29,30); that is, does this mean that type I/II keratin chains do not copolymerize with type III chains, yet type III chains can copolymerize with type IV (or VI) chains? The molecular basis of these phenomena is unknown. If the molecules are aligned differently and/or are of different axial dimensions, then it should be expected that the structures of the different IF systems will vary and that the various IF molecules should be incapable of copolymerization. To clarify these issues, we have now performed additional cross-linking experiments with type III vimentin IF and show that their molecular axial parameters are likely to be different from those deduced for KIF.

**Materials and Methods**

**Proteins**—Purified recombinant human vimentin expressed in bacteria and stored frozen as assembled IF at 1 mg/ml in phosphate-buffered saline, was a gift of Dr. Robert Goldman, Northwestern University Medical School. Human K5 and K14 were prepared from the basal cells of foreskin keratinocytes. Following separation of the epidermis by an overnight flotation on trypsin (31), the cells were plated for 4 h, during which time the basal cell population became attached to the plastic. These were then harvested and extracted to prepare the KIF cytoskeletons (10,21) highly enriched in K5 and K14. Following dissolution in SDS-gel buffer, the two keratin chains were preparatively isolated on a 5-mm-thick 7.5% polyacrylamide gel slab (12) usually freed of sodium, lithium, and SDS salts and then dried.

**IF Assembly in Vitro**—Vimentin was recovered by precipitation at pH 5, redissolved in 8 M urea (12), and reconstituted into 10 mM triethanolamine-HCl (pH 8.0) at 0.5 mg/ml containing 0, 10, or 150 mM KC1. In the high salt, IF assembly proceeds to completion; in the absence of salt, the vimentin exists in solution as a mixture of one- and two-molecule oligomers; in 10 mM salt, somewhat larger oligomers exist in solution (12) (see Fig. 1). K5/K14 KIF were assembled from equimolar mixtures by standard procedures (25–27). Equimolar mixtures of K14 and vimentin were mixed in 8 M urea buffer and reconstituted into 10 mM triethanolamine-HCl (pH 8.0).

**Cross-linking Procedures**—Cross-linking reactions were performed with the cleavable bifunctional reagent disulfosuccinimidyl tartrate (DST) (32) exactly as described previously (26,27). All reactions were performed for 30 min, and excess reagent was quenched with 0.1 M NH4HCO3. Initially, optimal conditions for cross-linking were determined with the amount of reagent varying between 0.1 and 1 mM. Reactions were assayed on 3.75–17.5% SDS-polyacrylamide gels to assess the degree of cross-linking. Chemical assays were also used to assess the degree of chemical modification of lysine side chains. Following cross-linking, protein was treated with 0.1 M iodoacetamide (35,5), which quantitatively modifies the one sulfhydryl group (to the S-carboxymethyl derivative) and all of the free ε-NH2 groups (to the N-carboxymethyl derivatives) (35). Amino acid analysis was then used to measure the amount of unmodified lysine; the DST adduct on single or cross-linked lysines is hydrolyzed back to free lysine, but the N-carboxymethyl derivative is not. Thus the amount of free lysine determined by amino acid analysis provides an estimate of the amount of DST adduct formation. These experiments established that 0.4 mM reagent gave the best yield of cross-linked products with the least possible chemical modification. Three different cross-linking reactions were done on vimentin protein in buffer without KCl, +10 mM KCl ("polymers"), or +150 mM KCl (IP). In the former, subsequent to reaction, the cross-linked proteins were recovered at pH 5, redissolved in SDS-gel electrophoresis buffer, and the one- and two-molecule species were isolated preparatively on a 3.0-mm-thick slab gel (29) and freed of glycine salts. Vimentin-K14 hybrid mixtures were also cross-linked with 0.4 mM DST, and the apparent two-molecule-sized oligomers, which were well resolved from the vimentin or K14 homotetramer-two molecule species (see Fig. 4), were similarly isolated by preparative gel electrophoresis. The dried proteins were resuspended in 70% formic acid (1 mg/ml), reacted with an equal weight of CNBr for 22 h at 23 °C, diluted 10-fold in water, and dried.

**Light Scattering Assays**—This was done by use of turbidity to measure the assembly of either vimentin IF or equimolar mixtures of K5/K14 KIF (0.5 ml) initiated by rapid removal of the urea buffer by passage through a 4 M KC1. Following dissolution in SDS-gel buffer, the two keratin chains were preparatively isolated on a 5-mm-thick 7.5% polyacrylamide gel slab (12) usually freed of sodium, lithium, and SDS salts and then dried. Assembly in vitro (10,22) in a Beckman DU-65 spectrophotometer at 310 nm at 1-min intervals for 1 h at 37 °C. The buffer used was 10 mM triethanolamine-HCl (pH 8.0) containing 1 mM EDTA and 1 mM dihydrogen orthophosphate. Vimentin assembly was initiated by the addition of 18 pl of 4 mM KCl (to 150 mM). KIF assembly was initiated by rapid removal of the urea buffer by passage through a 4 M KC1. Following dissolution in SDS-gel buffer, the two keratin chains were preparatively isolated on a 5-mm-thick 7.5% polyacrylamide gel slab (12) usually freed of sodium, lithium, and SDS salts and then dried. Assembly in vitro (10,22) in a Beckman DU-65 spectrophotometer at 310 nm at 1-min intervals for 1 h at 37 °C. The buffer used was 10 mM triethanolamine-HCl (pH 8.0) containing 1 mM EDTA and 1 mM dihydrogen orthophosphate. Vimentin assembly was initiated by the addition of 18 pl of 4 mM KCl (to 150 mM). KIF assembly was initiated by rapid removal of the urea buffer by passage through a
showed that IF assembly is not inhibited in the presence of 0.03-0.12 M urea.

Analysis of Cross-linked Peptides—The several DST-cross-linked products were digested to completion with trypsin (Sigma, bovine, sequencing grade) using 2% (w/w) enzyme for 6 h at 37 °C, cleaved with periodate, and the products recovered by HPLC.

In most cases, two well resolved peptide peaks, as well as certain minor peaks that apparently resulted from incomplete V8 digestion, were identified because they eluted well away from the elution time position of the intact cross-linked peptide. These peaks representing the "arms" of the cross-linked peptide were then recovered, dried, hydrolyzed in 5.7 N HCl in vacuo for 22 h, and their amino acid compositions determined on a Beckman 6300 analyzer.

Determination of Axial Parameters in Vimentin IF by Least Squares Fitting—Each of the characterized cross-links could be assigned by simple inspection (see Fig. 4; Ref. 26) to one of three modes of alignment in which two neighboring molecules are antiparallel: A11, in which their 1B rod domain segments are overlapped; A12, in which their rod domain 2 segments are overlapped; and A22, in which the entire molecules are largely overlapped. The degree of axial stagger between them is of the form Δ2(U,D), where U and D refer to up- and down-pointing molecules, respectively (26, 34-36). The numerical value of Δ2(U,D) can be calculated in terms of the positions of the 2 lysine residues, i1 and i2, involved in a cross-link between two protein chains, and has a value of i1 - i2 - 276 - L1 - L12 - L2 - 1, where L1, L12, and L2 are the axially projected lengths of the three linker segments measured in terms of h, the mean axial rise per residue in a coiled-coil (0.1485 nm) (26). The number 276 refers to the total number of residues in the four coiled-coil segments 1A, 1B, 2A, and 2B. The equation generated for each of 11 cross-links was then subjected to a least squares analysis in which the six unknown parameters, L1, L12, L2, A11, A22, and A12 were calculated (26).

RESULTS

Cross-linking of Vimentin Oligomers, Polymers, and IF with DST—Cross-linking of vimentin protein (0.5 mg/ml) in the a low ionic strength buffer of 10 mM triethanolamine-HCl (pH 8.0) with a range of concentrations of the bifunctional cleavable reagent DST shows that the reaction appears to be near complete in 30 min at 1-2 mM (Fig. 1). However, the appearance of a series of odd-numbered products indicates the occurrence of an unacceptable degree of cross-linking of random collision complexes above about 0.6 mM. With 0.4 mM reagent, densitometric analyses indicated that the reaction is about 50% complete, with about 10% of the protein as the two-chain or one-molecule species and about 30% of protein in a tetramer or two-molecule species, with trace amounts of protein in three- and perhaps four-molecule oligomers. The preponderance of the two-molecule species is expected from earlier data (19), although the present cross-linking experiments reveal the presence of minor but important amounts of one- and three-molecule species that previously could not be resolved on sucrose density gradients (19).

Using similar cross-linking conditions, vimentin protein in 10 or 150 mM KCl consists of mostly much larger species, too large to be resolved by the gradient polyacrylamide gel method utilized. In addition, titration experiments were performed in an attempt to quantitate the degree of chemical modification of the lysine side chains (Fig. 1B). There are 22 lysines/mol (55 kDa) of human vimentin (37). Following cross-linking of vimentin oligomers with 0.4 mM DST, the reactions were treated with iodoacetate at pH 9.5. Subsequent amino acid

![Fig. 1. Cross-linking of vimentin with DST. Panel A, 3.75-17.5% gradient polyacrylamide gel showing equal loads (2 μg) of cross-linked products of vimentin in solution in the presence of no KCl, 10 mM KCl or 150 mM KCl, as shown. In the former set, a range of DST concentrations was used as shown; at 0.4 mM DST, about half of the total protein exists as the one- and two-molecule oligomers. In experiments in which KCl was included in the assembly reaction, only 0.4 mM DST was used. Periodate treatment with 0.1 M sodium periodate. Markers consist of the mouse K10 chain cross-linked by glutaraldehyde (22), so that 1 = monomer, 2 = dimer, 3 = trimer, etc. Panel B, titration of DST-treated vimentin in the absence of KCl with iodoacetate at pH 9.5 provides an estimate of the degree of ε-NH₂ side chain modification. Amino acid analysis was then used to measure the amount of free lysine, which corresponds to the degree of reaction with DST.](image-url)
analyses revealed that there are 2.4 adducts/mol, corresponding to 11% ε-NH₂ lysine side chain modification; this compares with 18.9 and 20.3 adducts/mol at 1 or 2 mM DST, respectively. Accordingly, in view of the good yield of cross-linked products and relatively low degree of modification, 0.4 mM reagent was used routinely in this work. However, reaction of vimentin polymers or IF with 0.4 mM DST resulted in considerably more modification (4.2 and 7.2 adducts/mol, respectively).

Assembly Competence of Cross-linked Vimentin — When examined in an electron microscope following negative staining, vimentin forms long IF at 150 mM KCl, but only short subfilamentous structures at 0 or 10 mM KCl, as expected (Fig. 2, A–C). The one- and two-molecule cross-linked species obtained in a reaction in 0 mM KCl with 0.4 mM reagent were also examined. The resulting IF were somewhat shorter in length but more variable in width, ranging from 10 to 15 nm in diameter (Fig. 2, D and E), and thus were similar to unmodified vimentin IF. However, the two-molecule oligomer obtained by cross-linking with 1 mM reagent was not assembly-competent (Fig. 2F), indicating that the high degree of chemical modification interfered with normal assembly in vitro. In a similar experiment, vimentin IF were cross-linked with either 0.4 or 1 mM reagent, precipitated at pH 5, and redissolved in urea buffer. Following equilibration into buffer containing 150 mM KCl, short IF of 10-nm diameter were assembled with the former, but no IF structures were formed in the latter (Fig. 2, G and H). Together, these data provide strong support for the view that 0.4 mM reagent provides the most useful degree of reaction and, further, that the cross-linked oligomers are likely to assume a functionally appropriate conformation so that the positions of cross-linked lysines in them are likely to yield structurally relevant information.

Analysis of Lysine-Lysine Cross-Links — Fig. 3 shows the fractionation profiles of CNBr/tryptic peptides recovered from cross-linking reactions of the one- and two-molecule vimentin oligomers, as well as of the larger vimentin polymers formed at 10 mM KCl and of vimentin IF formed with 150 mM KCl. The peptide peaks that disappeared on treatment with periodate, and are thus candidate cross-linked species, are highlighted. There is a general shift of mass from a number of poorly resolved peaks that eluted early in the HPLC run, corresponding to mixtures of short polar peptides, to a number of well resolved new peaks that eluted at later times, corresponding to species of increased mass. Altogether, 38 peaks were recovered and purified. Each was digested with V8 protease, cleaved with periodate, and the peptide arms were isolated and their amino acid compositions determined. Most of the V8 peptides were quite short (generally < 10 residues) and obtained in substantial yield so that their simple amino acid compositions permitted unambiguous identification of the peptide along the vimentin chain of known sequence (37). In several cases, only one peptide arm was recovered, indicating an interchain cross-link involving the same lysine residue; this was particularly evident in the one-molecule species, as expected, since the fundamental coiled-coil molecule of vimentin IF is a homodimer (12). In this way, 38 cross-linked peptides were positively identified and consisted of 16 unique species (Table I). Many cross-links involving the same lysines were recovered in two or more of the reactions explored in this study, which provides strong evidence for the validity of the structural information contained in the data. Furthermore, three of the cross-linked species discovered here for vimentin, peptides 9, 11, and 13, have been found previously in experiments with the type III IF protein desmin, using the same (24) or different (23) cross-linking reagents and conditions, suggesting that vimentin and desmin IF are assembled from similar molecular alignments. We also noted that certain lysine residues were often cross-linked to multiple partners in different intra- or intermolecular alignments. This is particularly evident for position 2A-19 (found four times, a total of about 1.9 mol of cross-link/110 kDa of vimentin molecule). Several other commonly reacted positions found here in vimentin have also been reported for Kl/K10 and K 5/K14 IF (26, 27), including positions 1B-89/1B-90, 2B-23, 2B-83, and 2B-112. These may be key residues involved in defining higher orders of IF structure.

Of the 16 unique cross-links, five could be assigned to the intramolecular alignment of parallel in-register chains (Fig. 4, far left model), and 11 could be assigned to one of three modes of intermolecular alignment: the A₁₁ (6 cross-links, Fig. 4, second model from left) and A₁₂ (3 cross-links, Fig. 4, center model) modes suggest antiparallel staggered alignments at the two molecule level, or in vimentin polymers and IF; and the A₁₂ (2 cross-links, Fig. 4, second model from right) mode, which suggests an antiparallel alignment in near registration, occurred only in the vimentin polymers and IF. Significantly, these three types of alignment have been identified in earlier experiments with KIF (25, 26) and two (A₁₁ and A₁₂) with desmin IF (23, 24).

Calculation of Rod Axial Alignments and Linker Segments in Vimentin IF — Using the 11 unique intermolecular cross-links, separate equations were derived which relate directly to the positions of the lysines in adjacent molecules (Table II).
The equations are expressed in terms of six parameters ($A_{11}$, $A_{22}$, $A_{12}$, $L_1$, $L_{12}$, and $L_2$), each of which may be refined by use of standard least squares fitting methods. Although the ratio of equations to parameters is not high, the positions of the lysine residues involved in cross-link formation in vimentin oligomers is most fortuitous since the 11 original equations represent eight uncorrelated expressions. This is the same number as found previously in the analogous studies of the K1/K10 oligomers in which the raw number (21) of characterized cross-links was much higher than for vimentin. Furthermore, in the case of vimentin, many of the cross-links have at least one partner in or at a position that is close to the end of one of the three rod domain link segments (that is, L1-1, L1-5; L12-15; 2A-19, 2B-2). This provides better constraint on the lengths of the link segments than was possible for KIF, especially for L12. As with previous least squares analyses on keratin cross-links, the assumption has been made that the axial rise per residue in any particular link segment was constant. The optimal values after refinement are listed in Table III. These values are expressed in terms of $h_{\text{L}}$, which is equivalent to 0.1485 nm. Because the calculated axial values for the linker segments are significantly less than those predicted for KIF (Table III), the vimentin molecule length is substantially shorter (43.9 nm versus 46.2 nm). Furthermore, the axial staggers for the $A_{11}$ and $A_{22}$ alignment modes are significantly different from those in KIF, by an amount corresponding to approximately 19 and 37 residues, respectively; that is, the axial staggers between two molecules in vimentin and KIF are not conserved. The axial repeat length, determined from the staggers of $A_{11}$ and $A_{22}$, is 42.71 nm, which is 1.2 nm less than the molecule length. As seen for KIF, this results in a head-to-tail overlap of about 8 residues in a fourth mode of molecular alignment we have termed $A_{CN}$ (Fig. 4, far right model), and the extent of this overlap is remarkably similar to that adduced for the KIF (26, 27) (Table III).

Formation and Properties of Vimentin-K14 Hybrid Tetramers—The above data suggest that both the molecule length and axial staggers of neighboring molecules of vimentin IF are different from those in KIF. The following experiments were designed to test whether indeed a type I or type II keratin chain can form copolymers with a type III vimentin chain, as suggested in previous work (38). Purified human K14 and vimentin were mixed in 10 mM triethanolamine-HCl (pH 8.0) and cross-linked with 0.4 mM DST. Three different size species were found at each of the dimer and tetramer size products, compatible with one- or two-molecule oligomers, as well as a series of larger products (Fig. 5). As demonstrated in Fig. 1A, the major tetramer product of the reaction is likely to be the two-molecule vimentin species; thus the smaller tetramer product is likely to be a K14 two-molecule oligomer (10) and the middle-sized product a hybrid of vimentin and K14. This was confirmed by amino acid analysis; the upper band contained 3% glycine and 0.2% cysteine, consistent with vimentin only (37); the lower band contained 10% glycine and 1% cysteine, consistent with K14 only (39); and the middle species contained 7% glycine and 0.5% cysteine, consistent with a 1:1 mixture of the two proteins in the cross-linked tetramer product.

![HPLC fractionation of cross-linked peptides of vimentin](image)

**FIG. 3.** HPLC fractionation of cross-linked peptides of vimentin. The profiles are of CNBr/tryptic peptides of unreacted vimentin (panel A); one-molecule (panel B) and two-molecule (panel C) cross-linked oligomers; vimentin polymers assembled in the presence of 10 mM KC1 (panel D); and intact vimentin IF (panel E). The profiles of reactions B–D following cleavage of the cross-links by periodate were identical to profile A. Comparisons of profile A with profiles B–D revealed shifted peaks that were chosen as candidate cross-linked peptides that were harvested for further analysis (black). These peaks are numbered as in Table I. The broken line shows the acetonitrile gradient.
## TABLE I

### Cross-links in human vimentin oligomers, polymers, and IF

| Cross-linkage and heptad position | Sequence | Size of oligomer | Yield (mol/110 kDa) |
|----------------------------------|----------|-----------------|---------------------|
| **Intramolecular cross-links**    |          |                 |                    |
| 1. 2A-19 g                       | qqqyeSVAAKNLQEAeeewyk | 1,2,P,F          | 0.70, 1.51, 0.93, 1.56 |
| 2. L1-1                          | iliaeleQLKGGQQK | 1,2,P          | 0.43, 0.26, 0.55        |
| 3. 1B-42 g                       | EKLQEEem | 1,2,P,F | 0.14, 0.19, 0.22, 0.23          |
| 4. 1A-1 e                        | tneEVELEQEmndr | 1,2,P,F | 0.11, 0.26, 0.11, 0.26          |
| 5. L12-15                        | indeeielqiaqiqeqdqvdvdVSDKPDLtaalr | 1,2,P,F | 0.05, 0.06, 0.18, 0.16          |
| **Intermolecular cross-links in alignment mode A** | | | |
| 6. L1-5                          | GQGKSR | 2 | 0.08 |
| 7. L1-1                          | iliaeleQLKGGQQK | 2,P,F | 0.31, 0.16, 0.59 |
| 8. 1B-42 g                       | EKLQEEemlqr | 2,P,F | 0.19, 0.22, 0.56 |
| 9. 1A-17 g                       | FANYIDKVR | 2,F | 0.11, 0.33 |
| 10. L1-1                         | iliaeleQLKGGQQK | 2 | 0.11 |
| 11. 1A-17 e                      | KKLHDEeiqelqaqiqeqdvqddvSDKPDLtaalr | P | 0.17 |
| 2A-19 g                          | qqqyeSVAAKNLQEAeeewyk | | |
| **Intermolecular cross-links in alignment mode A** | | | |
| 12. 2B-23 e                      | QAKQEsneyr | 2,F | 0.37, 0.72 |
| 13. 2B-83f                       | lqdeIQAEMKeem | 2,F | 0.31, 0.61 |
| 14. 2B-2 e                       | nlqeaEWDKSK | F | 0.39 |
| 15. 2B-100h                       | eyqdlLNVLALDIEmtyr | P,F | 0.17, 0.39 |
| **Intermolecular cross-links in alignment mode A** | | | |
| 15. L1-5                          | GQGKSM | P,F | 0.14 |
| 16. 1B-90 f                       | KKLHDEeiqelqaqiqeqdvqddvSDKPDLtaalr | F | 0.14 |

*Heptad positions are indicated in italics.

Sequences identified by amino acid analyses are shown in uppercase letters; presumed sequences of the entire CNBr/trypsin peptide as isolated in the cross-link are shown in lowercase letters. Methionine residues at the end of peptides 3, 12, and 15 (underlined) were identified as homoserine.

1, 2, P, and F refer to one-molecule, two-molecule, polymers, and vimentin IF.

We noted two conservative sequence polymorphisms with respect to the published sequence of human vimentin: residue 2B-81 is an alanine instead of asparagine; residue 2B-101 is a leucine instead of methionine.

Following isolation by preparative gel electrophoresis, the cross-linked hybrid tetramer band was digested with CNBr and trypsin and resolved by HPLC. Subsequent cleavage with periodate revealed many minor peaks containing candidate cross-links between the K14 and vimentin chains (data not shown), of which 13 were isolated and partially characterized exactly as above (Table IV). Six peptides are likely to have formed between a K14-vimentin heterodimer, since lysines in the same residue positions on rod domain segments of the two chains were cross-linked together. Thus the coiled-coil rod domain segments of the two chain types may have become associated in an in-register alignment of parallel chains to...
Vimentin Intermediate Filament Structure

**Fig. 4.** Molecular alignments adduced in vimentin by DST cross-linking. Far left drawing, five cross-links were induced between two parallel in-register chains, which form the fundamental two-chain coiled-coil homodimer molecule for vimentin IF. A11 model, six cross-links were found to join two antiparallel molecules aligned in the A11 mode so that the 1B rod domain segments are largely overlapped. Two of these cross-links were found in experiments with desmin IF (23, 24). A12 model, three cross-links were found to join two antiparallel molecules in the A12 mode in which the 2B rod domain segments are largely overlapped, one of which was also found in desmin IF (23, 24). A22 model, two other cross-links were found to join two antiparallel molecules aligned in the A22 mode in which the two molecules are aligned almost exactly in register. Each of these modes was predicted for IF (35, 36), and they have been confirmed for Kl/K10 (26) and K5/K14 (27) KIF. However, the axial alignments described here for vimentin IF are different from those seen in KIF (Table III). A22 mode, when the A11 and A22 modes of alignment are directly compared, it is apparent that the end of the 2B rod domain segment of one molecule overlaps with the beginning of the 1A rod domain segment of a similarly directed molecule; this overlap defines a fourth mode of alignment, termed ACN.

**Table II**
Unique vimentin cross-links and equations used in the least squares analyses

The equations are derived, for example, as follows: 2B-83/L1-5 is given by (1 × A12) + (0 × A11) + (0 × A22) = (0.625 × L1) + (0 × L12) + (0 × L2) = −4 residues.

| Cross-link       | Mode | Value |
|------------------|------|-------|
| 2B-83/L1-5       | A12  | 1.0  0.625 0 0 -4 |
| 1B-90/2A-19      | A11  | 0 0 -1 |
| 1A-17/L12-15     | A22  | 0 0 0.063 |
| 1A-1/2A-19       | L1   | 0 0 0 |
| 1B-89/L1-5       | L12  | 0 0 -0.625 |
| 1B-89/L1-1       | L2   | 1 0 -0.125 |
| 1B-90/L1-1       | L12  | 1 0 -0.125 |
| 1B-42/1B-42      | L2   | 1 0 -1 |
| 2A-19/2B-112     | L12  | 0 0 1 1 |
| 2B-83/2B-23      | L2   | 0 0 1 -1 |
| 2B-2/2B-100      | L12  | 0 0 1 -1 |

**Table III**
Calculation of axial stagger in vimentin IF from cross-link data, and comparison with similar data from K5/K14 (Ref. 27) and K1/K10 KIF (Ref. 26)

| Structural feature | Vimentin Residues* | KIF Residues* |
|--------------------|--------------------|---------------|
| Molecular length   | 295.72             | 311.27        |
| Axial repeat       | 43.91              | 46.22         |
| Overlap (ACN)      | 8.09               | 6.83          |
| Length of L1       | 5.24 ± 0.41        | 0.78 ± 0.06   |
| Length of L12      | 6.06 ± 0.35        | 0.90 ± 0.05   |
| Length of L2       | 8.41 ± 0.30        | 1.25 ± 0.04   |
| A12                | −0.45 ± 0.40       | 0.43 ± 0.06   |
| A22                | −131.05 ± 0.38     | −19.46 ± 0.06 |
| A12                | 156.58 ± 0.76      | 23.25 ± 0.11  |
| Δz(1BU,1BD)        | −16.81             | −2.50         |
| Δz(2BU,2BD)        | −18.14             | −2.69         |
| Δz(1BU,2D)         | −40.69             | −6.04         |

*Measured in multiples of the mean axial rise in a coiled-coil conformation, h, (=0.1485 nm).

**Fig. 5.** Formation of vimentin-K14 hybrid molecules. A 3.75–17.5% gradient polyacrylamide gel of: lane 1, an equimolar mixture of vimentin and human K14 chains; lane 2, same, following cross-linking with 0.4 mM DST; lane 3, same as lane 2 but treated with 0.1 M sodium periodate. Markers are as in Fig. 1. The three tetramer species were individually eluted and analyzed to show that the central tetramer species (arrowhead) is a vimentin-K14 copolymer.
Cross-linked two-molecule vimentin species was added that would allow IF assembly to occur. 

Vimentin/K14 heterodimers is unlikely to represent a state and a cross-linked vimentin-K14 heterotetramer.

Hence the observed A12-like mode seen here for a pair of

Panel A, additions at 10 or 20 min are as in

aliquots added at 20 min included a K5/K14 mixture

of urea and allowed to assemble

(0.2;1 molar ratio of a K5/K14 mixture or the K14-vimentin hybrid tetramer to the vimentin did not increase the reaction rate. The addition of the same amount of hybrid tetramers at a later time not only halted further increases in IF assembly, but also apparently poisoned the reaction and caused disassembly instead. Assembly experiments were performed with K5/K14 KIF, with similar results (Fig. 6B); in comparison with controls, the hybrid tetramers failed to accelerate the reaction rate with increased protein concentration and also promoted KIF disassembly.

We have reported previously (38) that type III vimentin or desmin chains can copolymerize with a fraction of mouse epidermal keratin, which contains a mixture of the mouse K5, K6 (both type II), and type I K10/11 chains. We have repeated this work and documented by electron microscopy that IF are formed in such mixtures (data not shown). However, by use of amino acid analysis, it was clear from the glycine and cysteine contents that the IF formed on repolymerization are composed of type I/type II KIF and not type I/type III or type II/III copolymers.

Taken together, these data show that although type I/type III hybrid coiled-coil molecules can be formed in vitro, they are incapable of assembly into higher order structures.

**DISCUSSION**

**Packing of Molecules in Type III IF is Different from Type I/Type II KIF**—An extensive body of data has now established that IF are built from common structural principles, based on the conserved organization of their constituent protein chains and the conserved distributions of charged residues on the coiled-coil molecules they form. Nevertheless, there are significant variations in the sequences and properties of the different types of IF chains, so that it is less clear whether the molecules will pack in exactly the same way in the different types of IF. Indeed, indirect data suggest that there may be variations; the different chain types have different organizations of end domain sequences which to some extent govern the alignment of neighboring molecules, assembly and/or stability of IF: certain combinations of IF chain types do not coassemble in vitro in transfection or microinjection experiments, or when mixed in vitro; and differentiated cells can manipulate their various types of IF in different ways.

To clarify these issues, we have undertaken a detailed crosslinking study to ascertain as accurately as possible the possible axial dimensions and axial alignments of molecules in type III vimentin IF. These studies have confirmed and extended earlier cross-linking studies on type III desmin IF (23, 24). Our data reveal that vimentin molecules are likely to be significantly shorter than keratin molecules, largely because the vimentin linker sequences are less extended. Further, it is clear that although KIF and vimentin IF share common nearest neighbor molecule alignment modes (A_{11}, A_{22}, A_{12}), the precise dimensions of these alignments are different (Table III). The amount of stagger between the two molecules in either an A_{11} or A_{22} alignment is different in vimentin, so that in comparison with keratin molecules, they are displaced by about two to four additional heptads with respect to each other. However, measurement of the repeat length reveals...
that similarly directed vimentin molecules are overlapped (A_C) by approximately the same extent as for keratin molecules, so that the last 5–10 residues of the 2B rod domain segment overlap the first 5–10 residues of the 1A rod domain segment. The net result is that staggers between overlapped pairs of coiled-coil segments in vimentin molecules are significantly different from those in keratin molecules. Thus a two-dimensional surface lattice for vimentin is likely to be different from that proposed for keratin (Fig. 7). Nevertheless, certain other features of the lattice are in common with KIF. Assuming that the width of vimentin IF also consists of about 16 molecules/unit molecule length, based on quantitative electron microscopy methods (7), then the lattice will not close when folded, leaving a discontinuity spiralling along the axis of the filament. The exact dimensions of this seam will depend on the numbers of molecules in cross-section. We have postulated previously that IF may engage in dynamic exchanges of molecules along this discontinuity (26, 27). A second important feature of this surface lattice model is the existence of a repeat of 21.4 nm, corresponding to half of the axial repeat, which is significantly less than the 22.6-nm repeat of KIF (Table III). Indeed, our model now provides direct experimental confirmation that earlier measurements of axial repeats of shadowed type III IF (21 nm) (41, 42) and type I/II KIF (22.7 nm) (7) are different.

**Fig. 7. Two-dimensional surface lattice model for vimentin IF.** To conform with predicted mass measurements (7), this consists of 16 molecules in cross-section so that the model consists of alternating rows of antiparallel in-register (A_1) and antiparallel staggered (A_2) molecules. The next axial row begins with a 17th molecule, denoted here as 1'. There is a head-to-tail overlap of about 1 nm between similarly directed molecules (hatched throughout). A filament would have an axial repeat of 42.7 nm with equivalent points at axial intervals of 21.4 nm (heavy parallel diagonal lines), interrupted at the dislocation. The second diagonal line can be drawn across the lattice by arbitrarily connecting the same point on molecules and has a dislocation between molecules 16 and 1' denoted between the arrows. Interestingly, in a 16-molecule IF, the discontinuity will almost match when this lattice is folded in three dimensions. If there are fewer or more molecules, the discontinuity will vary in size (26).
chains (43–45) can occur because the KIF molecules possess the same axial parameters and can pack the same way; and (ii) that since type III vimentin, the type IV α-intermediate and NF-L chains, and type VI nestin chains can coassemble during neuronal cell differentiation in vivo (15), their molecules should also possess similar axial parameters, which are different from KIF. Further work will be necessary to test this hypothesis. Moreover, these incompatibilities in axial alignments may explain why, at least in part, certain cells have developed elaborate and separate type I/type II KIF and type III IF networks and regulate them differently (1).

We have demonstrated in this paper that many of the features of vimentin IF are similar to those of KIF. This is perhaps not surprising since the distributions of ionic charges along the various rod domain segments are well conserved between type III and type I/II KIF (4, 5), although minor differences in linker segment lengths are likely to change the exact packing dimensions. However, KIF and vimentin IF differ in a major way in the degrees of lateral stagger of neighboring molecules. Thus certain rod domain and/or end domain sequences that govern lateral packing of two molecules with respect to each other must be subtly different in the two systems. Previously, we have observed that the H1 (and possibly H2) end domain sequences immediately flanking the beginning and end of the rod domain of the type II K1 chain of a keratin heterodimer molecule are involved in determining the lateral registration of nearest neighbor keratin molecules (25). Sequences identical to the H1 region of type II keratin chains are not present on vimentin chains, but sequences that nevertheless should have equivalent functions on vimentin chains must now be identified.