

Sorting Out IF Networks: Consequences of Domain Swapping on IF Recognition and Assembly

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Abstract. Vimentin and keratin are coexpressed in many cells, but they segregate into two distinct intermediate filament (IF) networks. To understand the molecular basis for the sorting out of these IF subunits, we genetically engineered cDNAs encoding hybrid IF proteins composed of part vimentin and part type I keratin. When these cDNAs were transiently expressed in cells containing vimentin, keratin, or both IFs, the hybrid IF proteins all recognized one or the other or both networks. The ability to distinguish networks was dependent upon which segments of IF proteins were present in each construct. Constructs containing sequences encoding either helix 1B or helix 2B seemed

to be the most critical in conferring IF recognition. At least for type I keratins, recognition was exerted at the level of dimer formation with wild-type type II keratin, as demonstrated by anion exchange chromatography. Interestingly, despite the fact that swapping of helical domains was not as deleterious to IF structure/function as deletion of helical domains, keratin/vimentin hybrids still caused structural aberrations in one or more of the cytoplasmic IF networks. Thus, sequence diversity among IF proteins seems to influence not only coiled-coil but also higher ordered associations leading to 10-nm filament formation and/or IF interactions with other cellular organelles/proteins.

INTERMEDIATE filaments (IFs)¹ are composed of proteins (40–210 kd) which form coiled-coil dimers that further assemble in a hierarchy of higher ordered interactions, culminating in 10-nm cytoskeletal filaments (for reviews see Fuchs et al., 1987; Weber and Geisler, 1987; Aebi, 1988; Steinert and Roop, 1988; Parry, 1990). IF assembly is a self-driven process, dependent upon the primary amino acid sequence of IF polypeptides. Each IF subunit has a central α -helical domain (\sim 310 residues for cytoplasmic IFs; 356 residues for lamins) interrupted by three short nonhelical spacer segments (Hanukoglu and Fuchs, 1982, 1983; Geisler and Weber, 1982; Crewther et al., 1983; Steinert et al., 1983). While precise demarcation of domains depends upon the theoretical methods used, these domains are typically referred to as helix 1A, 1B, 2A, and 2B (for review see Conway and Parry, 1988). The first two linker segments, L1 and L12, vary slightly in length among IF proteins. The last linker, L2, is conserved in length, as are all four α -helical domains. The major feature driving assembly of the coiled-coil is a heptad repeat of hydrophobic residues, where the first (a) and fourth (d) amino acids of every seven (abcdefg) in the α -helical sequence are hydrophobic (McLachlan and Stewart, 1975; McLachlan, 1978). In addition, helices 1B, 2A, and 2B contain a periodic distribution of acidic and basic residues. The presence and location of these charged residues suggests that ionic interactions may play a role in as-

sembly of IF subunits into dimers, tetramers, and/or higher ordered structures (Parry et al., 1977; Henderson et al., 1982; McLachlan and Stewart, 1982; Conway and Parry, 1988). Combinations of apolar, acidic, and basic periodicities may result in larger unit periodicities encompassing nearly entire helical segments (Conway and Parry, 1988).

The classification of IF subunits into five distinct types is based upon sequence homologies within the central α -helical rod of different IF proteins (for review see Fuchs et al., 1987; Steinert and Roop, 1988; Conway and Parry, 1988). Different IF types typically share \sim 25–40% sequence identity in this segment, with the amino and carboxy ends of the rod the most highly conserved (\sim 90–99% identity) and the spacer domains the least conserved (\sim 5–20% identity). In contrast, the nonhelical end domains are highly variable in size and in sequence, even for individual members within a particular type. While some sequence variation can be tolerated for the *a* and *d* residues constituting the hydrophobic heptad repeat, substitutions at these sites are usually conservative. Most of the sequence variation in the α -helical domains occurs at residues *b*, *c*, and *f* on the outer surface of the coiled-coil (Conway and Parry, 1988). However, slight variations in the length of both acidic and basic repeats of different IF types have been noted, suggesting that coiled-coil interactions may be different for different IF classes (Conway and Parry, 1988).

Most IF types, including type III (vimentin, desmin, peripherin, and glial fibrillary acidic protein), can assemble into homopolymers (Steinert et al., 1981). In contrast, kera-

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

tins are subdivided into type I (K9-K19; pKi = 4.5-5.5; 40-56.5 kD) and type II (K1-K8; pKi = 5.5-7.5; 53-67 kD). Under most conditions, type I and type II keratins form heterodimers (Coulombe and Fuchs, 1990; Hatzfeld and Weber, 1990; Steinert, 1990). Under some conditions, homodimers have been described (Hatzfeld and Weber, 1990; Steinert, 1990), although these may not be competent for 10-nm filament formation (Hatzfeld and Weber, 1990). In tissue culture cell lines that express both vimentin and keratins (Osborn et al., 1980), there is segregation into two distinct IF networks. Thus, in spite of a high degree of structural homology among all IFs, sequences unique to different types seem to enable IF proteins to distinguish among themselves, thereby sorting out filament networks in vivo. The process of IF recognition also appears to take place in vitro (Hatzfeld et al., 1987). However, it has not been unequivocally resolved whether the process of IF sorting takes place at the level of the coiled-coil or at the level of tetramer formation.

A priori, a number of factors might account for IF sorting. One possibility is that a specific amino acid sequence(s) unique to each IF type confers IF subunit recognition. It is also possible that the acid and/or basic periodicity within each IF type might confer the ability of an IF subunit to either recognize self (type III) or specific partners (type I and II). Alternatively, a combination of acidic and basic periodicity and apolar heptad motifs might generate a unique pattern for each IF protein. To begin to distinguish between these possibilities, we have constructed cDNAs encoding hybrid IF proteins, consisting in part of the type III IF protein, vimentin, and in part the type I epidermal keratin, K14. To ensure that our hybrids did not perturb the heptad repeat of hydrophobic residues or the periodicity of acid and basic residues, we used site-directed mutagenesis to engineer convenient restriction endonuclease sites. This enabled us to swap either nonhelical domains, or one or more of the four individual α -helical segments. Using transient transfection experiments in mammalian cells and IF complex formation with bacterially expressed hybrid proteins, we delineated which of these segments are most important for subunit recognition and sorting out of IF networks. In addition, we demonstrated that for keratins, recognition takes place at the level of the coiled-coil, although swapping of IF domains also affects higher ordered interactions.

Materials and Methods

Construction of Vimentin/Keratin Hybrids

A full-length human vimentin cDNA clone, beginning 144 nucleotides upstream from the ATG initiation codon and extending to 36 nucleotides 3' from the polyadenylation signal, was obtained from Dr. Edward Gelmann (Sommers et al., 1989; for sequence see Ferrari et al., 1986). A full-length human keratin K14 cDNA was constructed from a genomic clone and a cloned cDNA as described previously (Albers and Fuchs, 1987).

Addition of sequences encoding the P-tag for mammalian expression vectors. cDNA sequences encoding the P-tag, *Drosophila hsp70* 3' noncoding sequences and polyadenylation signal were added 3' and in-frame to sequences encoding the entire human K14 up to amino acid residue #466 (#471 is the end) as described previously (Albers and Fuchs, 1987). These sequences were also added 3' and in-frame to sequences encoding the entire human vimentin up to amino acid residue #461 (#465 is the end). To facilitate cloning, site-directed mutagenesis was used to engineer a BamHI site 12 nucleotides 5' to the TAA stop codon of the vimentin cDNA. A BamHI site 5' to the P-tag and TGA stop codon of pS-K14P was engineered previously (Albers and Fuchs, 1987).

Making the Coding Segment of the Hybrids

V₆KKKKP. Sequences encoding the first 80 amino acid residues in the nonhelical head domain of human vimentin were spliced to K14 sequences beginning at amino acid residue 115 near the start of the α -rod domain using conventional cloning methods.

All others. The coding portions of all vimentin-keratin expression vectors except for pS-V₆KKKKP were constructed by engineering unique restriction sites into the sequences encoding the linker regions that dissect K14 and vimentin into four discrete α -helical domains (see Hanukoglu and Fuchs, 1983). In all cases, the Kunkel method of site-directed mutagenesis was used (Kunkel, 1985). Sites introduced into the vimentin cDNA were: (a) a KpnI site in linker L1, between helices 1A and 1B, at the corresponding position of the existing KpnI site in K14; and (b) a NotI site in linker L2, between helices 2A and 2B. A natural ClaI site existed in linker L12, between helices 1B and 2A, and this was also used in hybrid construction. Sites introduced into the K14 cDNA were: (a) a ClaI site in linker L12 between helices 1B and 2A, at the corresponding position of the natural ClaI site in the vimentin cDNA; and (b) a NotI site in linker L2 between helices 2A and 2B, at the corresponding position of the engineered NotI site in the vimentin cDNA. A natural KpnI site existed in linker L1, between helices 1A and 1B.

Below are the sequences in the linker domains of human K14 and vimentin, followed by the sequences of hybrid linkers generated when a helical domain of one IF protein is linked to a helical domain of the other. Number in parentheses indicates length of the linker segment. Underlined residues indicate a change in amino acid sequence as a consequence of cloning.

L1

KERATIN I RDWY - QRQ RPAE I KDYS P YFK (21)
 VIMENTIN LE Q - - LKGQ GKS R L GDL - YEE (18)
 KER/VIM I RDWY Q LKGQ GKS R L GDL - YEE (21)
 VIM/KER LE Q R Y - QRQ RPAE I KDYS P YFK (21)

L12

KERATIN R G Q V G G D V N V E M D A A P G V (18)
 VIMENTIN Q A Q I Q E Q H V Q I D V D V S K P (18)
 KER/VIM R G Q V G G D V N V I D V D V S K P (19)
 VIM/KER Q A Q I Q E Q H V Q I - D A A P G V (17)

L2

KERATIN A T N S E L V Q S G K S (12)
 VIMENTIN N R N N D A L R Q A K Q (12)
 KER/VIM A T N S A A A R Q A K Q (12)
 VIM/KER N R N N A A A Q S G K S (12)

Expression vectors. P-tagged cDNAs were used in mammalian expression vectors, but not in bacterial expression vectors. For expression in mammalian cells, P-tagged hybrid cDNAs were inserted into plasmid pJay1, containing the major early promoter and enhancer of SV-40, 5' upstream from a multiple cloning site (see Albers and Fuchs, 1987). For expression in bacteria, untagged cDNAs were inserted into plasmid pET-8c containing a T7 RNA polymerase promoter located just 5' from an NcoI site (Studier et al., 1990). Plasmids pET-K14 and pET-K5 were described previously (Coulombe and Fuchs, 1990). Hybrid clones with vimentin sequences at the 3' end contained sequences encoding all but four carboxy terminal residues. Hybrid clones with keratin sequences at the 3' end contained sequences encoding the entire carboxy terminus of K14. All plasmids were subsequently used to transform *Escherichia coli* strain BL21 (DE3), containing a cloned T7 RNA polymerase gene under control of the lacUV5 promoter (Studier et al., 1990). When induced with IPTG, the T7 polymerase gene is expressed, leading to induction of the target gene/cDNA inserted into pET-8c.

DNA Sequencing

To determine that each clone was correct and in the proper reading frame, all junctions were sequenced using the double-stranded sequencing protocol (Chen and Seeburg, 1985).

Cell Culture and Transfection of Hybrid Constructs into Mammalian Cells

PtK2 cells were cultured as described previously (Albers and Fuchs, 1987). MCF-7 and BHK cells were maintained in a 1:1 mixture of DME and Ham's F12 medium supplemented with 10% calf serum. DNAs were transfected into each cell line as described previously (Albers and Fuchs, 1987). PtK2

cells were routinely treated +/- colcemid (5×10^{-7} to 10^{-6} M; Boehringer-Mannheim Biochemicals, Indianapolis, IN) for 12–24 h before fixation (Franke et al., 1979). MCF-7 cells transfected with vimentin constructs were also subjected to colcemid analysis.

Immunofluorescent Labeling

After transfection, cultured cells on glass chamber slides were washed with PBS, fixed in methanol (-20°C for 10 min), and rinsed with PBS. P-tagged proteins were detected with a rabbit polyclonal anti-P antiserum (Wako Chemicals, Richmond, VA). Endogenous keratin networks were detected using either mouse monoclonal LE41 (anti-K8; Lane, 1982) or rabbit polyclonal anti-human K18 (McCormick and Fuchs, unpublished results). Endogenous vimentin networks in PtK2 and BHK cells were detected using a mouse monoclonal anti-porcine vimentin (anti-vim; Boehringer-Mannheim Biochemicals). Secondaries used were: FITC-conjugated goat anti-rabbit (Cappel Laboratories, Inc., Cochranville, PA; and Tago, Burlingame, CA), Texas red-conjugated goat anti-mouse (Tago), and Fluoroblock-conjugated goat anti-mouse (Biomedica Biochemicals, Foster City, CA).

Fast Performance Liquid Chromatography Purification of IF Proteins from Bacterial Extracts

Isolation of IF-containing inclusion bodies from total bacterial proteins, and subsequent purification of IF proteins by fast performance liquid chromatography (FPLC) anion exchange chromatography was achieved as described previously (Coulombe et al., 1990).

Complex Formation and Isolation

Pure preparations of K5 and hybrid IF proteins in 6 M urea buffers were combined in near equimolar amounts, but with slight hybrid excess, in a final volume of 1 ml and at a final concentration of 0.8–1.0 mg/ml. After 10 min at room temperature, mixtures were stored at 4°C until further processed. Complexes formed in 6 M urea buffer were separated from uncomplexed proteins using anion-exchange chromatography and the same conditions as above. Fractions were collected and analyzed by SDS-PAGE.

In Vitro Reconstitution of 10-nm Filaments

Anion exchange fractions were pooled, and complexes were concentrated and desalted by low speed centrifugation through Centricon 10 microconcentrator units (Amicon, Danvers, MA). Complex mixtures were then suspended in 6 M urea, 50 mM Tris HCl, 2 mM dithiothreitol, 0.3 mg/ml PMSF, pH 8.1, to a final concentration of 150 μg protein/ml. Samples were then dialyzed against 6.5 M urea, 10 mM Tris HCl, 10 mM β -mercaptoethanol, pH 7.20, for 3 h at room temperature, followed by 12 h at 4°C against 5 mM Tris HCl, 10 mM β -mercaptoethanol, pH 7.20, and finally 10 mM Tris HCl, 10 mM β -mercaptoethanol, pH 7.20 for 3 h at 4°C . Preparations were then stored at 4°C until negatively stained and examined under an electron microscope (model CM10; Philips Electronic Instruments, Inc., Mahway, NJ) as described previously (Coulombe et al., 1990). For quantitation of filament width, 36 filaments taken randomly from micrographs were analyzed.

Results

Preparation of Hybrid Vimentin-Keratin cDNA Constructs

The constructs prepared for this study are illustrated in Fig. 1. To maintain the periodicity of the heptad repeat of hydrophobic residues and the acidic/basic residues within the central rod domain, site-directed mutagenesis was used to engineer restriction endonuclease sites at the boundaries of each encoded α -helical domain of human vimentin and human type I keratin K14. Domains were then swapped using traditional cDNA subcloning procedures as outlined in Materials and Methods.

For transient gene transfection and expression of human vimentin-keratin hybrid cDNAs in a variety of cultured epithelial cells and fibroblasts, the SV-40 enhancer and major

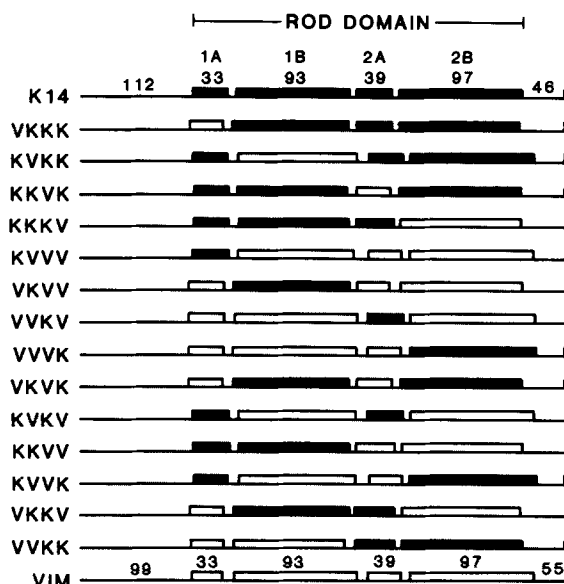


Figure 1. Vimentin and keratin hybrid constructs. Details of plasmid constructions are in Materials and Methods. (K14) The type I human epidermal keratin K14; (VIM) the type III human vimentin. Hybrid proteins are referred to as XXXX, and the series of X's corresponds to the four α -helical domains (1A, 1B, 2A, and 2B), which are either vimentin (V) or keratin (K). Rod domain is as shown, with demarcations taken from Hanukoglu and Fuchs (1983), with modifications from Coulombe et al. (1990). Nonhelical head (amino terminal) and tail (carboxy terminal) domains are indicated by thin lines, as are linker regions separating the helical segments. The numbers refer to numbers of amino acid residues in each domain. (■), K14 α -helical segments; (□), vimentin α -helical segments. In all cases, the IF identity of each head domain corresponds to that of helix 1A and the IF identity of each tail domain corresponds to that of helix 2B.

early promoter were used (Giudice and Fuchs, 1987). To distinguish expression of the hybrid from endogenous IF networks, a sequence encoding the antigenic portion of neuropeptide substance P was added in place of sequences encoding the last four residues (vimentin) or five residues (K14) of the IF proteins. For in vitro studies, we overexpressed cDNAs in bacteria and purified milligram quantities of hybrid IF proteins (Coulombe and Fuchs, 1990). In this case, P-tags were not necessary.

Constructs are referred to as pS-XXXXP or pET-XXXX (p, bacterial plasmid; S, SV-40 enhancer/promoter; ET, bacterial expression vector [Studier et al., 1990]; P, P-tag and the four X's in sequence refer to whether α -helical domains 1A [33 amino acid residues], 1B [93 residues], 2A [39 residues], and 2B [97 residues], respectively, are vimentin [V] or K14 keratin [K] [for demarcations see Hanukoglu and Fuchs, 1983; Coulombe et al., 1990]). Unless indicated, the type of IF sequence chosen for the nonhelical head (112 residues K14; 99 residues vim) or tail (46 K14; 55 vim) domains was also the same type used for helix 1A and 2B, respectively.

Expression of K14, K14-P, Vimentin, and Vimentin-P in MCF-7 Epithelial and BHK Fibroblast Cells

To examine the behavior of P-tagged K14 in epithelial and

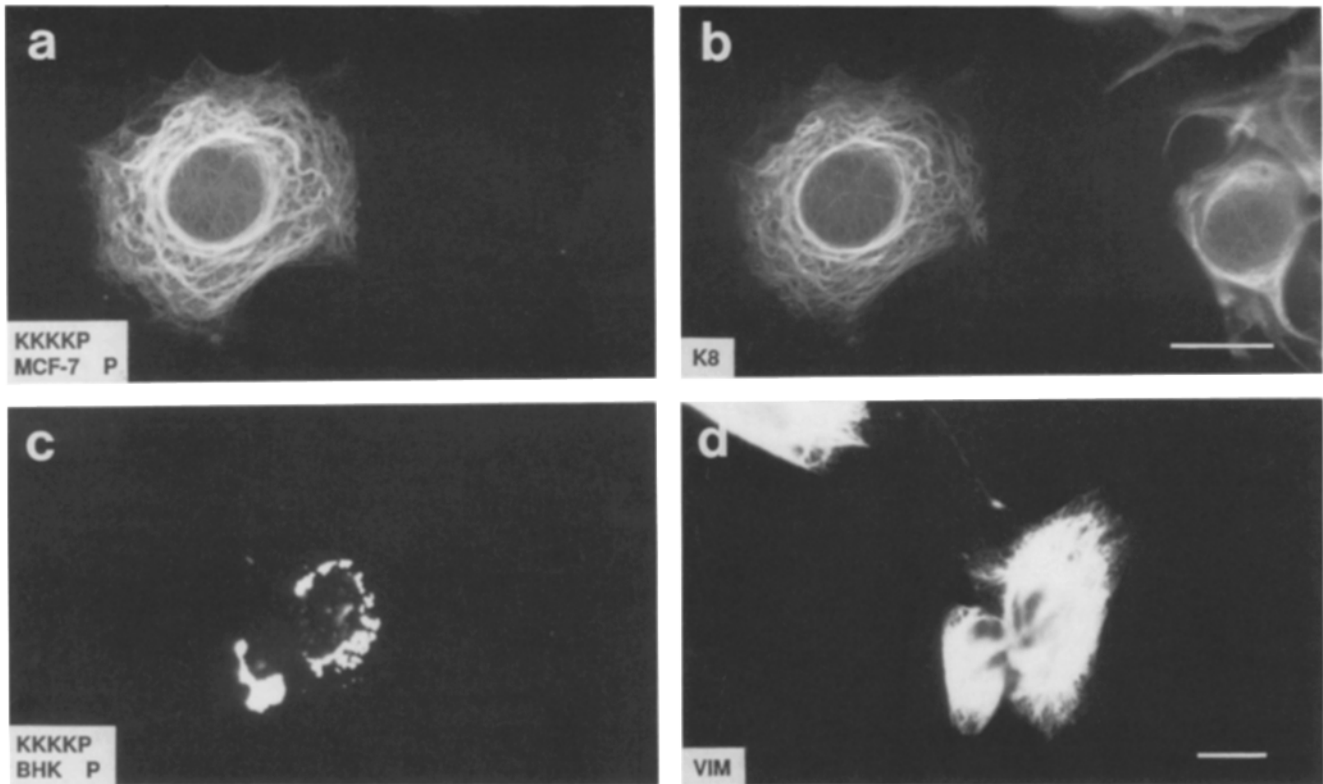


Figure 2. Transfection of MCF-7 and BHK cells with pS-K14P: P-tagged K14 integrates into keratin networks and doesn't recognize vimentin networks. MCF-7 simple epithelial cells (K8⁺, K18⁺, and K19⁺) and BHK fibroblasts (vimentin⁺) were transfected with pS-K14P. 65 h posttransfection, cells were fixed and subjected to indirect immunofluorescence as described in Materials and Methods. Transfected MCF-7 cell, costained with anti-P (a) and anti-K8 (b). Transfected BHK cells stained with anti-P (c) and anti-vim (d). Note: for this and subsequent figures, the hybrid expressed and the cell type involved are indicated on the first of each pair of frames. The antibody used for staining is indicated on each frame. Bars, 30 μ m.

fibroblast cells, we transfected pS-K14P into MCF-7 (K8⁺, K18⁺, K19⁺; Moll et al., 1982), PtK2 (K7⁺, K8⁺, K18⁺, K19⁺, vimentin⁺; Franke et al., 1978), and BHK cells (vimentin⁺; Steinert et al., 1981). 65 h after transfection, cells were fixed and subjected to double immunofluorescence (Fig. 2).

As expected from previous studies (Giudice and Fuchs, 1987; Albers and Fuchs, 1987), K14P integrated into the endogenous keratin filament network of MCF-7 cells (shown) and PtK2 cells (not shown) without any noticeable perturbation (Fig. 2 a, anti-P; Fig. 2 b, anti-K8). K14P did not recognize the vimentin network in BHK cells (Fig. 2 c, anti-P; Fig. 2 d, anti-vim), but rather accumulated in large cytoplasmic aggregates. These data are consistent with the knowledge that: (a) keratin and vimentin make distinct IF networks (Osborn et al., 1980); and (b) keratin filament formation requires both type I and type II keratins (Steinert et al., 1976; Franke et al., 1983).

When pS-Vim encoding wild-type human vimentin was transfected into MCF-7 cells, it formed an IF array, which upon close inspection, was distinct from the endogenous keratin IF network (Fig. 3 a, anti-vim; and Fig. 3 b, anti-K18; arrows denote region where the separate networks are visible). This was verified by treatment of transfected cells with colcemid (not shown), which causes the vimentin, but not keratin, network to collapse (Franke et al., 1979). In con-

trast to pS-Vim, pS-VimP encoding P-tagged vimentin did not form filaments in MCF-7 cells (not shown), and when introduced into PtK2 cells, it caused the endogenous vimentin network to collapse (Fig. 3 c-f). Slight perturbations were seen as early as 24-h posttransfection (Fig. 3 c, anti-P; and Fig. 3 d, anti-vim), and by 65 h, most networks were completely collapsed (Fig. 3 e, anti-P; and Fig. 3 f, anti-vim). At both early and late times after transfection, the keratin network remained largely unaffected (Fig. 3 g, anti-P; Fig. 3 h, anti-K8). Collectively, these findings indicate that either removal of sequences encoding the four COOH-terminal amino acids of vimentin and/or addition of sequences encoding the P-tag were sufficient to perturb the ability of human vimentin, but not human epidermal K14, to assemble into IF networks in vivo. The molecular basis for the deleterious effect of the P-tag on vimentin IF assembly is currently being investigated. Preliminary in vitro assembly studies have suggested that bacterially expressed human vimentin modified at the COOH terminus are not able to assemble into 10-nm filaments under standard vimentin assembly conditions. While these findings precluded examining the behavior of individual keratin domain exchange on vimentin filament network formation, the constructs did enable us to: (a) identify the domains responsible for vimentin and keratin IF recognition; and (b) examine the effects of domain swapping on keratin network assembly.

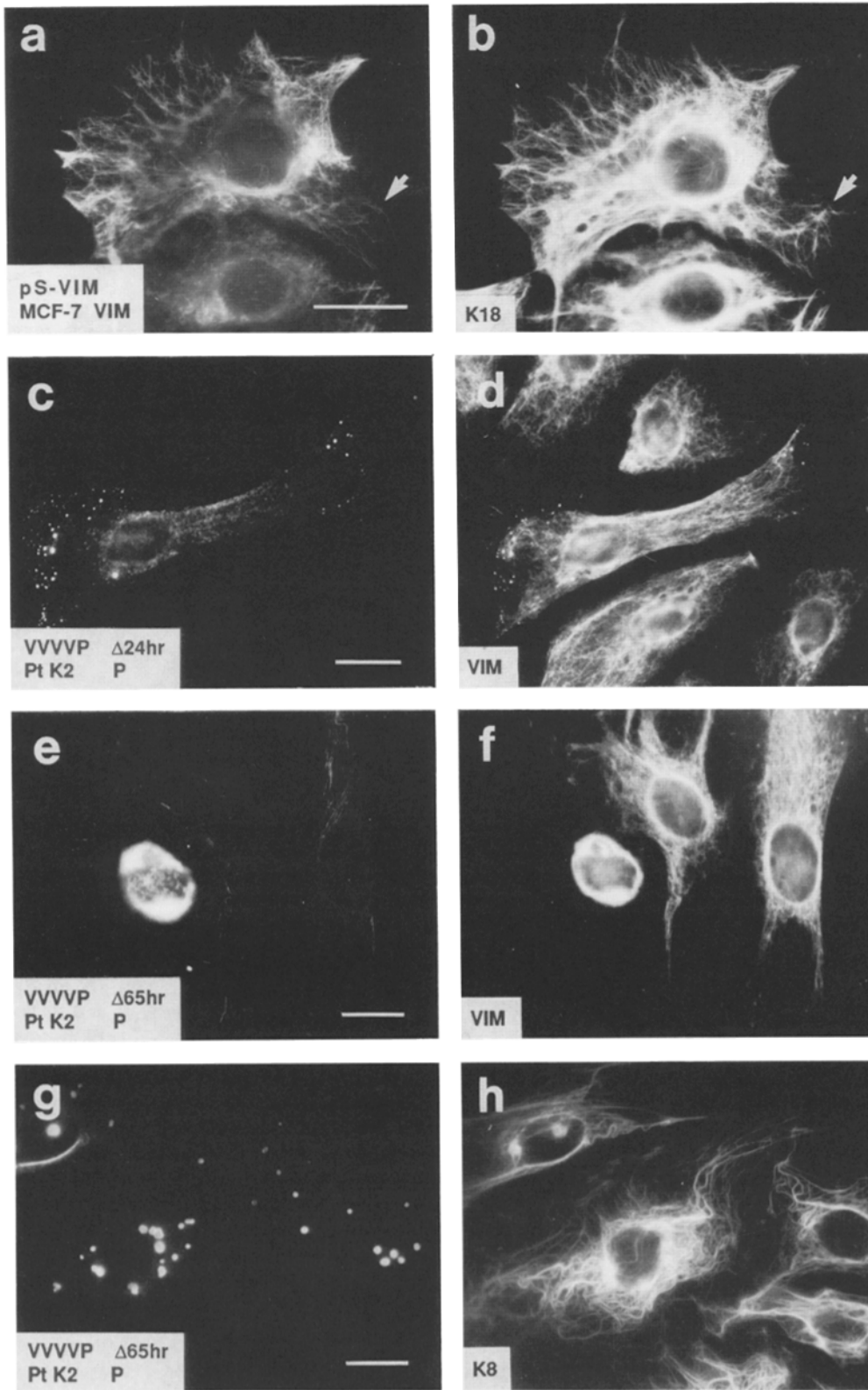


Figure 3. Transfection of MCF-7 and PtK2 cells with pS-Vim and pS-VimP: wild-type and P-tagged human vimentin recognize hamster vimentin networks, but P-tagged vimentin perturbs the network. MCF-7 cells (K8⁺, K18⁺, and K19⁺) and PtK2 cells (K7⁺, K8⁺, K18⁺, K19⁺, and vimentin⁺) were transfected with pS-Vim, encoding wild-type human vimentin and pS-VimP, encoding P-tagged human vimentin. 24 (c and d) or 65 (a, b, e, f, g, and h) h posttransfection, cells were fixed and subjected to indirect immunofluorescence (see Materials and Methods). pS-Vim-transfected MCF-7 cells stained with: (a) anti-vim; (b) anti-K18. pS-VimP-transfected PtK2 cells stained with: (c, e, and g) anti-P; (d and f) anti-vim; and (h) anti-K8. Note: for VVVVP and for other constructs containing the P-tagged COOH-terminal end of vimentin, the anti-vim recognizes both the hybrid and the endogenous vimentin protein. Bars, 30 μ m.

Hybrids with One α -Helical Domain of Vimentin and Three of Keratin

To examine the role of the nonhelical amino head domain and helix 1A in keratin recognition and network formation, we made VKKKP, containing the head and helix 1A domains of

vimentin (Fig. 1). When expressed in MCF-7 cells, VKKKP recognized and integrated into the keratin filament network (Fig. 4 a, anti-P; and Fig. 4 b, anti-K8). In doing so, however, it caused perinuclear collapse of keratin filaments, with some peripheral punctate aggregates. The deleterious effects could not be attributed to the vimentin head domain, since

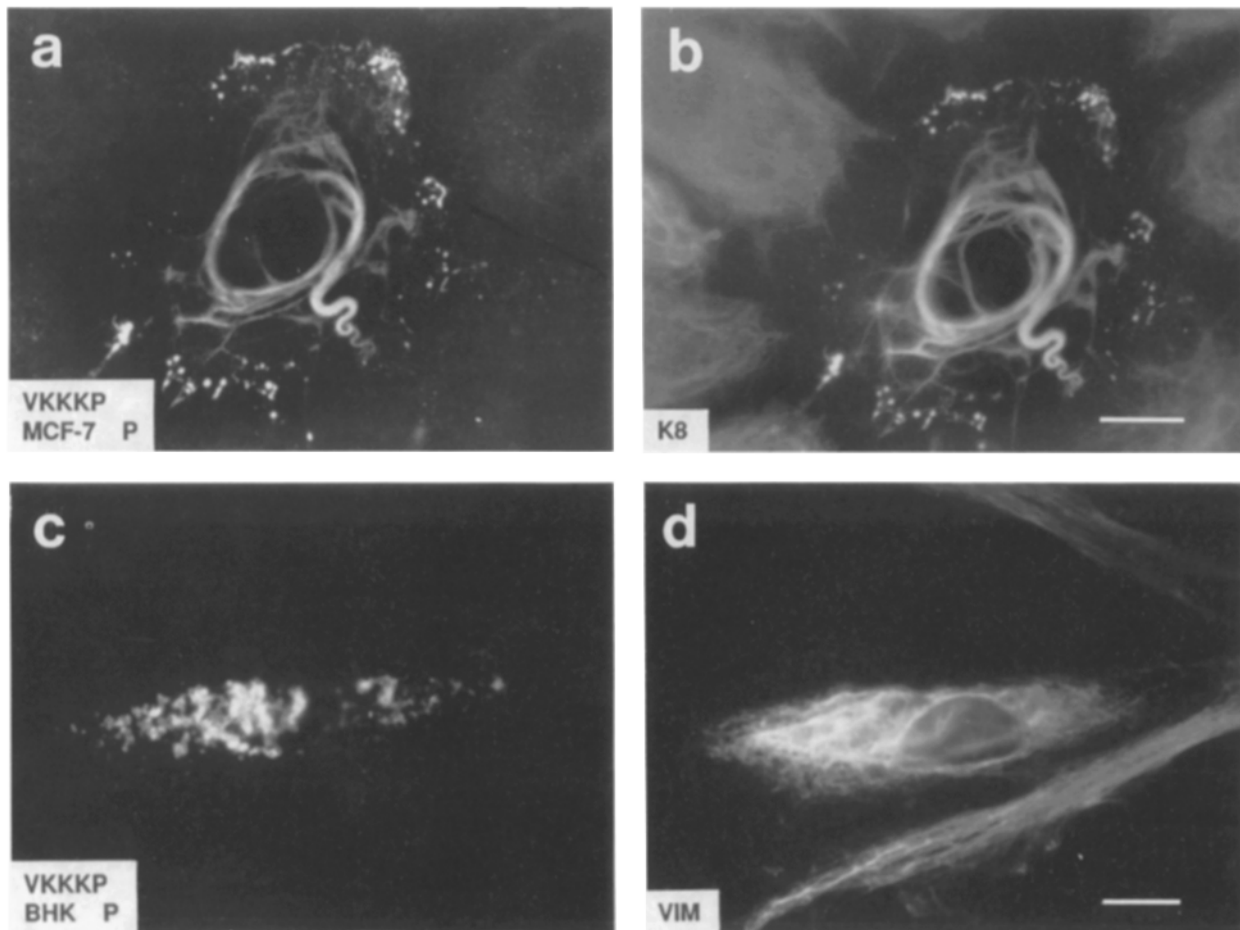


Figure 4. Transfection of MCF-7 and BHK cells with pS-VK444P: recognition and disruption of the keratin, but not the vimentin network. MCF-7 and BHK cells were transfected with pS-VK444P, encoding helix 1A of vimentin and the rest keratin. 65 h posttransfection, cells were fixed and subjected to indirect immunofluorescence as described in Materials and Methods. Transfected MCF-7 cell, costained with anti-P (*a*) and anti-k8 (*b*). Transfected BHK cells stained with anti-P (*c*) and anti-vim (*d*). Bars, 30 μ m.

a construct V₄K444P, with this segment of vimentin (V₄) and the rest keratin, integrated into the keratin filament network without noticeable perturbation (not shown). Thus, vimentin helix 1A was not able to fully compensate *in vivo* for K14 helix 1A. Interestingly, however, the phenotype generated with VK444P seemed to be less severe than that generated by Δ 150K14P, in which K14 head and helix 1A domains were deleted (Albers and Fuchs, 1989). Whether the inability to fully compensate stemmed from amino acid sequence differences in helix 1A (55% sequence identity between human vimentin and K14) or differences in sequences or spacing at the vimentin-keratin junction remains to be assessed.

To determine whether the vimentin head domain, including helix 1A, was sufficient for vimentin network recognition, we transfected pS-VK444P into BHK fibroblasts (Fig. 4 *c* and *d*). Most cells exhibited aggregates of VK444P (Fig. 4 *c*, anti-P) coexisting with a seemingly unperturbed endogenous vimentin network (Fig. 4 *d*, anti-vim). Occasionally, pS-VK444P caused collapse of the network (not shown), suggesting that this part of vimentin may have weak affinity for the vimentin network. Overall, however, the primary target for VK444P was the keratin filament network.

In contrast to VK444P, KV444P recognized and collapsed

the endogenous vimentin network in a majority of transfected BHK cells (Fig. 5 *a*, anti-P; and Fig. 5 *b*, anti-vim). However, in cells expressing both vimentin and keratin, this hybrid segregated primarily to the keratin network. Thus, in PtK2 cells, KV444P integrated into the keratin filament network (Fig. 5 *c*, anti-P; and Fig. 5 *d*, anti-K8), with only very weak or no staining of the vimentin network (not shown). Interestingly, the keratin filament network of many transfected PtK2 cells was indistinguishable from wild type (see two cells at left in Fig. 5, *c* and *d*), although perturbations in keratin network formation were still common in the pS-KV444P-transfected population (see cell at right in Fig. 5, *c* and *d*). While it was not possible to quantitate the amount of hybrid protein in each transiently transfected cell, the most severely affected cells were often those which stained the brightest with the anti-P antibody. Thus, it seems likely that the variation in phenotypes observed in this and subsequent transfected populations were because of variations in the ratio of hybrid to endogenous IF protein. The fact that more transfected cells were seemingly wild type when expressing KV444P rather than VK444P suggested that vimentin helix 1B may be more able than vimentin helix 1A to compensate for its keratin counterpart.

To investigate further the domains involved in IF recogni-

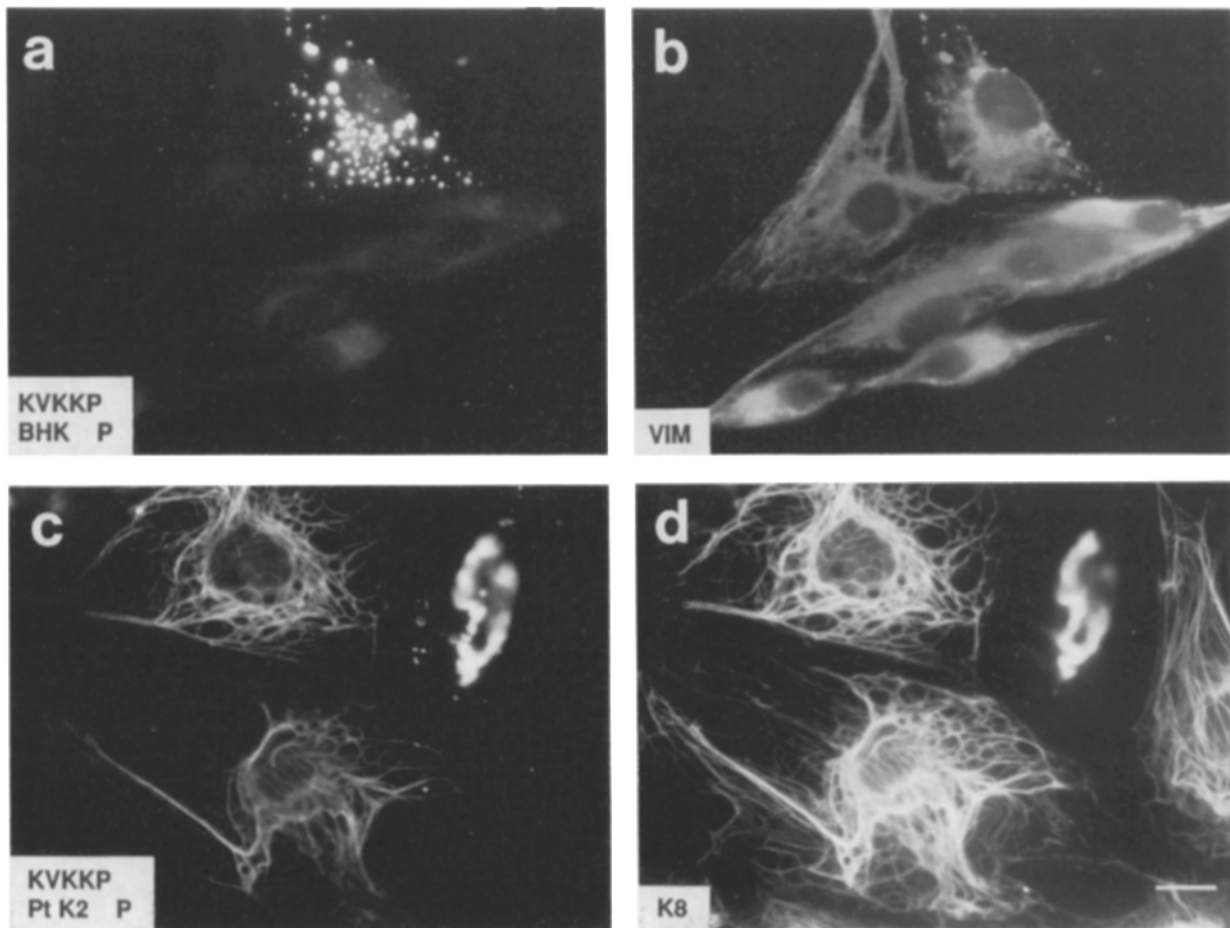


Figure 5. Transfection of BHK and PtK2 cells with pS-KVKKP: recognition and perturbations of both the vimentin and keratin networks. BHK and PtK2 cells were transfected with pS-KVKKP encoding helix 1B of vimentin and the rest keratin. 65 h posttransfection, cells were fixed and subjected to indirect immunofluorescence as described in Materials and Methods. pS-KVKKP-transfected BHK cells stained with anti-P (*a*) and anti-vim (*b*); PtK2 cells transfected with pS-KVKKP and stained with anti-P (*c*) and anti-K8 (*d*). Bars, 30 μ m.

tion and network formation, we made keratin constructs with the third or fourth α -helical domains exchanged with the corresponding segments of vimentin (Fig. 1). When expressed in BHK fibroblasts, KKVVP usually accumulated in aggregates, leaving the endogenous vimentin network intact (not shown). When expressed in either MCF-7 or PtK2 cells, KKVVP integrated into the keratin network, usually without perturbation (not shown). In contrast to KKVVP, KKKVP always recognized and disrupted the vimentin network of BHK cells (Fig. 6 *a*, anti-P; and Fig. 6 *b*, anti-vim), and frequently recognized and disrupted the vimentin network in PtK2 cells (not shown). As expected, this construct also recognized the keratin network (see examples of cells in Fig. 6 *c*, anti-P; and Fig. 6 *d*, anti-K8). In PtK2 cells, KKKVP recognized the keratin network more efficiently than the vimentin network. While the keratin network was often markedly perturbed, some transfected cells exhibited milder effects (see top examples in Fig. 6, *c* and *d*). Transfected MCF-7 cells also exhibited mild to severely perturbed networks (see bottom example in Fig. 6, *c* and *d*). Despite the sometimes deleterious effects of KKKVP, replacing the fourth α -helical domain of keratin with vimentin was still less severe than deleting it: a mutant keratin (C Δ 135K14P) missing the fourth α -helical domain of K14P completely disrupted the keratin

filament network in nearly all transfected PtK2 cells (Albers and Fuchs, 1987).

Collectively, our data on hybrids composed of one helix of vimentin and the rest keratin suggested that: (*a*) all four vimentin helices seemed to show some, but only partial, ability to compensate for their type I keratin counterparts; (*b*) vimentin helices 1B and 2A seemed better able to compensate than helices 1A and 2B; and (*c*) hybrids with vimentin helices 1B and 2B alone, but not helices 1A or 2A, were able to recognize the vimentin filament network, albeit with less affinity than for the keratin filament network.

Hybrids with One α -Helical Domain of Keratin and Three of Vimentin

To see whether a parallel existed between the domains required for keratin filament recognition and those involved in vimentin filament recognition, we made keratin-vimentin hybrids complementary to our vimentin-keratin hybrids. Analogous, but opposite to VKKKP, KVVVP recognized primarily vimentin rather than keratin networks. Thus in MCF-7 cells, KVVVP accumulated in cytoplasmic aggregates, with barely detectable associations with the endogenous keratin filament network (Fig. 7 *a*, anti-P; and Fig. 7 *b*, anti-K8). Punctate anti-P staining was usually concentrated

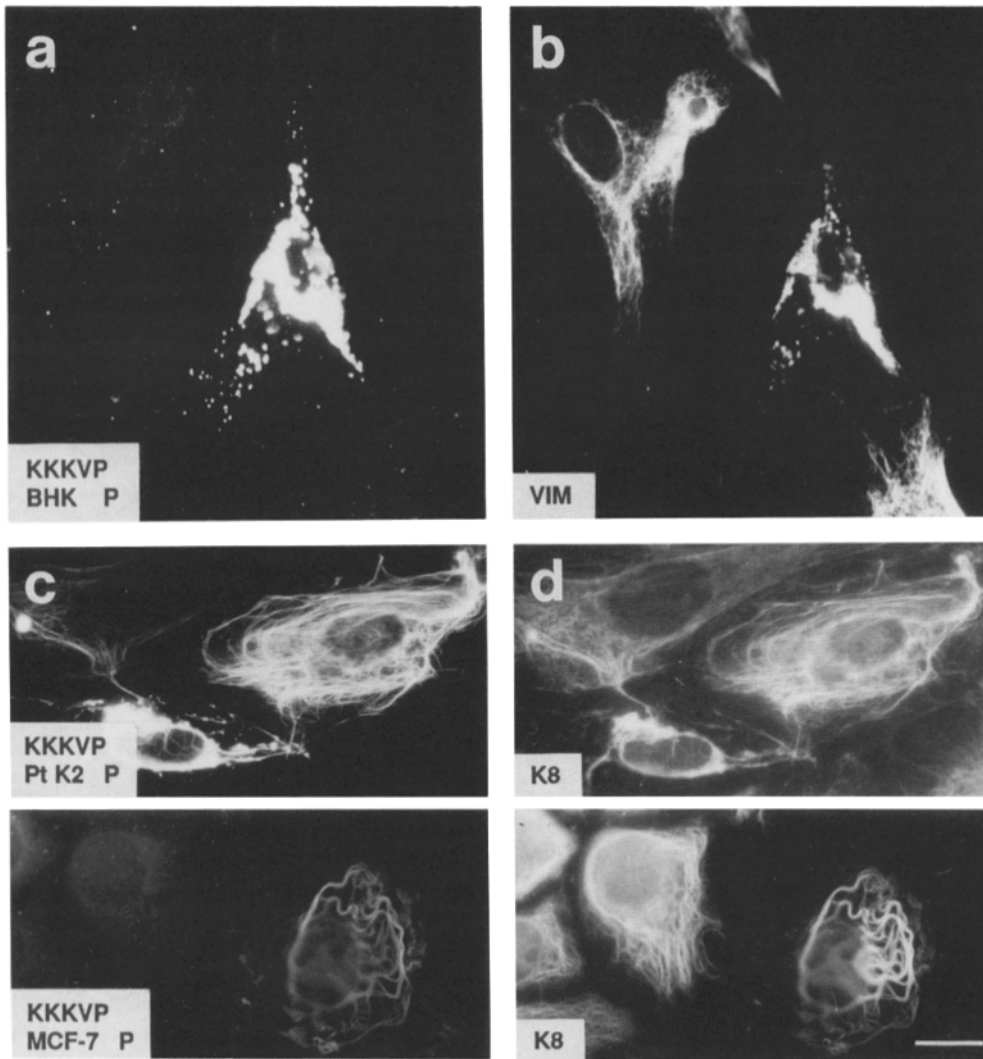


Figure 6. Transfection of BHK, PtK2, and MCF-7 cells with pS-KKKVP: recognition and perturbation of both vimentin and keratin networks. BHK, PtK2, and MCF-7 cells were transfected with pS-KKKVP encoding helix 2B of vimentin and the rest keratin. 65 h post-transfection, cells were fixed and subjected to indirect double immunofluorescence with anti-P and either anti-vim or anti-K8. pS-KKKVP-transfected BHK cell, costained with anti-P (a) and anti-vim (b). pS-KKKVP-transfected PtK2 cells (top pair) or MCF-7 cells (bottom pair) stained with anti-P (c) and anti-K8 (d). Bars, 30 μ m.

near the plasma membrane, and in these regions, the keratin network was somewhat retracted from the cell periphery (*arrowheads*, Fig. 7, a and b). Collectively, these observations suggest that KVVVP may cause subtle perturbations in the endogenous keratin network either through a weak interaction with keratin or through competition with keratin for binding to a membrane attachment site.

Similar to KVVVP, VKVVP showed strong recognition of the vimentin network in both BHK and PtK2 cells (not shown). In PtK2 cells, VKVVP recognized both networks, but anti-P staining was typically stronger over the vimentin network. In MCF-7 cells, VKVVP recognized and associated with the keratin network (Fig. 7 c, anti-P; and Fig. 7 d, anti-K8). While most VKVVP-expressing MCF-7 cells displayed a seemingly normal keratin filament network, the graininess of this anti-P staining was distinct from the uniform anti-P staining seen with KVKVP-expressing cells, suggesting that VKVVP may have decorated, rather than integrated into, the keratin filament network. Irrespective of this possibility, these results suggest that in contrast to helix 1A, helix 1B is sufficient for keratin recognition. Additional studies using *in vitro* complex formation confirmed this notion (see below).

Complementary to our results with KVKVP, VVKVP recognized vimentin networks in both BHK and PtK2 cells, but did not recognize keratin networks in MCF-7 or PtK2 cells (data not shown). In contrast, VVKVP not only recognized, but often disrupted, endogenous keratin filament networks. Keratin filament network disruption was frequent in MCF-7 cells which have no vimentin network, but perturbations of keratin networks were occasionally seen in PtK2 cells (Fig. 7 e, anti-P; and Fig. 7 f, anti-K8). Collectively, our results showed that: (a) helices 1B and 2B of keratin were the only keratin helices sufficient for keratin filament recognition; and (b) the hybrid containing keratin helix 2B was more disruptive to the keratin filament network than were the other hybrids containing only one helix of keratin and the rest vimentin.

Hybrids Composed of Half Vimentin and Half Keratin

Our results with vimentin-keratin or keratin-vimentin hybrids indicated that neither helix 1A nor helix 2A alone were sufficient for IF recognition. To examine whether exchange of both of these helices might confer IF recognition, we constructed VKVVP and expressed it in PtK2 cells (Fig. 8).

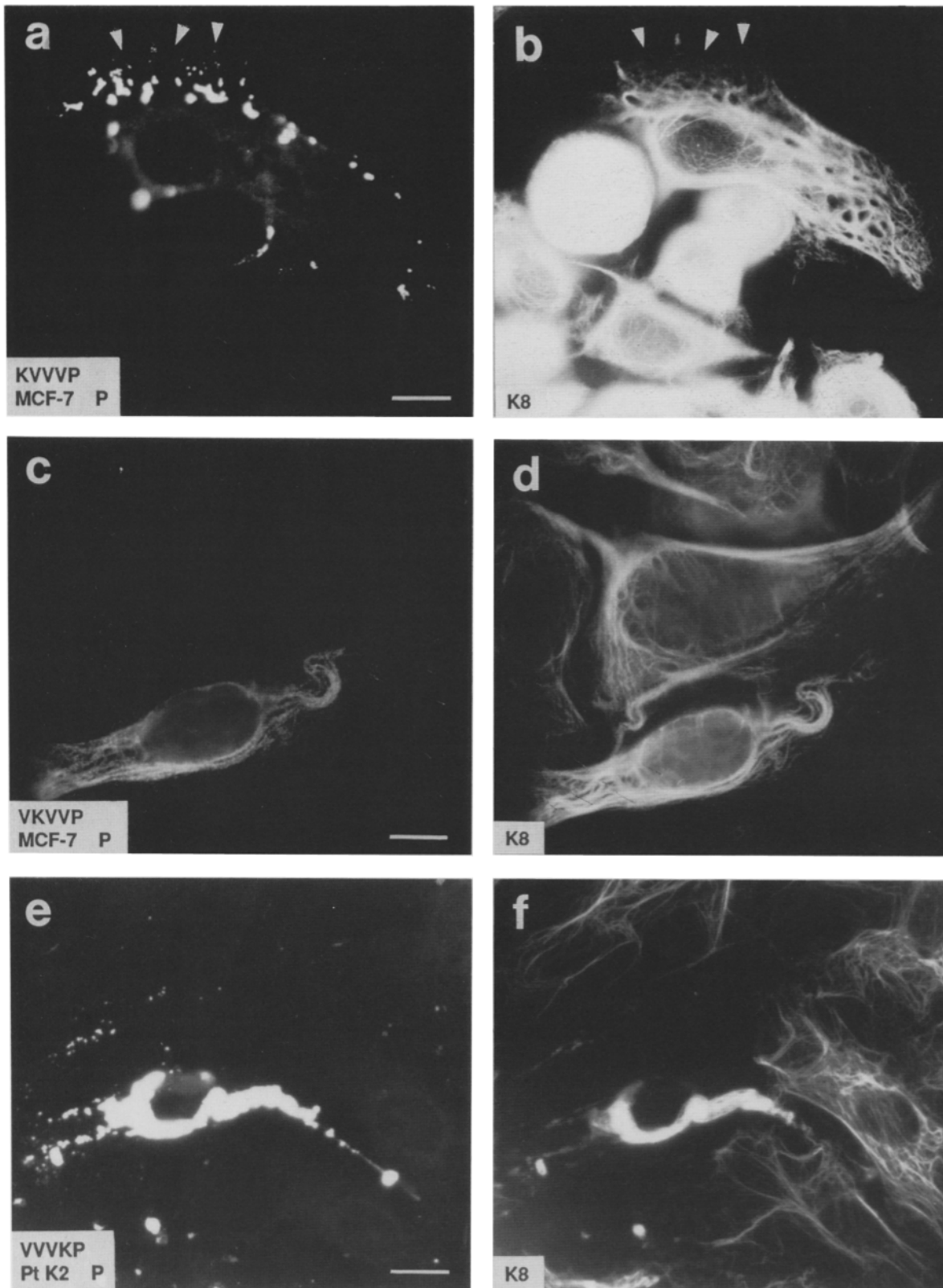


Figure 7. Transfection of MCF-7 and PtK2 cells with pS-KVVVP, pS-VKVVP, pS-VVKVP, and pS-VVVKP: keratin helices 1B and 2B, but not 1A or 2A, are each sufficient for keratin IF recognition. MCF-7 and PtK2 cells were transfected with pS-KVVVP, encoding helix 1A of keratin and the rest vimentin, pS-VKVVP encoding helix 1B of keratin and the rest vimentin, pS-VVKVP encoding helix 2A of keratin and the rest vimentin (not shown), and pS-VVVKP, encoding helix 2B of keratin and the rest vimentin. 65 h posttransfection, cells were fixed and subjected to indirect immunofluorescence as described in Materials and Methods. pS-KVVVP-transfected MCF-7 cells, costained with anti-P (*a*) and anti-K8 (*b*). pS-VKVVP-transfected MCF-7 cells stained with anti-P (*c*) and anti-K8 (*d*). pS-VVKVP-transfected PtK2 cells, costained with anti-P (*e*) and anti-K8 (*f*). Note that even though KVVVP did not recognize the keratin filament network efficiently, it caused at least partial withdrawal from the plasma membrane (note upper regions of the transfected cell). Note also that even though VKVVP is composed of mostly vimentin, it frequently labeled the keratin filament network without causing major perturbations in MCF-7 cells. In contrast to VKVVP, VVKVP had a deleterious effect on the keratin filament network. Bars, 30 μm .

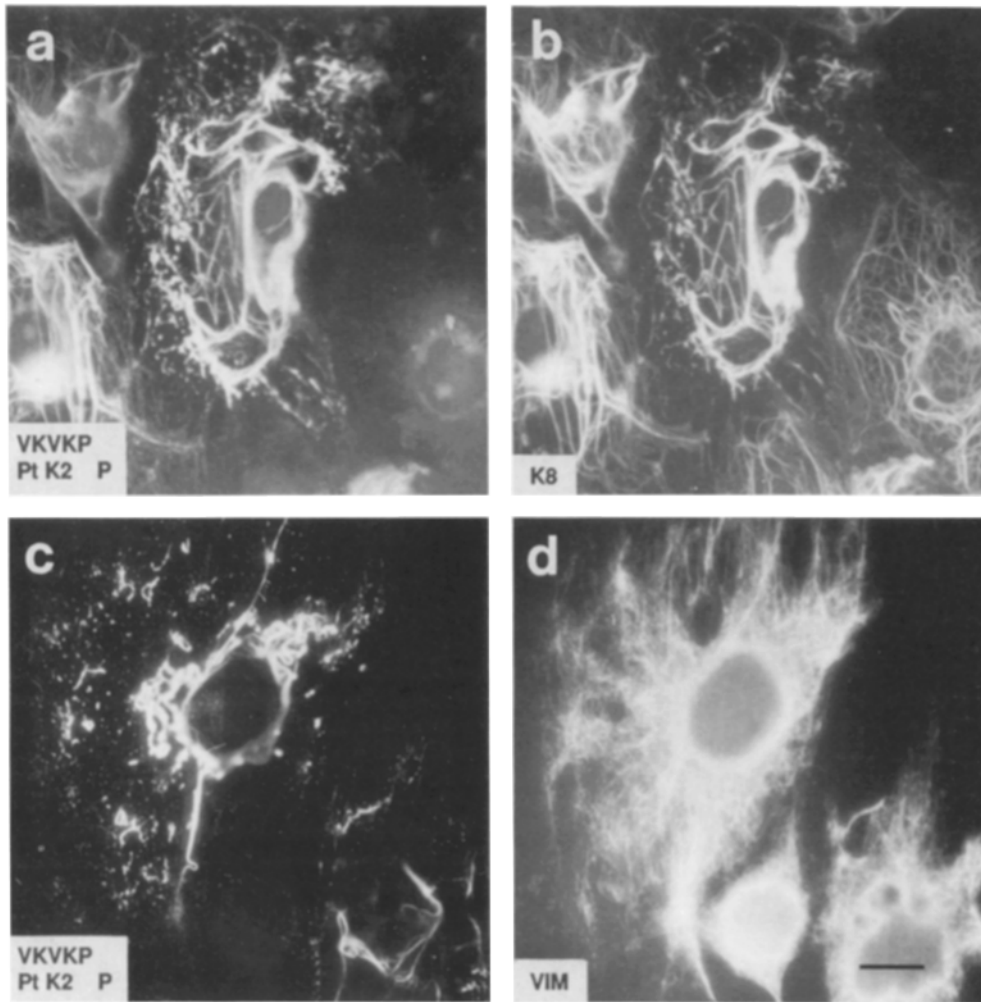


Figure 8. Transfection of pS-VKVVP into PtK2 cells: swapping helix 1A and 2A does not alter IF specificity. PtK2 cells were transfected with pS-VKVVP. 65 h posttransfection, cells were fixed and subjected to indirect immunofluorescence as described in Materials and Methods. pS-VKVVP-transfected PtK2 cells, costained with anti-P (*a*) and anti-K8 (*b*). Note that the hybrid IF protein recognized and disrupted the endogenous keratin network. pS-VKVVP-transfected PtK2 cells stained with anti-P (*c*) and anti-vim (*d*). Note that the hybrid IF protein did not recognize the vimentin network, but rather segregated into aggregates, leaving the vimentin network intact. Bars, 30 μ m.

Since VKVVP contained keratin helices 1B and 2B, we expected it to recognize and integrate into keratin filament networks in transfected epithelial cells. In all transfected PtK2 cells examined, the endogenous keratin network was collapsed or perturbed as a consequence of VKVVP expression (Fig. 8 *a*, anti-P; and Fig. 8 *b*, anti-K8). In MCF-7 cells, some transfected cells showed near normal keratin filament networks, although most were perturbed (not shown). In contrast, transfected PtK2 cells exhibited normal vimentin filament networks, which did not costain with anti-P (Fig. 8 *c*, anti-P; and Fig. 8 *d*, anti-vim). Occasionally in VKVVP-expressing BHK fibroblasts, the hybrid IF protein recognized and perturbed the endogenous vimentin network (not shown), suggesting that it had weak affinity for vimentin. Overall, however, despite 185 residues of vimentin, this hybrid did not interact efficiently with the endogenous vimentin network. Collectively, our results were consistent with our previous findings, and indicate that even though the hybrid VKVVP behaved as a keratin with respect to IF recognition, it was unable to assemble into a proper keratin filament network *in vivo*.

The behavior of KVKVP was complementary to that of VKVVP in transfected fibroblasts and epithelial cells (not shown). Thus, in PtK2 cells, KVKVP recognized vimentin, but not keratin networks. KVKVP caused collapse of a few

Table I. Summary of IF Network Recognition Studies

pS-XXXXXP	PtK2*	MCF-7	BHK*
KKKK	K ⁺⁺ /V ⁰	K ⁺⁺	V ⁰
VKKK	K ⁻ /V ⁰	K ⁻	V ⁰
KVKK	K ⁺ /V ⁻	K ⁺	V ⁻
KKVK	K ⁺⁺ /V ⁰	K ⁺⁺	V ⁰
KKKV	K ⁻ /V ⁻	K ⁻	V ⁻
KVVV	K ⁰ /V ⁻	K ⁰	V ⁻
VKVV	K ⁴ /V ⁻	K ⁺⁺	V ⁻
VVKV	K ⁰ /V ⁻	K ⁰	V ⁻
VVVK	K ⁻ /V ⁻	K ⁻	V ⁻
VKVK	K ⁻ /V ⁰	K ⁻	V ⁰
KVKV	K ⁰ /V ⁻	K ⁰	V ⁻
KKVV	K ⁻ /V ⁻	K ⁻	V ⁻
KVVK	K ⁻ /V ⁻	K ⁻	V ⁻
VVKK	K ⁻ /V ⁻	K ⁻	V ⁻
VVVV	K ⁰ /V ⁻	K ⁰	V ⁻

Summary of the recognition of IF networks by hybrid proteins in PtK2, MCF-7, and BHK cell lines. *K* and *V* indicate keratin and vimentin network recognition, respectively. (0) weak or no recognition (see text); (-) disruption of the network in >50% of transfected cells; (=) disruption of the network in >75% of transfected cells; (+) indicates recognition with little or no perturbation in >50% of transfected cells; (+ +) recognition with little or no perturbation in >75% of transfected cells. (*) All of the hybrid proteins which recognized the vimentin network caused a disruption of that network. The disruption may be because of the presence of the P-tag and/or the hybrid protein itself (see text). (4) Could not be evaluated (see text).

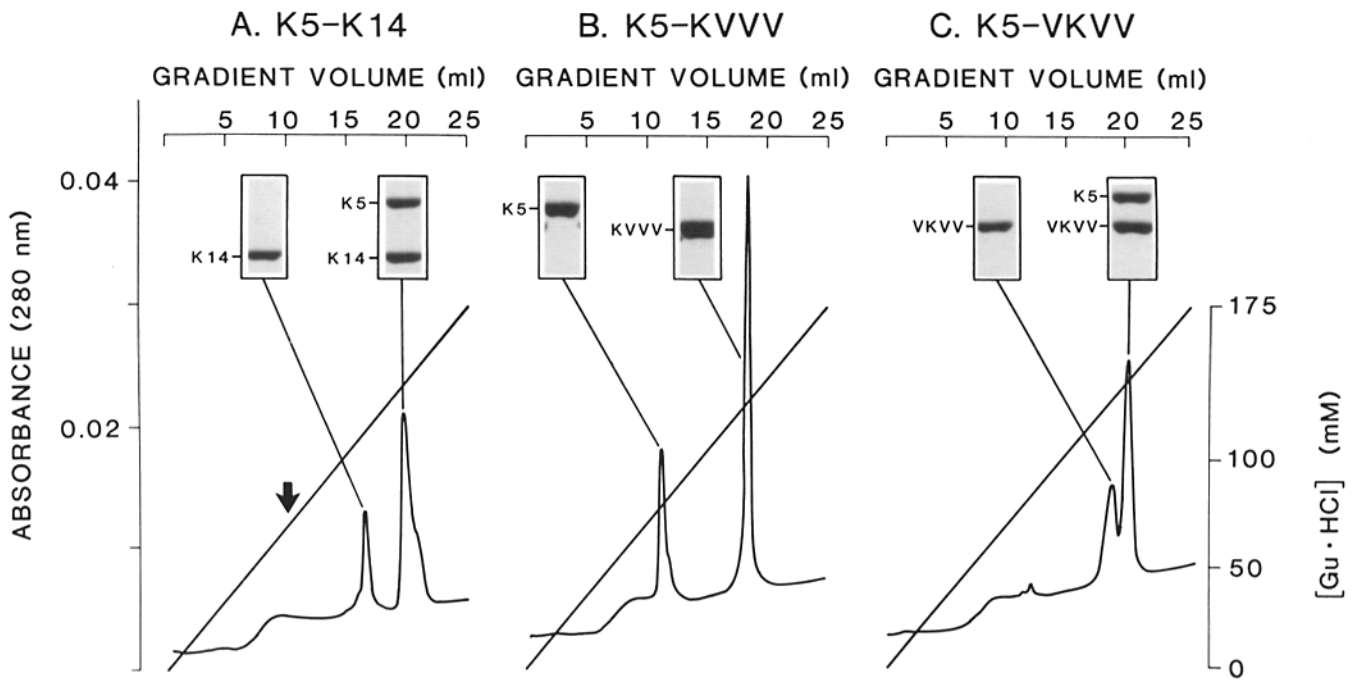


Figure 9. Complex formation between K5 and either K14, KVVV, or VKVV. K5 was combined with a molar excess of either K14 (a), KVVV (b) or VKVV (c) in 6 M urea buffer, and the complexes were resolved by anion exchange chromatography as described in Materials and Methods. Protein elution was monitored by ultraviolet absorption at A_{280} nm, and fractions (500 μ l) were collected. Aliquots (20 μ l) of each fraction were analyzed by electrophoresis through SDS-polyacrylamide gels, followed by staining with Coomassie blue (gel fractions from each peak are shown beside each UV absorbance peak). Peaks were identified as previously described (Coulombe and Fuchs, 1990). Arrow in a denotes where monomer K5 typically elutes. K14 in a was previously shown to be a monomer and the complex was shown to be a heterotetramer of two heterodimers (Coulombe and Fuchs, 1990).

transfected MCF-7 cells, consistent with the notion that the first and third α -helical domains confer weak affinity for their IF network, whereas the second and fourth domains confer stronger affinity.

We were intrigued by our earlier findings that both KVKKP and KKVKP often integrated without appreciable disruption into keratin filament networks *in vivo*. To determine whether the entire central portion of the vimentin rod domain might be able to compensate *in vivo* for that of K14, we made KVVVP and transfected it into MCF-7, PtK2, and BHK cells. In some transfected MCF-7 and PtK2 cells, KVVVP integrated into the keratin filament network with perturbations, but not major disruption (not shown). This hybrid recognized and disrupted the vimentin network in BHK cells and in PtK2 cells (not shown). Collectively, these findings were similar to those obtained with KVKKP and suggested that the central portion of the vimentin rod domain can substitute only partially for the corresponding segment of K14 in transfected cells.

Summary of Transfection Studies

Table I summarizes our results from the hybrid gene transfections.

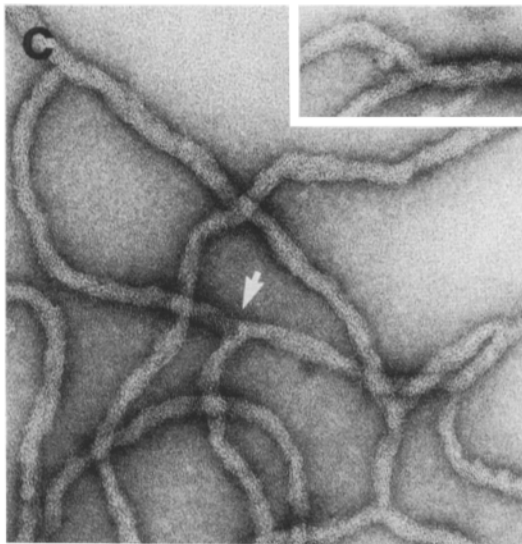
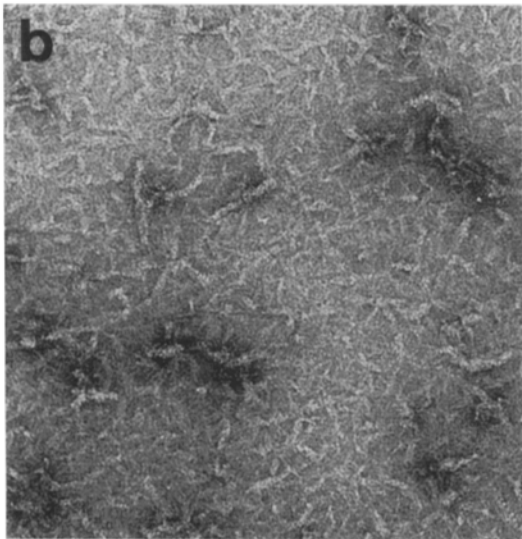
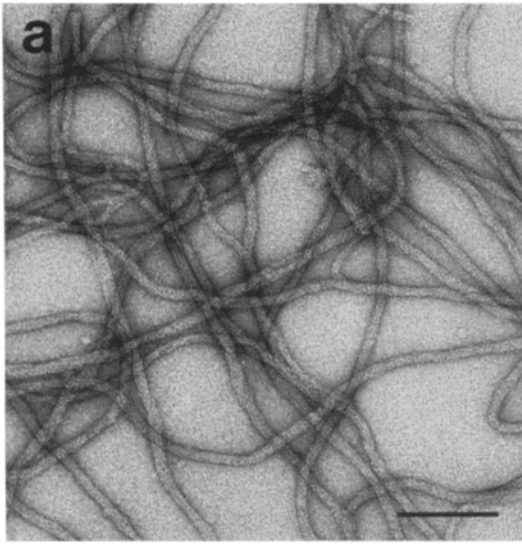
Keratin Filament Recognition Is Exerted at the Level of Complex Formation with K5

As demonstrated previously, wild-type K14 and K5 interact in the presence of 6 M urea to form tetramers which can bind to an anion exchange column, and be eluted as a single peak

with 130–135 mM guanidinium hydrochloride (Coulombe and Fuchs, 1990). SDS-PAGE analysis of the peaks revealed a 1:1 molar ratio of K5 and K14, as determined by densitometry scanning of the Coomassie blue-stained gel (Fig. 9 a). When K14 was present in excess, a monomer K14 peak eluted with 110 mM guanidinium hydrochloride. However, no monomer K5 peak (typically eluting with 70 mM guanidinium hydrochloride; arrow, Fig. 9 a) was present under these conditions, indicating that the equilibrium greatly favored formation of complexes between K5 and K14.

All of the hybrids that we examined which contained at least two consecutive α -helical segments of K14 formed complexes with K5 in the presence of 6 M urea (not shown). The concentrations of guanidinium hydrochloride necessary to elute different hybrid-K5 complexes varied slightly, consistent with the fact that the predicted p*K*i's of the hybrids were different from those of either K14 or vimentin. However, in all of these cases, the complex consisted of a 1:1 molar ratio of IF hybrid and K5, as judged by SDS-PAGE analysis. In addition, as for K5 and K14, the efficiency of complex formation was high.

In contrast to hybrids containing at least half K14, hybrids containing mostly vimentin sometimes showed a reduced ability to form complexes with K5. The efficiency at which hybrids and K5 formed complexes *in vitro* paralleled the ability of the hybrid to recognize keratin filament networks *in vivo*. Thus for example, KVVV exhibited no recognition of K5 in forming complexes under the conditions used here (Fig. 9 b), whereas VKVV complexed efficiently with K5



(Fig. 9 c). These results are consistent with our *in vivo* studies, and suggest that the ability of a hybrid protein to integrate into a keratin filament network is determined at the level of K5 recognition. Since the basic subunit structure for K5-K14 filaments is a heterodimer (Coulombe and Fuchs, 1990), hybrid recognition must be at the level of the coiled-coil.

A Helical Domain of a Different IF Type Allows Filament Elongation, Whereas Deletion of the Domain Blocks Elongation

The IF networks elicited by expression of vimentin-keratin hybrids seemed to be less aberrant than those obtained previously when α -helical domains were deleted, rather than substituted (Albers and Fuchs, 1987, 1989; Coulombe et al., 1990). This led us to wonder whether the effects of helix substitution on filament assembly might be less severe than those of helix deletion. To test this possibility, we chose to compare the behaviors of N Δ 150K14P (Coulombe et al., 1990) and VKKK when combined with wild-type K5 *in vitro*. Bacterial expression vectors were used to produce milligram quantities of these proteins, which after purification, were subjected to complex formation and filament assembly (see Materials and Methods).

Under the conditions used, wild-type K14 and K5 assembled into bona fide 10-nm filaments (mean diam 10.5 ± 0.8 nm) (Fig. 10 a). In contrast, when N Δ 150K14P was co-assembled with K5, only very short rodlets were observed (Fig. 10 b; mean diam 10.7 ± 0.6 nm; mean length 78 ± 4 nm; Coulombe et al., 1990). Thus, the absence of helix 1A and four residues of the L1 linker in N Δ 150K14P severely restricted filament elongation. Interestingly, when VKKK was combined with wild-type K5, it assembled efficiently into filamentous structures (Fig. 10 c). The filaments differed from wild-type keratin filaments in several ways. Most notably, the diameter of the filaments was nearly twice the normal size (18.4 ± 2.1 nm). In addition, a number of apparent branchpoints were seen (arrowheads, Fig. 10 c). Some of these branchpoints seemed to arise from an intertwining of filaments, as shown in Fig. 10 c (inset). While additional

Figure 10. *In vitro* assembly of K14-K5, N Δ 150K14P-K5, and VKKK-K5: deletion of keratin helix 1A blocks filament elongation, while substitution with vimentin helix 1A does not. Purified K14, N Δ 150K14P, and VKKK were each combined with K5 in 6 M urea buffer, and complexes in a 1:1 molar ratio were resolved by anion exchange chromatography as outlined in Materials and Methods. Complexes were dialyzed against keratin assembly buffer and examined by EM. (a) K14-K5 filaments; (b) N Δ 150K14P-K5 filaments; VKKK-K5 filaments. Note that only 10-nm rodlets formed with N Δ 150K14P. Note also that filaments formed with VKKK are wider than wild-type keratin filaments. Note also the presence of extensive branching (c, arrowhead). A possible explanation for branching is that the wider filaments are composed of keratin-hybrid IFs with a propensity to coil about each other. A suggestive example of this is illustrated in c (inset). Alternatively, the wider filaments may be composed of a greater number of protofibrils, or may have other abnormalities. Bar, 100 nm.

studies will be necessary to determine at a molecular level how these structures differ from wild-type 10-nm filaments, these findings indicate that the presence of vimentin helix 1A in the hybrid restored elongation in the assembly process.

Discussion

Sorting Out Intermediate Filaments

In 1987 Hatzfeld et al. reported that a radiolabeled keratin segment encompassing all of the rod domain, ~30% of the head domain and ~10% of the tail domain of the type I keratin K18 could recognize and bind to its type II partner keratin K8 when resolved by SDS-PAGE and transferred to nitrocellulose paper. Moreover, when a similar K8 rod segment was proteolytically cleaved into two fragments, either half could specifically recognize and bind to K18, although the complexes formed by these halves seemed to be somewhat weaker than the complexes of intact keratins. These K8 segments only recognized K18 and did not associate with K8 or with vimentin. At the time, the complexes formed were suspected to be heterotetramers of K8/K18 homodimers. Although more recent evidence suggests that the complexes may have been either heterodimers or heterotetramers of two heterodimers (Coulombe and Fuchs, 1990; Hatzfeld and Weber, 1990; Steinert, 1990), nonfunctional homodimers of K8 and K18 have been generated under *in vitro* conditions (Hatzfeld and Weber, 1990), and consequently the nature of the interaction remains unknown. Nevertheless, these data demonstrated for the first time that the ability of a type II keratin to recognize its type I partner resided in at least two different domains.

Our studies have confirmed and extended these earlier studies by Hatzfeld et al. (1987). An important feature of our studies is that we have conducted both *in vivo* and *in vitro* investigations of IF protein recognition. The *in vitro* studies have enabled us to evaluate IF protein recognition under conditions where we do not generate keratin homodimers (Coulombe and Fuchs, 1990; Coulombe et al., 1990; and this study). We have demonstrated that recognition between hybrid proteins and K5 takes place at the level of coiled-coil formation, at least *in vitro*, and most likely *in vivo* as well. In addition, we have assayed both type I keratin and vimentin domains for IF recognition, and similar to the type II keratin K8 (Hatzfeld et al., 1987), recognition resides in at least two separate domains. For vimentin and K14, we have narrowed the two major domains to helices 1B and 2B. The parallels between vimentin, K14 and K8 suggest that these principles may be generally applicable to the IF protein family.

A priori, it might seem reasonable to expect that segregation of vimentin and keratin networks would be dependent solely upon a specified percentage of sequence from one IF protein or another. Since helices 1B and 2B are larger than helices 1A and 2A, this possibility is consistent with our data. However, any model for IF recognition must also take into account our finding that neither VKVKP (keratin recognition) nor KVKVP (vimentin recognition) exhibited altered specificity despite the presence of 184 and 197 residues, respectively, from a different IF protein. Even when the contribution of the nonhelical head domain is not considered, these hybrid proteins still contained ~85 foreign residues of central rod domain, which is close to the number of residues

present in either helices 1B or 2B. Thus, if a specific length of the central rod domain is important, it would seem that it is an uninterrupted stretch of α -helical sequence which is essential in determining IF specificity. Further studies will be necessary to delineate the limit amount of helix 1B and 2B required for efficient IF recognition.

Since all IF proteins have helices 1B and 2B that are predicted to be α -helical and that contain heptad repeats of hydrophobic residues throughout, it is not surprising to find a considerable amount of sequence homology in these regions. Taking into account conservative substitutions, vimentin and K14 share ~50% sequence homology in these domains. The most striking differences between vimentin and K14 in helices 1B and 2B are the location and nature of the charged residues. That ionic interactions might be important in promoting coiled-coil formation has been suggested previously by McLachlan and Stewart (1975), who noted in the course of Fourier transform analyses and model building that there is a periodic distribution of charged side chains in α -tropomyosin. Fourier transform analyses of segments of the coiled-coil domains of type I and type II keratins and vimentin have also revealed a periodicity of alternating bands of positively and negatively charged residues, which could play a role in stabilizing the coiled-coil (Parry et al., 1977; McLachlan, 1978; McLachlan and Stewart, 1982; Conway and Parry, 1988). For vimentin and K14, ~25–30% of the residues in helices 1B and 2B are charged in one of the two IF proteins and either neutral or oppositely charged in the other. Approximately 30% of these are in positions e or g of the heptad, i.e., two positions flanking the hydrophobic residues (a and d). It is interesting to note that the periodicity of the repeating unit of basic and acidic residues varies for vimentin, type I, and type II keratins (Conway and Parry, 1988). Elucidating the precise role that this periodicity might play in dimer formation will depend upon more extensive exchange and site-directed mutagenesis, which was beyond the scope of the present study.

The Consequences of Domain Swapping on Keratin Network Formation and Filament Assembly

Through the course of our transfection studies, we noticed that our hybrid proteins usually exhibited less severe effects on endogenous PtK2 or MCF-7 keratin filament networks than our prior K14 mutants (Albers and Fuchs, 1987, 1989), where varying amounts of the COOH or NH₂-terminal portions of the K14 rod domain had been deleted. Previously, we had shown that the major structural consequence of deleting the COOH- or NH₂-terminal segments of the rod domain was a severe restriction in filament elongation (Coulombe et al., 1990). Our *in vitro* studies with VKKK have shown that in contrast to deletion, substitution of the NH₂-terminal portion of the keratin rod domain with the corresponding segment from vimentin does not interfere greatly with filament elongation. While we have not yet determined why the hybrid-K5 filaments are so atypically wide, our studies do suggest that mutations that affect filament elongation are more disruptive to overall keratin network formation *in vivo* than mutations that affect other aspects of filament structure. This said, it is clear from both our hybrid gene transfection studies and our *in vitro* assembly studies that helical domain swapping is not without consequence to IF network and filament formation.

A priori, it might be expected that the degree to which domain swapping might affect IF network formation might be dependent upon both the size of the domain and the degree of sequence divergence within it. Thus, we were surprised that helix 1A, which is relatively short and shares 55% sequence identity between vimentin and K14 polypeptides, had a rather disruptive effect on IF network formation, while helix 1B, which is almost three times longer and shares less (38%) sequence identity, often showed very mild effects on keratin network formation. While additional studies will need to be conducted to elucidate the underlying molecular basis, our findings suggest that different regions of the rod domain may be more sensitive to domain swapping than others.

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