The Structure of Vimentin Linker 1 and Rod 1B Domains Characterized by Site-directed Spin-labeling Electron Paramagnetic Resonance (SDSL-EPR) and X-ray Crystallography*

Received for publication, December 19, 2011, and in revised form, May 30, 2012. Published, JBC Papers in Press, June 26, 2012, DOI 10.1074/jbc.M111.334011

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Background: The complete structure is not known for any intermediate filament (IF) protein.

Results: Linker 1 and rod 1B in human vimentin were characterized using electron paramagnetic resonance spectroscopy and x-ray crystallography.

Conclusion: The rod 1B adopts two functional conformations that mediate formation of an anti-parallel “A11” tetramer.

Significance: Understanding vimentin structure provides insight into all IFs and the related human pathologies.

Despite the passage of ~30 years since the complete primary sequence of the intermediate filament (IF) protein vimentin was reported, the structure remains unknown for both an individual protomer and the assembled filament. In this report, we present data describing the structure of vimentin linker 1 (L1) and rod 1B. Electron paramagnetic resonance spectra collected from samples bearing site-directed spin labels demonstrate that L1 is not a flexible segment between coiled-coils (CCs) but instead forms a rigid, tightly packed structure. An x-ray crystal structure of a construct containing L1 and rod 1B shows that it forms a tetramer comprising two equivalent parallel CC dimers that interact with one another in the form of a symmetrical antiparallel dimer. Remarkably, the parallel CC dimers are themselves asymmetrical, which enables them to tetramerize rather than undergoing higher order oligomerization. This functionally vital asymmetry in the CC structure, encoded in the primary sequence of rod 1B, provides a striking example of evolutionary exploitation of the structural plasticity of proteins. EPR and crystallographic data consistently suggest that a very short region within L1 represents a minor local distortion in what is likely to be a continuous CC from the end of rod 1A through the entirety of rod 1B. The concordance of this structural model with previously published cross-linking and spectral data supports the conclusion that the crystallographic oligomer represents a native biological structure.

Three different protein families make up the filamentous cytoskeleton of the mammalian cell cytoplasm: thin filaments composed of actins, microtubules composed of tubulins, and intermediate filaments (IFs) composed of intermediate filament proteins. In contrast to the small number of actin and tubulin proteins, the IF family is composed of scores of members, expressed in both tissue-specific and differentiation-specific patterns (1, 2). Further emphasizing their diversity, IFs can be either homopolymeric or heteropolymeric, but the majority of IFs are constructed from one or two IF proteins. IF proteins are not soluble under physiologic conditions, and they spontaneously assemble into IFs upon dialysis from chaotropes, such as 8 M urea (3, 4). Thus, it is not surprising that whereas the x-ray crystal structures of actin and tubulin are known (5–7), no complete IF protein has had its structure solved by crystallographic or other means. The general predictions of IF protein structure, deduced from their amino acid sequences based on cDNA sequences that began to emerge in the 1980s, have only recently been subject to revision based on both spectroscopic and crystallographic structural data.

Analysis of IF protein sequences has consistently shown that the central region is composed of amino acids conforming to a heptad repeat pattern (a-b-c-d-e-f-g) with non-polar amino

* This work was supported, in whole or in part, by National Institutes of Health (NIH) Grant EY015560 (to P. G. F.); NEI, NIH, Core Facilities Grant P30-EYO12576 (to the University of California Davis); NIH Grant AG029246 (to J. C. V.), and NIH Grant U54-GM094597 (to G. T. M., J. F. H., and the Northeast Structural Genomics Consortium). Work at the University of California Davis was conducted in a facility constructed with support from Research Facilities Improvement Program Grant C06 RR-12088–01 from the National Center for Research Resources, NIH.

The abbreviations used are: IF, intermediate filament; SDSL-EPR, site-directed spin-labeling electron paramagnetic resonance; CC, coiled-coil.

§ The atomic coordinates and structure factors (code 3UF1) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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acids preferentially located at the a and d positions (8–10). From this simple pattern, an α-helical coiled-coil (CC) structure (11) was predicted to form. Based on this pattern and prediction, the middle of all IF proteins became known as the central rod domain; head and tail domains that vary widely in both size and primary sequence are located at either end of the central rod (12–15). Short but highly conserved motifs are present at each end of the central rod domain, the “helix initiation motif” at the N terminus and the “helix termination motif” at the C terminus (16, 17). Using site-directed spin-labeling electron paramagnetic resonance (SDSL-EPR) spectroscopy, Aziz et al. (18) recently generated the first insights into the structural organization of the head and tail domains of an IF protein.

In silico analysis of the central rod domain shows that the heptad repeat is interrupted by non-conforming amino acid sequences at three places (10, 19, 20). Thus, for the past 25 years, the central rod domain has been described as four α-helical CC domains (1A, 1B, 2A, and 2B) separated by small non-CC linker domains (linkers L1, L1-2, and L2) named by the regions they connect (1, 14, 15, 21). Originally, the predicted non-helical nature of linker secondary structure suggested that linkers served as flexible connectors between the CC domains. However, in contrast to the prediction that linkers are flexible, our SDSL-EPR characterization of linker L2 revealed a rigid, rapidly assembling structure in which both chains were aligned in parallel (21).

Despite widespread acceptance of the predicted structure of IFs, very little informative experimental data existed until the early 2000s. Using a “divide and conquer” strategy, Strelkov and co-workers (22–24) solved x-ray crystal structures for protein segments extracted from both the N- and C-terminal regions of the central rod domain of vimentin. Surprisingly, the resulting structure of the highly conserved rod 1A domain showed an α-helix but not a CC (Protein Data Bank entry 1GK7). However, based on the curvature of the observed α-helix, the authors predicted that the region does form a CC during the earliest stages of IF assembly (24).

At the other end of the rod domain, rod 2B was found to form a long α-helical CC (Protein Data Bank entry 1GK4). Near the middle of the structure, the evolutionarily conserved “stutter,” a 3-residue shift in the register of the hydrophobic heptad repeat pattern in the primary sequence in this region, was found to cause only a slight deviation from a canonical left-handed CC structure (24). One heptad earlier than the predicted C terminus of rod 2B, the CC-forming α-helices in this structure begin to separate, which was interpreted to represent the actual end of the CC region (22, 24). One of the parallel CC dimers in this structure (the AB dimer) deviates substantially from proper 2-fold rotational symmetry and interacts with another CC dimer (the EF dimer) in an anti-parallel geometry; this interaction buries extensive solvent-accessible surface area in the interface between CC dimers (2443 Å² per CC dimer in Protein Data Bank entry 1GK4). The resulting tetrameric bundle contains both parallel and anti-parallel α-helices. However, the third CC dimer in the asymmetric unit of this crystal structure (the CD dimer) does not make any similar interaction. Moreover, these x-ray crystal structures were not derived from intact IF proteins assembled into a filament, and thus it is possible that the observed structures and oligomeric organizations may differ from the dominant conformations in vivo, especially near the non-physiological termini of the crystallized protein constructs.

Perhaps the most surprising structural result reported to date is the recently solved crystal structure of a protein construct containing vimentin rod 2A and L2 (Protein Data Bank entry 3KL7) (25). Instead of folding into a short parallel CC as expected, the peptides form an anti-parallel tetramer composed of parallel dimers that adopt a standard CC structure at vimentin positions 305–334.

Based on this structure, the authors concluded that the previously predicted rod 2A and L2 regions exist as a single pair of parallel helices in the assembled filament and not a CC structure. This conclusion supports a hypothesis advanced by David Parry (26) that the amino acid sequences of rod 2A and L2 conform better to a right-handed CC structure with a hendecad (11-residue) repeat rather than a canonical left-handed CC structure with a heptad repeat. However, the aforementioned crystal structure of rod 2A and L2 (25) shows that, near the region previously predicted to be the beginning of coil 2B (residues 290–300), the parallel α-helices gradually adopt a canonical left-handed CC structure with a heptad repeat. The CC structure begins over positions 302–305 and extends through the end of the crystallized construct at residue 334 (25). The final seven residues in this construct overlap the CC region observed in the earlier crystal structure of rod 2B, providing evidence that the parallel CC in this region of human vimentin extends continuously from residue 305 through 405. Therefore, the most recent crystallographic data confirm our previous conclusions from SDSL-EPR that vimentin adopts a non-CC structure between residues 291 and 301 (21). Thus, independently, the available SDSL-EPR and crystallographic data consistently show that residues 291–302 are not assembled into a CC structure, contrary to earlier predictions.

The structures of the predicted non-CC regions L1 and L1-2, which precede and follow rod 1B, have not been described by any method. Our prior SDSL-EPR data support a CC structure in at least part of rod 1A and rod 1B, which flank the L1 segment (27, 28). In this report, we present SDSL-EPR and crystallographic analyses of protein constructs containing the end of L1, the beginning of rod 1B, and most, if not all, of rod 1B. Collectively, these data demonstrate that the non-CC region of L1 is likely to be shorter than predicted (29), whereas rod 1A and 1B form one continuous left-handed CC structure with the L1 segment representing only short perturbation. Our crystal structure demonstrates that rod 1B forms a parallel CC, which dimerizes with itself in an anti-parallel geometry to form a tetrameric α-helical bundle that is likely to be a key feature of higher order vimentin oligomerization and IF structure in vivo. This interaction is centered near residue 191, as predicted by our prior EPR studies.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis and Purification for Spin Labeling—**

Vimentin mutants were constructed using site-directed mutagenesis, and recombinant proteins were produced in Escherichia coli. Vimentin readily forms inclusion bodies; these
were isolated, and the recombinant vimentin was purified and spin-labeled as described in detail previously (18, 21, 27, 28). In short, site-directed mutagenesis was used to introduce cysteine residues at specific sites in a vimentin expression construct (originally provided by Roy Quinlan (University of Durham, Durham, UK)) using Bio-Rad iProof DNA polymerase and mutagenic oligonucleotides. Coding sequence changes were confirmed by automated DNA sequencing. Mutant vimentin protein was produced by bacterial overexpression using a pT7 vector and E. coli BL21AI (Invitrogen). Inclusion bodies were purified using lysozyme/DNase, high/low salt washes, and chromatography (AKTA FPLC, GE Healthcare). Site-directed spin labeling was performed by first treating the purified protein with 100 μM tris-(2-carboxyethyl)phosphine hydrochloride (Invitrogen) followed by spin labeling with 500 μM O-87500 (1-oxyl-2,2,5,5-tetramethyl-3-pyrroline-3-methylmethanethiosulfonate-d15, Toronto Research Chemicals). The unincorporated label was separated from spin-labeled protein by chromatography over a Source S column (AKTA FPLC). Protein concentrations were measured by the BCA method (Pierce). Purified spin-labeled proteins were stored at −80 °C.

In Vitro Filament Assembly and Electron Microscopy—Filament assembly was generally conducted by dialyzing the spin-labeled protein from 8 M urea into filament assembly buffer (10 mM Tris, pH 7.5, 160 mM NaCl) overnight at 37 °C. Electron microscopy of the negatively stained samples was performed to verify the filament assembly of each mutant. Following dialysis, 10 μl of the sample was removed and stained with 1% uranyl acetate on Formvar-coated carbon grids and then observed using a Phillips CM-120 electron microscope with a Biotwin Lens (FEI Co. (Hillsboro, OR); made in Eindhoven, Netherlands) operated at an 80-kV acceleration voltage. Images were acquired with a Gatan MegaScan 794/20 digital camera (2000 × 2000) or a Gatan BioScan 792 (Gatan, Pleasanton, CA).

EPR Spectroscopy of Site-directed Spin Labels—EPR measurements of the spin-labeled proteins were conducted on a JEOL X-band spectrometer fitted with a loop-gap resonator (18, 21, 27, 28). Spectra were collected from ~5–7 μl of purified, spin-labeled, dialyzed protein, at a final protein concentration of 25–100 μM, loaded in a sealed, quartz capillary tube. Spectra were obtained by a single scan of 120 s over 100 G at a microwave power of 4 milliwatts at room temperature (unless otherwise specified). Modulation amplitude (0.125 millitesla) was optimized to the natural line width of the attached nitroxide as described previously (18, 21, 27, 28). Normalization of the spectra to the same number of spins was done by normalizing each spectrum to the same integrated intensity/amplitude. To improve the fidelity of the calculation, each sample was double-integrated after its solubilization in 2% SDS. Low temperature spectra were collected from samples frozen at −100 °C. For increased low temperature sensitivity, samples in 5 mM Tris, pH 7.5, were mixed with the appropriate volume of 10× IF assembly buffer (100 mM Tris, pH 7.5, 1.6 M NaCl) in an Eppendorf tube. After 25 μl of the mixture was rapidly pipetted into a capillary, it was centrifuged in a low speed bench top centrifuge to collect the assembling filaments at the bottom. Mixing, pipetting, and spinning were repeated to generate two capillaries for each sample. Both capillaries were placed in the low temperature cavity for spectral data collection.

Protein Expression and Purification for Crystallography—The vimentin protein was targeted by the Northeast Structural Genomics Consortium as part of a project aimed at determining the three-dimensional structures of proteins involved in signaling networks associated with human cancer (30). Ligation-independent cloning was used to introduce a PCR product encoding residues 144–251 of human vimentin between the Ndel and Xhol sites in vector pET15_NESG to produce construct HR4796B-144-251-14.3, which is available from the PSI Materials Repository. The resulting protein with an N-terminal affinity purification tag with sequence MGHHHHHHHSHSM was expressed in E. coli BL21(DE3) cells harboring the rare tRNA expression plasmid pMGK. The cells were induced overnight at 17 °C using 1 mM IPTG after growth at 37 °C to midlog phase in M9 minimal medium (31) supplemented with selenomethionine and containing 10 μg/ml kanamycin and 100 μg/ml ampicillin. Cells were lysed by sonication in Binding Buffer (50 mM Tris-HCl, 500 mM NaCl, 40 mM imidazole, 1 mM tris-(2-carboxyethyl)phosphine hydrochloride, and 0.02% NaN3, pH 7.5) and centrifuged at 27,000 × g for 40 min at 4 °C. The supernatant was loaded onto an AKTAxpress purifier (GE Healthcare) for an automated two-step immobilized affinity chromatography and gel filtration purification procedure. Briefly, the His-tagged proteins were bound to a 5-ml HisTrap HP column (GE Healthcare) and eluted into an internal storage loop in 50 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole, 0.02% (w/v) NaN3, pH 7.5. The major peaks were automatically injected onto a Superdex 75 gel filtration column (GE Healthcare) equilibrated in low salt buffer (20 mM Tris-HCl, 100 mM NaCl, 5 mM DTT, pH 7.5). The peak fractions were concentrated to 10.6 mg/ml and frozen in liquid N2 in 50-μl aliquots. Per liter of culture, this procedure yielded 100 mg of purified protein, which had acovalent molecular mass of 14.4 kDa based on MALDI-TOF mass spectrometry on a Voyager DE Pro mass spectrometer (Applied Biosystems, Foster City, CA) compared with a theoretical value of 14.6 kDa (data not shown).

Analytical Gel Filtration and Static Light Scattering—A thawed aliquot of the concentration protein crystallization stock was subject to analytical gel filtration on a Shodex 802.5 column (Showa Denko, Tokyo, Japan) running at 4 °C in 100 mM NaCl, 0.025% (w/v) NaN3, 100 mM Tris-Cl, pH 7.5. The column eluant was monitored using static light scattering and refractive index detectors (miniDAWN™ TREOS and Optilab® T-rEX, Wyatt Technologies, Santa Barbara, CA).

Crystallization—Crystallization was performed in 1 + 1-μl hanging drop vapor diffusion reactions at 293 K. Crystallization conditions were identified via high throughput robotic screening of 1536 different conditions at the Hauptmann-Woodward Institute (Buffalo, NY) (32). Manual optimization yielded a final precipitant solution containing 18% (w/v) PEG 3350, 0.15 M ammonium sulfate, 0.1 M Tris-HCl, pH 8.0. Before flash freezing in liquid nitrogen, the crystals were cryoprotected using the well solution with ethylene glycol added to 15–20% (v/v).

X-ray Data Collection and Structure Determination—X-ray data were collected using a wavelength of 0.979 Å from a single crystal maintained at 100 K on beamline X4A at the National
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Synchrotron Light Source at Brookhaven National Laboratory. The diffraction images were integrated and merged using DENZO and SCALEPACK, respectively (33). The structure was solved by single wavelength anomalous dispersion using PHENIX (34). Selenium positions were found by AutoSol to generate an initial protein model. Manual rebuilding in COOT (35) was guided by inspection of 2Fo − Fc electron density maps. Protein models were refined with translation, libration, and screw rotation (TLS) displacement of a pseudo-rigid body (36). During the final stage of refinement, strong pairwise NCS restraints (coordinate sigma of 0.05 and B-factor weight of 10) were maintained between residues 146–245 in subunits A and C and also maintained between residues 146–248 in subunits B and D. The programs HELANAL (37) and TWISTER (38) were used to measure α-helix and CC geometry, respectively. Molecular graphics figures were prepared using PyMOL (39).

RESULTS

SDSL-EPR Analyses of L1 and the N Terminus of Rod 1B in Human Vimentin—Fig. 1 shows a schematic of the vimentin central rod domain, along with pertinent amino acid sequences from L1 and the flanking CCs. Boundaries are based on in silico predictions of IF secondary structure, which identified rod 1B as beginning at Gly147 and terminating at Ala1247 (29). Each end of rod 1B is flanked by a predicted non-helical linker region. Positions 139–146, including the helix-distabilizing residues Gly140 and Gly142, are described as linker 1 (L1). JPRED secondary structure analysis of positions 120–180 identifies positions 141–146 as non-α-helical and non-CC (not shown), whereas the remainder of the sequence is generally predicted to be both, by Jpred analysis (47). Similarly, JPRED analysis of the sequence of 181–250 predicts α-helical and CC structure at all positions. We performed SDSL-EPR at positions 146–168 spanning the predicted N terminus of the rod 1B CC. Consistent with our previous results, most of the resulting protein variants were able to form IFs after mutation to cysteine and attachment of the spin label, with exceptions noted in Table 1.

Fig. 2 shows the spectra obtained from vimentin variants spin-labeled at positions 146–168, after dialysis against low ionic strength Tris buffer at pH 7.5. Under these conditions, filaments are not formed, and the spectra report the local secondary, tertiary, and quaternary structure of the vimentin species formed in that environment. Qualitative inspection of the spectra in Fig. 2 shows that positions 146, 150, 154, 157, 158, 161, and 164 exhibit the characteristics of a or d residues in a heptad CC structure. These positions show extensively broadened spectra, consistent with the prediction that the corresponding spin labels in the partner vimentin chains are packed in close proximity at the CC interface. Comparing the shapes of the curves from the samples spin-labeled at positions 150–154, the spectra from the hypothesized d (150) and a (154) positions are readily identified as different from the spectra at positions 151, 152, and 153 (which represent e, f, and g heptad positions). This qualitative identification of a or d positions overlaps the predicted phasing of heptad repeats in this region: 147 a, 150 d, 154 a, 157 d, 161 a, and 164 d. Furthermore, this heptad phase is consistently in register with the phase we identified in earlier experiments at downstream positions in rod 1B (i.e. 171 d, 175 a, 178 d, etc.) (28). However, the spectra obtained at positions 146, 147, and 158 do not fit this simple pattern.

To evaluate whether the line broadening observed for the putative a and d positions is attributable to close proximity of

| Residue | WT aa | Heptad | dβ/d | IFs | ΔIFs |
|---------|-------|--------|------|-----|------|
| 140     | G     | f      | 0.37*| Y   | Y    |
| 141     | Q     | g      | 0.41*| Y   | Y    |
| 142     | G     | a      | 0.60*| Y   | Y    |
| 143     | K     | ?      | 0.43*| Y   | Y    |
| 144     | S     | ?      | 0.45*| Y   | Thin |
| 145     | R     | ?      | 0.38*| Y   | Y    |
| 146     | L     | ?      | 0.46  | Rare, ULFs | Y   |
| 147     | G     | a      | 0.53  | Y   | Y    |
| 148     | D     | b      | 0.4   | Y   | ND   |
| 149     | L     | c      | 0.41  | Y/N | ND   |
| 150     | Y     | d      | 0.57  | Short | ND  |
| 151     | E     | e      | 0.4   | Y   | ND   |
| 152     | E     | f      | 0.41  | N-1 | ND   |
| 153     | E     | g      | 0.39  | N   | ND   |
| 154     | M     | a      | 0.48  | Y   | ND   |
| 155     | R     | b      | 0.35  | Y   | ND   |
| 156     | E     | c      | 0.39  | Y   | ND   |
| 157     | L     | d      | 0.45  | N   | ND   |
| 158     | R     | e      | 0.38  | N   | ND   |
| 159     | R     | f      | 0.35  | Y   | ND   |
| 160     | Q     | g      | 0.37  | Y   | ND   |
| 161     | V     | a      | 0.44  | Y   | N    |
| 162     | D     | b      | 0.37  | Short | N   |
| 163     | Q     | c      | 0.38  | Short | ND  |
| 164     | L     | d      | 0.47  | Y   | ND   |
| 165     | T     | e      | 0.37  | Y   | ND   |
| 166     | N     | f      | 0.36  | Y   | ND   |
| 167     | D     | g      | 0.38  | Y   | ND   |
| 168     | K     | a      | 0.44  | Y   | ND   |

* Previously published (28).
these positions in the protein structure as opposed to restriction of the probe’s motion, we collected low temperature spectra and calculated $d_{1/d}$ values (see Table 1). This calculation provides a model-independent assessment of the proximity of spin-labeled side chains in a semiquantitative manner. As established previously, $a$ and $d$ positions typically produce values near 0.5, whereas all other positions in a heptad repeat CC typically produce values under 0.4.

The $d_{1/d}$ values calculated for the region 147–161 clearly show a heptad repeat pattern with consistent phase starting at residues 150 (d) and 154 (a). The $d_{1/d}$ value for residue 158 reveals a value inconsistent with a heptad $a$ or $d$ position, indicating that the broadened room-temperature spectrum at this site is not attributable to spin-spin interaction but rather to a very rigid molecular environment. On the other hand, the calculated $d_{1/d}$ value for position 146 is consistent with spin-spin interaction at this site, suggesting that this residue is likely to be packed in the intersubunit interface although it is not in proper register with the heptad repeat pattern shown by the immediately C-terminal residues in rod 1B. Moreover, the $d_{1/d}$ value observed at position 147 is slightly elevated compared with a typical heptad $a$ or $d$ position, which would be consistent with tight steric interactions at this site upon mutation of this Gly residue to a Cys residue and attachment of a spin label. These observations suggest that a departure from canonical CC geometry at residues 146 and 147 at the N terminus of rod 1B leads to close interhelical packing of both of these adjacent residues.

The crystallographic studies reported below support this interpretation.

Similar spectroscopic results were observed in our previously published SDSL-EPR study encompassing positions 120–145 at the C terminus of rod 1A. The final residue fitting the regular heptad repeat pattern was Gly$^{142}$, which was in register to be a heptad $a$ position at the C terminus of the rod 1A CC. A spin label at this position gave a significantly elevated $d_{1/d}$ value (0.6) compared with typical heptad $a$ or $d$ positions, just like Gly$^{147}$ at the N terminus of rod 1B in the current study. Furthermore, the observed $d_{1/d}$ ratios at positions 143–145 failed to fit a heptad repeat pattern, suggesting that the elevated $d_{1/d}$ value at position 142 reflected a departure from canonical CC geometry at the C terminus of rod 1A. Overall, the qualitative spectral results and $d_{1/d}$ values at residues 143–146 suggest that this L1 linker segment is a relatively rigid transition between the CCs formed by rods 1A and 1B. Although L1 is likely to be more flexible than the CC regions it connects, it is not disordered and instead seems likely to represent a short, stutter-like distortion in what is otherwise a very long and regular CC structure spanning rods 1A and 1B.

**Supporting Mutagenesis Studies of the L1 Region**—To provide further insight into the structure adopted by the L1 linker, we created various substitution, deletion, and insertion mutants, and we assayed the resulting proteins for filament assembly (Fig. 3). To investigate the hypothesis that the presence of glycines 140, 142, and 147 was evidence of a sterically constrained
local environment, we began by altering Gly\textsuperscript{142}. The G142A and G142F mutants were both able to assemble into IFs. The ability of the G142F mutant to assemble argues that L1 is not critically constrained and that the presence of Gly\textsuperscript{140}, Gly\textsuperscript{142}, and Gly\textsuperscript{147} is not solely the result of selection for a residue without a side chain.

A different approach to assess the packing and phasing of the residues that make up L1 was taken by individually deleting each residue from 140 to 147. These single amino acid deletions were well tolerated with minimal perturbations in IF assembly (Fig. 3 and Table 1) (additional data not shown). Surprisingly, even the double deletion mutant combining \textit{Gly140} and \textit{Gly147} also assembled into filaments. As controls for these deletion experiments, we deleted either Val\textsuperscript{161} or Asp\textsuperscript{162} within the canonical CC region of rod 1B (\textit{a} and \textit{b} heptad positions, respectively) and observed no IF assembly. Thus, deletion of residues at the end of rod 1A, in linker 1, or at the start of rod 1B produces strikingly different effects than deletion of residues within the CC region. The contrasting tolerance for deletions in the L1 region possibly supports the inference from SDSL-EPR spectroscopy that it does not adopt a canonical CC structure.

Finally, we attempted to extend the canonical heptad repeat pattern at the N terminus of rod 1B, but most such constructs interfered with filament assembly. To extend the interfacial hydrophobic stripe in proper register with the CC formed by rod 1B, we made a double mutant in which Leu\textsuperscript{146} is changed to Gly and Gly\textsuperscript{147} is changed to Leu. This L146G/G147L protein failed to form IFs (Fig. 3). Similar results were observed by Herrmann \textit{et al.} (46) in an attempt to form a continuous CC structure linking rod 1B to rod 1A; the addition of three properly phased aliphatic amino acids to L1 also prevented IF assembly. However, it is notable that IFs form after deletion of Gly\textsuperscript{147} (Fig. 3), which places Leu\textsuperscript{146} in an \textit{a} heptad position relative to the canonical CC that starts with Tyr\textsuperscript{150}, a \textit{d} position. Although further investigation will be required to understand the operative sequence constraints in L1, mutagenesis experiments in this region of the protein suggest that it does not adopt a canonical CC structure, consistent with the SDSL-EPR results presented above and the crystallographic results presented below.

\textbf{The Crystal Structure of Tetrameric Rod 1B from Human Vimentin}—A semiautomated procedure employing nickel-nitrilotriacetic acid affinity chromatography followed by gel filtration chromatography was used to purify a soluble rod 1B peptide containing residues 144–251 of human vimentin plus an 11-residue N-terminal affinity tag with sequence MGHHHHHHHHH. The concentrated protein stock solution was characterized using analytical gel filtration chromatography monitored by in-line refractive index and static light-scattering detectors (Fig. 4). In a buffer at pH 7.5 with \sim 100 mM ionic strength, the vast majority of the protein elutes as an isolated peak with a broad trailing edge. Debye analysis shows a molecular mass of 64–70 kDa at the top of this peak, which progres-
The crystal structure of the vimentin rod 1B peptide (Figs. 5–7 and Tables 2 and 3) shows a homotetrameric assembly matching the intersubunit interaction geometry predicted from our earlier solution EPR experiments (28). The crystal structure in space group P2_1_2_1_2_1 was refined at 2.8 Å resolution to working and free R-factors of 23.4 and 28.4%, respectively (Table 2). The atomic model contains residues 144–251 fragment of human vimentin.

The interfaces of the AB and CD dimers, and 144–248 in protomer D. In addition, the final five residues protomer A, 148–249 in protomer B, 144–246 in protomer C, and 144–249 in protomer D. The protein construct analyzed here, which produced the crystal structure analyzed in this paper, contains a 10-residue sequence of the rod 1B peptide, which produces a series of

The N-terminal segments of protomers C and D visualized in chains C and D. The N-terminal segments of protomers A and B (Fig. 6, and Table 2). Each protomer forms a single α-helix (e.g. as shown in Fig. 6), with a length varying from 151 to 161 Å (as measured by the distance between terminal Ca atoms). The single tetramer in the asymmetric unit of these crystals (Fig. 5, A and B) is likely to represent the dominant physiological oligomer of rod 1B, commonly known as the A11 tetramer (40, 41). The interfaces of the AB and CD dimers, which form asymmetrical parallel CCs (described in detail below), bury ~5400–5600 Å² of solvent-accessible surface area per dimer (Table 3), whereas ~4000 Å² is buried in the interfaces between the CC dimers in the likely physiological tetramer (the sums of the A-C, A-D, B-C, and B-D interfaces in Table 3). An initial refinement was performed in the absence of any non-crystallographic symmetry restraints. This refinement showed that the A and C subunits adopt very similar conformations (Fig. 6A) and that the B and D subunits also adopt very similar conformations (Fig. 6B) but that the A/C subunits adopt a significantly different conformation from the B/D subunits (Fig. 6C). Therefore, non-crystallographic symmetry restraints were applied selectively to the A/C and B/D subunit pairs to improve the convergence of the final refinement (Table 2).

The rod 1B tetramer comprises a symmetrical dimer formed by a pair of asymmetrical parallel α-helical CC dimers (Fig. 5). The A and B subunits form one of the two CC dimers in the tetramer, whereas the C and D subunits form the other. Least squares superposition of these CC dimers demonstrates that the asymmetrical CC formed by the AB subunits has a conformation very similar to that formed by the CD subunits (Fig. 5C) (i.e. consistent with the observation reported above) and that the A and C protomers adopt equivalent conformations (Fig. 6A) as do the B and D protomers (Fig. 6B). However, least squares superposition of the A and B protomers shows that they adopt substantially different conformations from one another (Fig. 6C), as do the C and D subunits (data not shown). The non-equivalence of their conformations is demonstrated clearly by superposition of the AB dimer on itself based on alignment of the A and B subunits (Fig. 5D). This non-equivalence produces substantial asymmetry in the structure of the parallel CCs formed by rod 1B, which is a critical structural feature determining its higher order oligomerization behavior and therefore its biological function. This asymmetry is also reflected in the rotation angle of 147°, yielding least squares superposition of the A and B protomers or the C and D protomers forming the parallel CC dimers (Table 3), which is significantly different from the 180° angle that would relate the protomers in a symmetrical CC dimer.

The asymmetry in the CC architecture is encoded in the sequence of the rod 1B peptide, which produces a series of localized bends and kinks that differ in the α-helices formed by protomer A versus B (Figs. 6 (C–G) and 7A). (Note that the stereochemical features discussed here are all shared by protomers A and C as well as protomers B and D, as demonstrated by the fact that the AB dimer has the same conformation as the CD dimer, as shown in Fig. 5C.) These biologically important differences in protomer conformation must be stabilized by their mutual interactions, probably primarily by those across the interface of the parallel CC dimer. Superposition of four sequential segments of protomers A and B (Fig. 6, D–G) demonstrates that substantial differences in the trajectory of the α-helix axes occur at residues 174–178, 198–204, and 213–217. Analysis of the local α-helical bending angle (Fig. 7A) shows systematic differences in the A/C versus B/D protomers at all of these sites, although there is not a tight correspondence between differences in the computationally computed bending angle and the sites of significant divergence in α-helix trajectory. Ultimately, the differential bending in the A/C versus B/D...
Stereochemical Analyses of the Rod 1B Crystal Structure—

Despite the obvious conformational differences between the A/C versus B/D protomers (Fig. 6) and the resulting deviation from proper 2-fold symmetry within the AB and CD dimers (Fig. 5D), they show comparatively minor deviations from the idealized parallel CC architecture first described by Crick (11). Every seventh residue makes locally symmetrical interhelical contacts from Tyr150 (heptad position d) and Met154 (heptad position a) through Leu234 and His238. The CC radius (Fig. 7E) varies only from ~4.4 to 5.6 Å throughout the structure. The CC pitch (Fig. 7F) varies more significantly, but only from ~90–170 Å from residue 155 to 225, indicating relatively standard local CC geometry throughout this region. The pitch increases dramatically after residue 225, where the parallel α-helices pack together essentially without coiling around one another. This region of the structure represents the only significant departure from canonical CC geometry, except for the overall deviation from proper 2-fold symmetry produced by the differential bending of the A/C versus B/D protomers.

The asymmetrical CC dimers formed by subunits AB and CD interact with one another with proper 2-fold symmetry (i.e., a rotation angle of 180° between the equivalent A and C protomers and B and D protomers, as indicated in Table 3). Therefore, the rod 1B tetramer has nearly perfect 2-fold symmetry although its CC building blocks are not symmetrical. This architecture is determined by the genetically encoded distortions from proper symmetry within the parallel CCs, which produce a mutually complementary interaction surface that involves completely different residues in the A/C versus B/D subunits (Table 3).

The fact that the A and B protomers in one CC dimer make very different intersubunit interactions from one another can be seen visually by superimposing the ABCD tetramer on itself via least squares alignment of either the equivalent A and C subunits or the non-equivalent B and D subunits. When the A and C subunits are aligned, the entire tetramer superimposes well (Fig. 5E). In contrast, when A and B are aligned, clear con-
formational differences are observed between the parallel CC dimer pairs (Fig. 5F), and importantly, the second CC dimer in each tetramer is found on opposite surfaces of the CC dimer containing the protomer used for superposition (Fig. 5F). A subunit makes the same contacts with the D subunit as the C subunit makes with the B subunit (as detailed in Table 3), reflecting the proper 2-fold symmetry of the tetramer. However, the conformationally equivalent A and C subunits interact with one another in a manner completely different from that in which the conformationally equivalent B and D subunits interact with one another. The A and C subunits bury $\sim 1300 \text{ Å}^2$ of solvent-accessible surface area per subunit in their mutual interface, via interactions between 11 residues in each subunit related by proper 2-fold symmetry (Table 3). In contrast, the B and D subunits do not make any contacts at all outside of the non-physiological N-terminal affinity tag (Table 3). The asymmetrical structure of the parallel CC dimers forming the tetramer underlie these dramatic differences in the intersubunit interactions between the A and C versus between the B and D subunits, which are responsible for the formation of a stable tetramer with proper 2-fold rotational symmetry.

Note that the structural asymmetry in the constituent parallel CCs is required to produce a stable interaction of this kind without higher order oligomerization. If the A and B subunits had equivalent conformations and formed a parallel CC dimer with proper 2-fold symmetry, the capacity for opposite surfaces of this dimer to interact with themselves would produce higher order polymerization and potentially unlimited growth of the resulting $\alpha$-helical bundle. Therefore, the tetrameric organiza-
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TABLE 2

Statistics from the crystal structure of rod 1B from human vimentin

| Parameters | Values |
|------------|--------|
| General information | PDB ID: 3UF1 |
| | NESG ID: HR4796B |
| | Native protein length: 466 |
| | Length of solved fragment: MGHHHHHHH + 144–251 |

Crystal parameters

- Space group: P2,2,2 |
- a: 61.0 Å |
- b: 86.8 Å |
- c: 114.9 Å |
- Z: 4 molecules/asymmetric unit

Data quality

- Beam line: X4A at Brookhaven NSLS |
- Collection date: August 10, 2010 |
- Wavelength: 0.979 Å |
- Resolution range: 20.0–2.8 Å |
- Total reflections: 817,538 |
- Observed reflections: 28,240 |
- Mean redundancy: 13.5 |
- Completeness (%): 98.8 |
- r.m.s. deviation, bond angles: 1.08° |
- r.m.s. deviation, bond length: 0.009 Å |
- Rsym: 0.085 (0.422)* |
- Mean redundancy: 15.3 (10.4) |
- Completeness (%): 98.8 (93.0) |
- (I/σI): 22.2 (3.0) |

Refinement

- Resolution range: 20.0–2.8 Å |
- No. of reflections: 15,190 |
- Rwork: 23.4% (34.1%) |
- Rfree: 28.4% (40.0%) |
- No. of protein atoms: 3646 |
- No. of non-protein atoms: 0 |
- Overall mean B factors |
  - Subunit A: 76 Å² |
  - Subunit B: 78 Å² (subunit B) |
  - Subunit C: 84 Å² (subunit C) |
  - Subunit D: 85 Å² (subunit D) |
  - r.m.s. deviation, bond length: 0.009 Å |
  - r.m.s. deviation, bond angles: 1.08° |
- Ramachandran plot |
  - Most favored: 96.2% |
  - Additional allowed: 3.1% |
  - Generously allowed: 0.7% |

* Friedel pairs are counted separately in this number.

Table 3

| Subunits | Packing interfaces in the tetrameric structure of rod 1B from huma |
|----------|---------------------------------------------------------------|
| A−B     | 5938 Å² | 1 Å | A | 150 |
| C−D     | 97      | 5939 Å² | 1 Å | C | 28 |

Fig. 8 shows the interhelical interactions at the N termini of both CC dimers in the asymmetric unit of the crystal structure, extending from the first visualized residues through residue 160 in the region with canonical CC structure. Consistent with our SDSL-EPR results (25 and Table 1), Tyr150 and Met154 lie at the hydrophobic interface created by the association of two α-helices in parallel CC geometry. In the CD dimer, in which the α-helical subunit D is extended by residues from the N-terminal hexahistidine affinity tag, the side chains of Leu146 also make hydrophobic contact across the intersubunit interface. This contact, which was predicted by our SDSL−EPR studies (Fig. 2 and Table 1), is one residue out of register compared with the regular CC structure that starts at residue 150 (a d position in the heptad repeat). As discussed above, a regular CC would have an interhelical contact at residue Gly147 (which would be an a position in a regular heptad repeat). The lack of a side chain at this site interferes with canonical interhelical CC packing and probably contributes to the stutter-like distortion that puts Leu146 in contacts across the intersubunit interface at the adjacent site. The first residues visualized in the AB dimer are Leu146 and Ser144 (in the A and B subunits, respectively), presumably due to the disordering influence of the truncation of the native protein sequence before residue Ser144. Nonetheless, residue Leu146 in subunit B still points across the interhelical axis toward subunit A. Therefore, the crystallographically observed conformations are consistent with our SDSL−EPR observations suggesting that L1 adopts a relatively rigid stutter-like structure involving an interhelical contact at residue Leu146, a contact that is out of phase with the...
DISCUSSION

The data in this report extend the structural characterization of the human IF protein vimentin to cover ~90% of the central rod domain. We present SDSL-EPR spectroscopy data establishing linker L1 (L1) as a very short discontinuity between the parallel \( \alpha \)-helical CC structures formed by rod 1A and rod 1B on either side. We present EPR data supporting the formation of CC structure by a part of L1 and x-ray crystallography data confirming this inference and demonstrating the formation of CC structure throughout the entirety of rod 1B. As judged against historic predictions, the crystal structure confirms the close proximity of the side chains of residue Glu\(^{191} \) in the interface between two CC dimers interacting in an anti-parallel geometry, consistent with our previous SDSL-EPR characterization of rod \( \alpha \)A, which encompassed positions 120–145 (27), identified CC structure by qualitative and quantitative methods. Slightly puzzling was the higher than expected \( d_1/d \) value (0.6) obtained at Gly142, which was predicted to be an \( \alpha \) heptad position. Superficially, the observed \( d_1/d \) value indicated a closer than typical distance between spins labels at such a position, and we conservatively concluded that the data were not consistent with a canonical CC structure at this site.

Additional insight into this observation is provided by reconsideration of previous SDSL-EPR and x-ray crystallography data in the context of the new spectroscopic and crystallographic data presented in this paper. Our previous SDSL-EPR characterization of rod \( \alpha \)A (28) identified non-CC and CC structures in residues 280–305. We observed high \( d_1/d \) values for the positions at the beginning of the rod 2B CC, including a value of 0.59 at Ala\(^{302} \). The recently solved crystal structure of the 265–330 fragment of human vimentin (25) demonstrates that forming vimentin. As a result of these data, the structure of linker 1-2 is the only remaining region of the vimentin rod domain that has not been characterized by crystallographic or spectroscopic means.

The SDSL-EPR data presented in this paper establish a high \( d_1/d \) ratio as being a signature characteristic of the termini of the regular CC structures in the vimentin rod domain. Our previous SDSL-EPR characterization of rod \( \alpha \)A, which encompassed positions 120–145 (27), identified CC structure by qualitative and quantitative methods. Slightly puzzling was the higher than expected \( d_1/d \) value (0.6) obtained at Gly142, which was predicted to be an \( \alpha \) heptad position. Superficially, the observed \( d_1/d \) value indicated a closer than typical distance between spins labels at such a position, and we conservatively concluded that the data were not consistent with a canonical CC structure at this site.

Additional insight into this observation is provided by reconsideration of previous SDSL-EPR and x-ray crystallography data in the context of the new spectroscopic and crystallographic data presented in this paper. Our previous SDSL-EPR characterization of linker L2 (28) identified non-CC and CC structures in residues 280–305. We observed high \( d_1/d \) values for the positions at the beginning of the rod 2B CC, including a value of 0.59 at Ala\(^{302} \). The recently solved crystal structure of the 265–330 fragment of human vimentin (25) demonstrates
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this position to be the start of the CC formed by rod 2B, consistent with our conclusions from SDSL–EPR (21). Therefore, we propose that a new spectroscopic “benchmark” has been established (i.e. that a particularly high \( d/j/d \) value marks the transition point to and from canonical CC structures). Furthermore, based on this new benchmark, we identify position 142 as the end of the CC formed by rod 1A.

Similarly, based on our new proposed benchmark, we conclude from the SDSL–EPR data presented in this paper (Fig. 2 and Table 1) that position 147 represents the beginning of the CC formed by rod 1B. Our assignment of Gly147 as an “a” heptad position is entirely consistent with the downstream phasing of the heptad repeat pattern starting at residue 150 in our crystall structure of rod 1B (Figs. 5–8 and Tables 2 and 3), our current SDSL–EPR data covering positions 146–168 (Fig. 2 and Table 1), and our earlier SDSL–EPR data covering positions 169–193 (28).

Early studies predicting the locations of the linker and CC regions in vimentin typically proposed that the CC regions are rigid, whereas the linker regions are substantially more flexible (14, 15, 45). It was hypothesized that this flexibility accommodated slight shifts in packing during filament assembly. Given the widespread prevalence of this structural model, our SDSL–EPR characterization of linker L2 was striking in that it found this region to be a rapidly assembling and relatively rigid structure (21). Our characterization of linker L1 presented in this paper (Fig. 2 and Table 1) leads to a similar conclusion (i.e. that L1 forms a relatively rigid structure rather than a flexible loop). Further research will be required to establish whether linker L1–2 forms a qualitatively similar structure or a flexible loop connecting rod domains 1 and 2, as proposed in previous literature (15). It has not escaped our notice that the increased CC pitch at the end of rod 1B is similar to the situation within the recently solved rod 2A/linker 2 (26).

The crystal structure presented in this paper (Figs. 5–8 and Tables 2 and 3) demonstrates that the rod 1 peptide adopts two very different conformations. This structural polymorphism is likely to be critical for its biological function. One rod 1B subunit in each conformation is used to assemble an asymmetrical parallel CC dimer that itself dimerizes to form a tetramer with proper 2-fold rotational symmetry (Fig. 5). All four subunits form \( \alpha \)-helices that assemble into parallel CC dimers with relatively canonical geometry (Fig. 7). As labeled in this report (and Protein Data Bank entry 3UF1), subunits A and C adopt equivalent conformations, as do subunits B and D. However, these two subunit pairs differ substantially in their local bending angle at three sites, producing a substantial difference in overall conformation (Fig. 6). Therefore, the parallel CC dimers formed by combining one subunit in each conformation (i.e. A with B and C with D) do not have 2-fold rotational symmetry. Symmetrical CC dimers forming a symmetrical interface would tend to grow without limit based on propagation of equivalent intersubunit structural interactions. Therefore, the structural asymmetry in the AB and CD dimers, which represents a remarkable example of evolutionary exploitation of the structural plasticity of polypeptides, explains the ability of these dimers to form a tetramer with proper 2-fold rotational symmetry that does not polymerize into higher order oligomers.

Despite the importance of this asymmetry in determining the functional oligomerization properties of rod 1B, we are unaware of any previous predictions of structural asymmetry in this region of vimentin, which is the longest predicted CC region in the molecule.

Although this asymmetry is necessary to prevent high order polymerization of rod 1B, further research will be required to determine whether it is a dominant feature in actually controlling IF diameter. Published results on vimentin constructs with truncated head or tail domains make it highly unlikely that the asymmetry in rod 1B is the only determinant of IF caliber (42, 46). Our data suggest that the head domain, rod 1B, and the tail each have a role in limiting assembled vimentin filaments to 10 nm in diameter.

In summary, this report provides a combination of spectroscopic and crystallographic evidence supporting formation of a CC structure by rod 1B in human vimentin. The crystal structure presented in this paper combined with earlier spectroscopic data (28) demonstrates that rod 1B assembles into a so-called A11 tetramer (41, 43, 44), meaning an anti-parallel alignment of two parallel CCs formed by segments of rod 1. This alignment of rod 1B domains is also supported by cross-linking experiments (40). IF proteins are among the most abundant in eukaryotic cells, and mutations in them have been implicated in more than 85 different human diseases. Therefore, there is a compelling need to characterize normal IF structure so that the pathogenic effects of mutations can be understood. The ability to obtain a crystal structure of a likely physiological oligomer of rod 1B suggests that it may be possible to obtain crystal structures of larger segments of vimentin in physiologically relevant conformations. If so, the combination of x-ray crystallography and SDSL–EPR can be used to provide comprehensive characterization of the atomic structure of an IF protein in an intact filament, which would provide an important foundation for understanding of the effects of mutations on IF structure and function. The data presented herein underscore the value in using multiple independent methods to characterize the structure of this class of proteins that have complex conformational properties.

Acknowledgments—Technical assistance with electron microscopy was provided by G. Adamson (Electron Microscopy Laboratory, Department of Medical Pathology and Laboratory Medicine, University of California Davis School of Medicine). We thank J. Steggall (California Department of Food and Agriculture, Sacramento, CA) for help running the program TWISTER under Mac OSX. We also thank Northeast Structural Genomics Consortium scientists T. Acton for supervising cloning; M. Abishadze for performing crystal optimization; and A. Lauricella, J. Luft, and G. DeTitta for conducting high throughput microbatch crystallization screening.

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