The Conserved H1 Domain of the Type II Keratin 1 Chain Plays an Essential Role in the Alignment of Nearest Neighbor Molecules in Mouse and Human Keratin 1/Keratin 10 Intermediate Filaments at the Two- to Four-molecule Level of Structure*

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A number of fundamental questions pertaining to the registration and packing of the constituent coiled-coil molecules in keratin intermediate filaments, and to the regions of the sequences that are responsible for these levels of organization, remain to be elucidated. In this study, small assembly-competent oligomers of mouse and human keratin 1/keratin 10 keratin filaments were cross-linked by the formation of disulfide bonds catalyzed by the copper-phenanthroline reaction. By isolation and characterization of cross-linked peptides, it has been possible to establish two major modes of molecule alignment: an antiparallel arrangement of half-staggered molecules with their 2B segments overlapping and an antiparallel arrangement of molecules in close axial registration. These data confirm earlier models based on theoretical considerations (Crewther, W. G., Dowling, L. M., Steinert, P. M., and Parry, D. A. D. (1983) Int. J. Biol. Macromol. 5, 267–274). Interestingly, these models place the conserved H1 and H2 end domain segments, which flank the ends of the rod domains of the type II keratin 1 chain, in alignment with either the ends of the rod domains and/or with the L2 segment near the center of the rod domains, of the nearest neighbor molecules. Competition experiments with synthetic peptides suggest that the conserved H1 (and possibly H2) subdomain sequences unique to type II keratin chains play pivotal roles in the registration of neighboring molecules in keratin filaments. The data thus afford a molecular explanation for why keratin filaments require a type II chain for assembly in vivo and in vitro.

Intermediate filaments (IF) are ubiquitous constituents of the cytoskeletons of most types of eukaryote cell types. In general, however, IF are most abundant in advanced differentiated cells, such as the neuronal IF of neurological tissues, and keratin IF (KIF) of terminally differentiating epithelial cells. For example, suprabasal epidermal cells express large amounts (about 70% of total cellular mass) of keratin 1/keratin 10 (K1/K10) KIF. To date, only a few details of the organization of protein chains in IF have been resolved. The first level of organization involves the formation of a two-chain coiled-coil molecule of two compatible chains (2, 3). In the special case of KIF, this is a heterodimer consisting of a type I and a type II chain, such as for K1 (type II) and K10 (type I) KIF (4, 5). The second step seems to involve the formation of a series of small oligomers of this molecule. Several earlier studies have suggested that two molecules become aligned to form a four-chain complex or “building block.” However, very recent studies from this laboratory have shown that two-, three-, and four-molecule oligomers are commonly observed when epidermal KIF begin assembly in vitro (6, 7). Although all observers agree that the first two molecules become aligned antiparallel to each other, confusion has existed as to whether they lie in register (8–11) or are staggered (2, 6, 12–14). Recent studies showed that the staggered mode of alignment is the thermodynamically most stable form in solution (6). However, the degree by which the adjacent molecules overlap is unknown. Resolution of this question is important because it has critical implications for the higher levels of molecule alignment and even if the different types of IF organize their molecules in the same way (15). Because of this lack of data, current models on the intermediate-to-complete levels of molecular organization are very uncertain, although there is evidence for entwining strands of protofilaments and/or protofibrils (16), and limits on the total numbers of molecules in assembled IF have been made from quantitative mass measurements (15, 17–19).

The initial purpose of the present work has been to resolve this question on the exact degree(s) of nearest neighbor molecule overlap in K1/K10 KIF. We have noted that these chains have a favorable distribution of cysteine residues such that disulfide bond formation can be induced without significant chain rearrangement. Once crosslinked, peptides were isolated and characterized to locate the precise position of the disulfide bonds. In this way, we have deduced two modes of molecule alignment. Most interestingly, these alignments have allowed us to realize the importance of the H1 and H2 sequences in the non-helical regions flanking the ends of the rod domains that stabilize and/or drive the exact molecule alignments observed.

MATERIALS AND METHODS

Isolation of Proteins—Freshly prepared newborn BALB/c mouse epidermal cells were plated for 4 h (20). Those which attached in this
time, consisting mostly of basal cells, contain almost exclusively the K5 and K14 chains. Those which did not attach in this time, mostly suprabasal keratinocytes, contain primarily the K1 and K10 chains in their unprocessed forms. Both populations of cells were extracted to prepare profilaggrin-free cytoskeletons by established procedures (5, 6, 21). These were then extracted in a buffer of 8 M urea, 50 mM Tris-HCl (pH 8.0), 1 mM DTT, and 1 mM EDTA, clarified by centrifugation at 225,000 × g, and the concentration determined spectrophotometrically, assuming E1% 1 cm = 4.8. Human foreskin epidermis was extracted in a buffer of 0.1 M Tris glycine, pH 8.3, containing 1% SDS and 1 M NaCl and clarified by centrifugation at 225,000 × g. In this way, all the keratin protein chains from the inner living cell layers remained in solution. The keratins were then recovered free of SDS by ion-pair extraction and redissolved in the urea buffer. These human proteins consist mostly of unprocessed K1 and K10 chains as well as traces of K5 and K14.

In vitro KIF reassembly (0.06–1.5 mg/ml) was performed by dialysis (2 h each) into solutions of decreasing urea concentrations, from 6 to 4 to 2 to 1 and to 0 M, containing 5 mM Tris-HCl (pH 7.6), 1 mM DTT, and 1 mM EDTA (5). In some experiments, the proteins in urea solution were quickly transferred to this assembly buffer by passage through a 5 × 1 cm column of Sephadex G-25. For all batches of KIF used, the assembly competence was checked by a centrifugation assay (6); the batch of proteins used was only if at least 95% of the KIF was pelletable after assembly at a concentration of 1 mg/ml. For assembly below the critical concentration (Cc), the initial protein concentration (C0) in urea was about 60 pg/ml, which ended up at about 15 pg/ml after the assembly procedure, just below the Cc required for K1/K10 KIF reassembly (7). In some experiments, both native sulfhydryl and modified forms of the human K1 and K10 chains were separated by preparative slab gel electrophoresis, electroeluted, concentrated, and freed of SDS by ion-pair extraction (21, 23).

The nonepidermal keratins K7, K8, K17, K18, and vimentin (24) were also isolated from the cytoskeletons of cultured HeLa cells by differential reassembly from low ionic strength KIF assembly buffer (for KIF) or this buffer containing 0.15 M NaCl (for vimentin IF) (25).

Cross-linking Procedures—Cross-linking experiments were done with both human and mouse K1/K10 chains. Solutions at 45 µg/ml were quickly freed of DTT by passage through a 1-cm column of Sephadex G-25 and then oxidized with the copper-phosphorothionate complex essentially as described (26). The efficacy of the cross-linking reaction was monitored by electrophoresis on 3.75–7.5% gradient polyacrylamide gels (5). Oligomers containing the most abundant one-, two-, three-molecules species were separated preparatively on 3-mm-thick slab gels, cut out, recovered by electroelution as above, and at about 15 pg/ml after the assembly procedure, just below the Cc, for K1/K10 KIF reassembly (7). In some experiments, both native sulfhydryl and modified forms of the human K1 and K10 chains were separated by preparative slab gel electrophoresis, electroeluted, concentrated, and freed of SDS by ion-pair extraction (21, 23).

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Processing of Cross-linked Peptides—Following oxidation, the K1/K10 protein solutions were made to 0.1 M N-ethylmaleimide acetate (pH 8.5) and digested with TPCK-trypsin (Sigma, type XIII) using a 2% (w/w) of enzyme at 37 °C for 6 h. Further enzyme (1% w/w) was added for another 6 h. An aliquot corresponding to about 0.1 nmol of keratin chain monomers was then resolved by reverse-phase HPLC (Beckman Model 128/168 with System Gold software) using a 50-ml gradient of 0–40% acetonitrile in 0.1% TFA.

A second aliquot was treated with 10 mM DTT to reduce the disulfide bonds and run in an identical fashion. The two profiles were compared with each other to identify shifted peaks that indicate potential disulfide bond cross-linked peptides in the former that had been cleaved in the latter. Subsequently, the major portion of the oxidation reaction was resolved preparatively by HPLC and the candidate cross-linked peaks were collected and freeze-dried. Each was separately redisolved in 0.1 M Tris-HCl (pH 8.5) in 10 mM DTT and alkylated with iodoacetate as above, rerun by the same HPLC method, and the two peaks which represent the two arms of the disulfide bond cross-linked peptide fraction were resolved and harvested. A portion of each peptide was hydrolyzed in 5.7 N HCl at 108 °C in vacuo for 16 h and its amino acid composition determined on a Beckman 6300 analyzer.

Construction of Synthetic Peptides—The following peptides, corresponding to specific sequence domains of the keratin chains of known sequences (27, 28), were synthesized (denoted by the single letter amino acid code):
Fig. 1. Locations of cysteine residues in the mouse and human K1 and K10 chains. Arrows indicate the positions of cysteines adduced from published sequences along a stick diagram representing the secondary structure of the chains. The arrow in parenthesis marks the optional cysteine in the V2 subdomain of the human K1 chain.

Fig. 2. Cross-linking of Human K1/K10 KIF by Cu-P. Following cross-linking at \( C_0 < C_c = 45 \mu g/ml \), the products were resolved on a 3.75-7.5% polyacrylamide gel. –DTT, cross-linking reaction. +DTT, cross-linking reaction incubated with 10 mM DTT to hydrolyse induced disulfide bonds. M, markers of isolated mouse K10 cross-linked extensively with glutaraldehyde.

The results of this study would provide valid data on the alignment of nearest neighbor molecules in intact assembled KIF. The initial intent was then to determine separately the sequence locations around the cross-links in each of the cross-linked oligomeric species. However, we found that subsequent to separation of the oligomers, the multiple steps involved in tryptic digestions, fractionation of peptides by HPLC under acidic conditions, freeze-drying, followed by further HPLC fractionations, etc. resulted in considerable degrees of disulfide bond exchange and even lanthionine formation (β-elimination of one sulfur atom to form an unbreakable cross-link). Accordingly, further analyses of cross-links were performed on the unfractionated mixture of cross-linked oligomers.

Characterization of Disulfide Cross-links in Nearest Neighbor Molecules—As described under “Materials and Methods,” the mouse and human cross-linked KIF molecules were digested with trypsin to completion. A portion was fractionated by HPLC. A similar portion was treated with DTT to reduce the disulfide-cross-linked peptides and then resolved in the same way in a separate run. By comparison of the two profiles, it is readily apparent that several peaks in the former were lost in the latter, indicating potential crosslinked species (Figs. 3 and 4). A total of 10 such peptide fractions were reproducibly found for mouse KIF (Fig. 3). However, most batches of human KIF protein yielded five peaks, whereas one experiment yielded only three peaks. Subsequent analysis of the peaks (see below) and amino acid analyses of the fractionated K1 and K10 chains isolated from separate foreskins revealed that certain individuals in the human population possess a K1 molecule with only 3 cysteine residues, having lost the cysteine in the V2 subdomain (see Fig. 1) as an apparent normal polymorphic sequence variant. Experiments performed on individuals homozygous for this 3-cysteine variant (the epidermis of one foreskin in five tested) yielded the three disulfide bond cross-linked peaks of Fig. 4B, whereas most heterozygous individuals yielded five cross-linked peaks seen in Fig. 4C, having two additional peptides.

Each peptide was then isolated preparatively by an identical HPLC run, reduced and alkylated with iodoacetate, and the two peaks resulting from the two arms of the cross-linked peptide were again separated by HPLC for amino acid analy-
sis. In some cases, only one peak was found, due to the peptide being cross-linked to itself. Because the yields of these single peptides were quite high (>0.3 mol/195 kDa), it is highly unlikely they arose from cross-linking of homo-oligomers known to be present in the KIF solutions (Fig. 2). Furthermore, because of these high yields the peptides could be recovered very pure, so that the resulting analyses yielded simple amino acid compositions which in most cases afforded direct location of the peptides along the K1 or K10 chains. This was aided by the finding that all peptides contained only 1 cysteine/mol, and by the fact that there are few cysteines in these keratin chains and sequences of the intact proteins are known. Peptides Mo2 and Mo8 gave one identifiable peptide, but their partners were reproducibly complex and thus could not be found in these keratins; they may be disulfide-interchange species or species cross-linked to other minor proteins in the keratin preparations and were not further studied. The likely sequences for all of the other mouse and human cross-linked peptides are shown in Table I.

There Are at Least Two Modes of Alignments of Nearest-neighbor Molecules—With a view to the most recent ideas on nearest neighbor alignments of molecules in K1/K10 KIF (6, 7), the data of Table I permitted construction of the models illustrated in Fig. 5 and were as follows. Model A, K1–K10 heterodimer (peptides Mo3, Mo5, Mo10, and Hu3). This means that the K1 and K10 chains exist as a dimer and are aligned in parallel and in close axial register. This is expected and re-confirms the now accepted structure of the two-chain coiled-coil molecule for KIF (3–5, 36–38). Disulfide cross-links across the V1 regions within this one-molecule species (peptides Mo10 and Hu3) are consistent with the recent view that these glycine-rich domains adopt a compact folded glycine-loop configuration (39). Model B, two molecules staggered and aligned antiparallel so that their segments 2 are adjacent (peptides Mo1, Mo4, Mo6, Mo7, and Hu1). These peptides arise due to the multiple cysteines in the 2B region and are entirely consistent with the recent view that the first two molecules during KIF assembly are aligned antiparallel and staggered (7). Indeed, these data establish that the two molecules are almost half-staggered, consistent with the views of a variety of earlier suggestions (2–7, 40–42), but different from suggestions for alignments in the type III glial fibrillary acidic protein (13). Model C, two molecules are aligned in register but antiparallel (peptides Mo9, Hu2, Hu4, and Hu5). Since this mode of alignment is unstable in aqueous solution at the two-molecule level of assembly (6), these peptides could only have arisen from the three- and/or four-molecule species in solution (6, 7). Of particular note is the high yield, in relation to the amounts of the three- and four-molecule oligomers in solution, of the peptides Mo9 and Hu5 due to cross-linking of the single cysteines in each of the H1 and H2 subdomains. In order for these disulfide cross-links to have formed, the H1 subdomains must adopt a more folded conformation than implied in Fig. 1 to permit close apposition of the thiol groups (Fig. 5). This alignment model is also consistent with earlier views (40, 41). Given the distributions of cysteine residues as they are in the mouse and human keratin chains, no disulfide cross-links could form that would suggest an alignment of molecules with the 1B segments of the rod.

fig. 4. Identification of cross-linked peptides in human KIF. Experimental details are exactly as described in the legend to Fig. 3. However, in this case, we found that different batches of human keratins yielded different peaks, such that preparations containing the K1 chain having only 3 cysteines/mol gave three cross-linked peaks (B), whereas the four-cysteine variant of K1 yielded five cross-linked peaks (C).

Table I

| Number | Cross-linkage | Yield | Model (see Fig. 5) |
|--------|---------------|-------|-------------------|
| Mo-1   | K1 H2 Cys* to K1 2A Cys* | 0.00  | B                 |
| Mo-2   | Uninterpretable |       |                   |
| Mo-3   | K1 2B Cys* to K10 2B Cys* | 0.46  | A                 |
| Mo-4   | K1 2B Cys* to K1 2B Cys* | 0.11  | B                 |
| Mo-5   | K1 2B Cys* to K10 2B Cys* | 0.28  | C                 |
| Mo-6   | K1 2B Cys* to itself*   | 0.59  | B                 |
| Mo-7   | K10 2B Cys* to itself*  | 0.98  | B                 |
| Mo-8   | Uninterpretable         |       |                   |
| Mo-9   | K1 H1 Cys* to K1 H2 Cys* | 0.22  | C                 |
| Mo-10  | K1 V1 Cys* to K10 V1 Cys* | 0.95  | A                 |
| Hu-1   | K10 2B Cys* to itself*  | 0.35  | B                 |
| Hu-2   | K1 V1 Cys* to K1 V2 Cys* | 0.20  | C                 |
| Hu-3   | K1 V1 Cys* to K10 E1 Cys* | 0.29  | A                 |
| Hu-4   | K1 V2 Cys* to K10 E1 Cys* | 0.11  | A                 |
| Hu-5   | K1 H1 Cys* to K1 H2 Cys* | 0.22  | C                 |

*The yields of these peptides are too high to be accounted for by the presence of some homodimer molecules in these protein samples (estimated to be 5–10% of protein).
Accordingly, several additional experiments were performed to assess their roles in KIF assembly.

A Synthetic H1 Peptide Disrupts Preformed KIF to the Two- to Four-Molecule Stage of Assembly—We have recently documented that a synthetic peptide corresponding to the native H1 sequence of the mouse K1 chain can cause rapid disassembly of preformed mouse or human K1/K10 KIF in vitro (44, 45). In the present study, this peptide could also disassemble K5/K14 as well as the K7/K8/K17/K18 KIF of simpler epithelial cells. For example with the K5/K14 KIF, a 1:1 molar ratio of the peptide:KIF reduces the KIF within 60 min to short subfilamentous particles (Fig. 6B), individual ones of which are 60–70 nm long (Fig. 6C). When such a reaction was then centrifuged at 100,000 x g for 30 min, 90–95% of the total protein remained in solution, indicating very efficient disassembly indeed. (In contrast, <5% of untreated KIF remained in solution following centrifugation). By cross-linking with glutaraldehyde, the largest species in solution consisted of two and three molecules (four to six chains) (Fig. 6D, lanes 2 and 3, representing two different experiments). Similar results were obtained with the K7/K8/K17/K18 KIF of simpler nonepidermal keratins (data not shown). However, the H1 peptide had no effect on preformed type III vimentin IF prepared from HeLa cells (data not shown). Accordingly,
reassembly experiments were performed by mixing either SCM-K10 with normal K1 (Fig. 7B) or SCM-K1 (using the 3-cysteine variant) with normal K10 (Fig. 7, C and D). In the former, all of the protein appeared in a disordered state, whereas in the latter, 60–70-nm-long subfilamentous particles are evident. Cross-linking of these reactions with glutaraldehyde revealed only a small amount of one-molecule alignments had occurred in the former (Fig. 7E, lane 1), but substantial amounts of two- and three-molecule (but no four-molecule) alignments had occurred with the latter (Fig. 7E, lane 2). These data show that the introduction of 3 charged residues in place of the normal cysteines in the human K10 chain (2 of which are in the rod domain, Fig. 1) severely inhibits the formation of stable heterodimer one- and two-molecule species, whereas the insertion of the 3 new charged residues in the human K1 chain (none of which are in the rod domain, one each of which is in H1 and H2 (Fig. 1), limits KIF assembly at the two- to four-molecule stage.

The Synthetic H1 Peptide Interacts with Two Specific Acidic Regions along the Rod Domains—In view of the molecule alignments adduced in Fig. 5, a series of experiments was devised to test whether the basic H1 domain interacts with acidic regions along the rod domains of the KIF chains. There are two sequence regions common to both the type II class of

the H1 peptide disrupts most if not all KIF formed from many different combinations of keratin chains and is apparently specific to KIF.

Similar experiments were not possible with the synthetic H2 peptide, since it was insoluble in the KIF assembly buffer.

These experiments were repeated by adding to preformed mouse K1/K10 KIF the H1 (Fig. 6E) and H2 (Fig. 6F) peptides that had been modified by conversion of their sulfhydryl groups to the S-carboxymethyl (SCM) derivatives. Note that although the KIF are notably shorter and contain some abnormally wide or twisted entities, the net alteration in KIF integrity is minor. Samples were examined on Formvar-coated grids, except in C, where a holey carbon-film grid was used to achieve higher resolution of the small oligomers remaining after clarification by centrifugation. Bar, 100 nm.

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Fig. 6. Disassociation of preformed mouse KIF in vitro by use of synthetic H1 peptides. A, normal-appearing human K1/K10 KIF reassembled in vitro using isolated two-molecule disulfide-bond cross-linked oligomer from Fig. 2. B and C, the H1 peptide (2 mg/ml in KIF assembly buffer) was added to a final molar concentration ratio of 1:1 with respect to 1 mg/ml suspension of preformed mouse K5/K14 KIF. C, the above solution was clarified at 100,000 × g for 30 min (whereupon >95% of total protein remained in solution) to better visualize the oligomers in solution, individual ones of which are about 70 nm long (arrows), corresponding in size to two- to four-molecule oligomers. D, chain composition of human K1/K10 KIF disassembled in 60 min by a 1:1 molar ratio of the synthetic H1 peptide. Following clarification by centrifugation at 100,000 × g, the products of two separate experiments were cross-linked with glutaraldehyde and resolved on a 3.75–7.5% polyacrylamide gel. Lanes 1 and 2 two different experiments showing that the maximum size of oligomers remaining in solution correspond to two- or three-molecule species, respectively. Lane M, marker ladder of crosslinked mouse K10 chain (7). E and F, SCM-derivatized forms of the H1 and H2 peptides, respectively, were mixed in a 1:1 molar ratio to preformed mouse K5/K10 KIF for 60 min. Although the KIF are notably shorter and contain some abnormally wide or twisted entities, the net alteration in KIF integrity is minor. Samples were examined on Formvar-coated grids, except in C, where a holey carbon-film grid was used to achieve higher resolution of the small oligomers remaining after clarification by centrifugation. Bar, 100 nm.

Critical Roles of the Cysteine Residues in the H1 and H2 Subdomains—To further assess the roles of the cysteine residues of individual keratin chains in human KIF, in vitro assembly experiments were performed by mixing either SCM-K10 with normal K1 (Fig. 7B) or SCM-K1 (using the 3-cysteine variant) with normal K10 (Fig. 7, C and D). In the former, all of the protein appeared in a disordered state, whereas in the latter, 60–70-nm-long subfilamentous particles are evident. Cross-linking of these reactions with glutaraldehyde revealed only a small amount of one-molecule alignments had occurred in the former (Fig. 7E, lane 1), but substantial amounts of two- and three-molecule (but no four-molecule) alignments had occurred with the latter (Fig. 7E, lane 2). These data show that the introduction of 3 charged residues in place of the normal cysteines in the human K10 chain (2 of which are in the rod domain, Fig. 1) severely inhibits the formation of stable heterodimer one- and two-molecule species, whereas the insertion of the 3 new charged residues in the human K1 chain (none of which are in the rod domain, one each of which is in H1 and H2 (Fig. 1), limits KIF assembly at the two- to four-molecule stage.

The Synthetic H1 Peptide Interacts with Two Specific Acidic Regions along the Rod Domains—In view of the molecule alignments adduced in Fig. 5, a series of experiments was devised to test whether the basic H1 domain interacts with acidic regions along the rod domains of the KIF chains. There are two sequence regions common to both the type II class of

Fig. 7. The sulfhydryl groups of the H1 and H2 domains in the human K1/K10 chains are essential for KIF assembly. Human keratins were converted to their SCM derivatives with iodoacetate and preparatively separated. KIF were then assembled from mixtures of unmodified chains (A), SCM-K10 and normal K1 (B) or SCM-K1 and normal K10 (C and D). A–C were observed over Formvar-coated grids and D over a holey carbon film grid. Arrows designate 60–70-nm oligomers. Bar, 100 nm. E, polyacrylamide gel of products recovered from B and D. M, marker of cross-linked oligomers of the mouse K10 chain.
chains (K1) and type I class of chains (K10) that contain multiple adjacent acidic residues, in the L2 region (peptides 3 and 4), and at the end of the 2B rod domain segment (the canonical LLEGE sequence; peptides 5 and 6). In addition, there is one other acidic region in K10 (residues 91–97 of the 1B segment of its rod domain, peptide 7) and one in K1 (residues 72–79 of the 2B rod domain segment, peptide 8), sequences that are conserved within the type I and type II families, respectively, but are not common to both chain types.

Synthetic peptides of each of these six acidic regions were tested separately or in combination and mixed with preformed mouse K1/K10 KIF. Two assays to assess the degree of KIF disassembly were used: centrifugation at 100,000 × g for 30 min and electron microscopy as above. Peptides corresponding to the L2 region of the K1 (peptide 3) (Fig. 8A) or K10 (peptide 4) (data not shown) chain in either a 1:1 or 10:1 molar ratio did not seriously affect the stability of preformed KIF in solution (KIF length > 5 μm). It was found that a 1:1:1 molar ratio of peptide 3 together with H1 caused only minor changes to KIF morphology (Fig. 8B; KIF length was reduced to 1.6 ± 0.5 μm) and no change in the yield of protein in KIF; that is, the L2 peptide had ameliorated the destructive effect of the H1 peptide alone. As expected, a net molar excess of the H1 peptide promoted KIF disassembly. Next, a 5:1 molar ratio of peptide 5, corresponding to the last 21 canonical residues of the 2B segment, destroyed KIF structure, resulting in small clumps of apparently disordered protein (Fig. 8C), exactly as reported recently (46). Only traces of KIF protein could be pelleted, indicating almost total dissolution. The shorter 9-residue version of this peptide (6) required about a 10:1 molar excess to disassemble KIF to a similar degree, both by electron microscopy (Fig. 8D; subfilamentous particles < 100 nm) and centrifugation. As found above for the H1 peptide, equimolar amounts of the L2 peptide could protect KIF from disassembly by either peptide 5 or 6, although the KIF were somewhat shorter (Fig. 8E with peptide 5; KIF length 1.1 ± 0.6 μm) and the yields of protein remaining in KIF were >90%. Interestingly, 1:1:1 comixtures of peptides 1 and 5 with KIF could also provide significant protection against KIF disassembly (Fig. 8F; KIF length 0.8 ± 0.5 μm), and 85–90% of total protein could be pelleted. In clear contrast to these findings, peptides corresponding to the unique acidic sequences of K10 (1B segment, peptide 7) and K1 (2B segment, peptide 8) did not promote significant disassembly of KIF (Fig. 8G, KIF length > 6 μm). Furthermore, neither peptide 7 nor 8 could protect KIF from disassembly in comixtures with the H1 peptide (Fig. 8H for peptide 7; subfilamentous particles < 100 nm). Control experiments with the other basic peptides 9 (having the LLEGEIRIM motif cutoff), or peptides 10–12 from unrelated proteins, did not have a significant effect on preformed KIF at molar ratios of up to 10:1 and could not protect KIF from disassembly by either the peptide 1 or 5 (data not shown). Therefore, protection from disassembly by the H1 and canonical LLEGE peptides relied not only on acidic charge characteristics but specific sequences as well. These data suggest there are specific interactions between the H1, L2, and LLEGE sequences.

**DISCUSSION**

*There Are at Least Two Modes for Alignments of Neighboring Molecules in KIF—* The purpose of the present experiments was to determine the nearest neighbor alignments of molecules in the earliest stages of KIF assembly. Resolution of this question would likely impose severe constraints on the higher orders of molecule alignments within KIF. For this study, we have used K1 and K10 chains that have 3–8 cysteines/chain, more than in either the K8/K18 or K5/K14 systems, but fewer than in wool keratin chains (47). Since cysteine residues are likely to find a partner for disulfide bond formation, we have assumed that the cysteines of the K1/K10 chains would be juxtaposed so as to form the most thermodynamically stable disulfide bonds within the context of maximal stability of intact KIF structure. Recent studies have suggested that substitution of a cysteine in a non-native location in the 1A rod domain segment of K14 promotes failed KIF assembly *in vitro*, abnormal KIF organization in cultured cells and clinical pathology in living epidermal tissue (48). Moreover, since the sulfhydryl groups must lie within 0.3 nm for disulfide bond formation, the axial displacement of 2 adjacent cysteine residues must be ±2 residues. Thus the data would provide accurate assessments of nearest neighbor molecule alignments.

Our strategy was to cross-link the small oligomers formed during KIF assembly in subcritical protein concentrations where assembly progresses only as far as the four-molecule stage (7). Our control experiments showed that separated one-, two-, and three-molecule disulfide-bond cross-linked oligomers of human KIF chains could assemble *in vitro* into long KIF at concentrations above C0 (Fig. 8A). Furthermore, the experimental conditions employed here do not permit molecule rearrangements in solution (5, 6). Thus the molecule alignment information deduced by this strategy is valid and can be extrapolated to intact KIF. It was demonstrated many years ago (32, 35) that solutions of keratin chains, that are now known to consist of small oligomers (7), are assembly-competent when cross-linked by oxidation to disulfide bonds; however, their SCM derivatives are not (35), thereby indicating the critical importance of the thiol groups and the charge characteristics of the keratin chains.

By cross-linking the juxtaposed cysteine residues of mouse and human K1/K10 KIF to disulfide bonds catalyzed by the Cu-P reaction, we have been able to establish two distinct modes of molecule alignment (Fig. 5). One involves an anti-parallel alignment of half-staggered molecules, having the segments 2 aligned in register. The second involves an antiparallel alignment of in-register molecules. The present studies directly confirm previous hypotheses, since, interestingly, each of these had been proposed earlier on the basis of a variety of work including x-ray diffraction analyses of the well-ordered KIF of the porcupine quill (49, 50), alignments of amino acid sequences (2), theoretical considerations of ionic interaction scores (40, 41, 49, 50), the identification of a cross-linked peptide arising from a transpeptidation reaction (42), and limited proteolytic digestions coupled with electron microscopy (6, 7). A third model having the segments 1 of two antiparallel molecules aligned half-staggered was also a highly favored form in these aforementioned earlier studies but was not seen in the present work due to the disposition of cysteines along the rod domains. Further cross-linking experiments using other clevable cross-linking reagents are in progress in an attempt to confirm the presence of this third model.

Implications for higher order organization with KIF of these alignment models will be discussed in more detail elsewhere (61). Interestingly, a recent study with type III desmin IF reported the recovery of three cross-links that conform to this third model (43). Similarly, this mode of alignment was observed in paracrystals formed with the type III glial fibrillary rod domain segment (13). These developments encourage us that this mode of alignment is also likely for KIF. These data would therefore seem to support the notion that the molecules of both type III and type I/II KIF are aligned in the same...
FIG. 8. The H1 subdomain of the K1 chain in one molecule interacts specifically with two conserved acidic regions along the rod domain of a neighboring molecule. All reactions were performed for 60 min prior to stopping with uranyl acetate stain. Mixture of synthetic peptide 3 corresponding to the L2 region of the K1 chain did not interfere with the structural integrity of preformed mouse KIF at a 1:1 molar ratio (A). A 1:1:1 comixture of the H1 peptide and L2 peptide with preformed KIF has only a minor affect on KIF integrity (B). Mixtures in a 5:1 molar ratio of peptide 5 promoted catastrophic disassembly of preformed KIF (C), but a 10:1 molar ratio of peptide 6 was required to achieve similar disassembly (D). The dissociation could be ameliorated by a 1:1:1 comixture of KIF with peptides 3 and 5 (E) or 1 and 5 (F), providing neither peptide in each case was present in excess. However, peptide 7, corresponding to an alternative acidic rod domain sequence region, did not disassemble preformed mouse K1/K10 KIF (G) and could not protect KIF from disassembly by peptide 1 (H). Bar, 100 nm.
The Critical Roles of the H1 (and Possibly H2 Subdomains) for KIF Assembly and Structure—One of the most striking implications of the present work is that it has provided insights into the role in KIF structure of least part of the end domains of the chains. KIF are unique in that they require both a type I and type II chain for assembly in vitro (32, 51) and in vivo (52), for the formation of the obligatory type I-type II heterodimer (4, 5). There are two important differences between these two chain types. One is that the rod domain segment 2 of type II chains is less acidic than in type I chains (53). The other is that type II chains only possess sequences immediately adjacent to the beginning and end of their rod domains which have been termed H1 and H2 domains, respectively (1, 28, 54), because these amino acid sequences have been conserved within the type II family. Indeed, a review of the GenBank and NBRF data files show that the H1 sequences represent one of the most conserved regions within the entire IF family of proteins. Secondary structure analyses using available algorithms (55) reveal for H1 (36 residues long) a likelihood of a turn (or loop)-$\beta$-strand-$\beta$-loop-$\alpha$-helix conformation, leading into the coiled-coil rod domain segment 1A. Similarly, the much shorter H2 (20 residues long) is also predicted to adopt a turn-$\beta$-strand-turn conformation (data not shown here). The $\beta$-strand region of H1 is similar in size to that of H2. Such sequences are therefore likely to adopt a folded conformation. Indeed, in order for a disulfide bond to form between the cysteine in H1 (3rd residue) and H2 (4th residue) in an antiparallel alignment, the H1 sequences must be folded back to be juxtaposed with cysteine 4 of H2 (Fig. 5). Thus it is possible that in the alignment of model C of Fig. 5, the H1 and H2 sequences dock with each other to form a $\beta$-sheet motif. In addition, the H1 sequences adjacent to the rod domain are basic in charge. Our models place the H1 and H2 sequences of one molecule adjacent to the canonical LLEGEE acidic region at the end of the rod domain of its nearest neighbor (Fig. 5, Model C) or the middle of the rod domain near the L2 region of its nearest neighbor (Fig. 5, Models B and D). The juxtaposition of such oppositely charged sequences thus raises the obvious possibility that the basic H1 subdomain can also dock with these acidic regions in the alignment of model D of Fig. 5 by simple ionic interactions. Several experiments have been performed to test these ideas.

In a previous study (45), we showed that the likely structure and charge characteristics of the H1 subdomain are critical features for KIF assembly and stability in solution: an H1 peptide corresponding to the H1 subdomain sequence promoted rapid disassembly of preformed K1/K10 KIF. We show in the present paper that the same synthetic H1 peptide is also capable of disassembling KIF containing the K5/K14 (Fig. 6, B and C) or K7/K8/K17/K18 chains of simpler epithelia, but not IF assembled from type III vimentin chains. Thus the phenomenon is a specific characteristic of most if not all KIF. This disassembly process indicates that KIF assembled in vitro are even more flexible and dynamic in solution than previously thought (5, 17, 23, 56). This disruption of KIF by the peptide can be explained by competition with the same sequence region of the intact chains, displacement of the intact chain and/or molecule, leading to collapse of KIF structural integrity. The SCW derivatives of the H1 and H2 peptides did not promote KIF disassembly (Ref. 45; Fig. 6, E and F), indicating the critical importance of the charged properties of the peptides. This was confirmed by experiments with the intact human K1 in which its sulfhydryl groups on the H1 and H2 subdomains had also been converted to their SCM derivatives; when mixed with normal K10, KIF assembly was inhibited (Fig. 7, C and D). Analysis on polyacrylamide gels of these two reactions showed that the KIF had been reduced to oligomers containing only two or three molecules (Figs. 6D and 7E). This establishes that the H1 subdomain plays an essential role in KIF structure at the two- to three-molecule stage of assembly. Interestingly, two types of acidic peptides could ameliorate the effect of the H1 peptide, corresponding to the canonical LLEGEE sequence at the end of the rod domain of all IF chains and of sequences in the vicinity of the L2 region of the K1 and K10 chains. This protection is apparently specific since other acidic peptides, corresponding to two regions unique to type I and type II keratin chains (peptides 7 and 8), could not afford protection (Fig. 8). We interpret this to mean that the H1 region is not normally aligned or associated with these regions in KIF. Also, other neutral (peptide 9) and basic (peptides 10–12) peptides could not provide protection against disassembly by the H1 peptide. Accordingly, the data support the view that these acidic sequences interact directly and specifically with the basic H1 peptide sequences. Parenthetically, the LLEGGE peptides, but not L2 peptides, could also promote KIF disassembly (Fig. 8, C and D), exactly as described earlier (46), although in this case, the peptides resulted in apparent total disruption of KIF.

Other data exist to support these modes of interaction of the H1 subdomain in KIF. We have demonstrated that an L to P substitution in the $\beta$-strand motif of the H1 subdomain in human K1 causes epidermolytic hyperkeratosis (45). This mutation is likely to change the structure of the H1 subdomain. A synthetic peptide containing this mutation is markedly less effective in disassembly of KIF (45). Similarly, we note that a replacement of the first E of LLEGEE by a G in the K5 chain, thereby reducing the acidic nature of this canonical sequence, is the proximal cause of another disease, epidermolysis bullosa simplex (57).

There are several conclusions from these data. The first is that the H1 sequences have been built into the type II chains for the purpose of registration of the first two and/or three molecules during KIF assembly. This view thus provides a molecular explanation for the rate limiting step in KIF assembly (7); the alignment of a third (and/or fourth) molecule antiparallel and in register with respect to the more abundant two-molecule species appears to be the most difficult equilibrium step during assembly. Also, the critical importance of these sequences in the proper registration of the first two or three molecules explains why the H1 sequences have been conserved throughout evolution of the entire type II family and why a mutation that significantly changes likely structure of the H1 subdomain is the proximal cause of a serious pathology (45). Moreover, this conclusion means that a type II chain is an obligate component of the coiled-coil molecule. This line of reasoning does not preclude the possibility that a type II homodimer could successfully dock with the usual type I-type II heterodimer molecule. Nonetheless, greater structural and functional specificity is built into a heterodimer than a type II homodimer, since previous data have established that heterodimers are thermodynamically more stable than homodimers (5, 6). Interestingly, in differentiating cell types that switch from one set of keratin chains to another, e.g. K5/K14 of basal epidermal cells to K1/K10 of terminal differentiating epidermal cells, the type II chain (K1) is always expressed prior to its type I partner (K10) (58), implying that type II homodimer molecules can be successfully accommodated into the cytoskeleton (59). Perhaps the present data...
afford a molecular explanation for this order of expression. We were unable to perform similar direct experiments with the H2 peptide, but assume that because its amino acid sequence and thus likely structure has been well conserved, it also may have an obligatory role in KIF assembly and stabilization.

We have been able to take advantage of the absence of cysteine residues in the rod domain of the human K1 chain (as compared with the mouse K1 chain and mouse and human K10 chains) to explore the critical roles of the cysteines in the H1 and H2 domains for molecular alignments at the two- to three-molecule levels of KIF structure (Fig. 7). We believe that the other cysteines in the rod domains of the chains have evolved to aid in chain and/or molecule alignments in KIF assembly; interestingly, there is a conserved cysteine near the center of rod domain segment 2B in mouse and human K10s, and in mouse K1, and there are cysteines one-third and two-thirds along this same domain. This view may explain why two- and three-molecule disulfide bond-cross-linking oligomers are still assembly competent (e.g. Fig. 6A4), whereas the insertion of extra charges may interfere with ionic interactions (2, 37, 38). On the other hand, available data have shown that the inapparant substitution of a cysteine in the rod domain in the K14 chain can promote severe pathology (48). We did not find any disulfide bonds involving the cysteine at residue 88 of the 2B rod domain of the K10 chains, but we suspect these too are important probably at higher levels of molecule alignments. Cysteine residues in the V1/V2 domains readily form disulfide bonds in high yield to confirm the alignments described here and further suggest these unusual sequences fold into a more compact glycine loop conformation (39). The structural/functional significance of an optional cysteine residue in V2 of the human K1 chain is not clear at this time.

Although we have been unable to perform direct experiments on the H2 subdomain, the conservation of these sequences throughout the type II keratin family is striking and points to its critical importance for KIF structure. To date only one study has involved experiments with the H2 domain. Mutant K8 chains with deletions from its carboxyl termini (60), including H1 subdomain and its role in KIF.

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