In vitro Assembly Properties of Human Type I and II Hair Keratins

Yuko Honda¹, Kenzo Koike², Yuki Kubo³, Sadahiko Masuko¹, Yuki Arakawa¹, and Shoji Ando⁴*

¹Department of Anatomy and Physiology, Faculty of Medicine, Saga University, Nabeshima 5-1-1, Saga 849-8501, Japan, ²Beauty Research Center, KAO Corporation, Bunka 2-1-3, Sumida-ku, Tokyo 131-8501, Japan, ³Department of Biomolecular Sciences, Faculty of Medicine, Saga University, Nabeshima 5-1-1, Saga 849-8501, Japan, ⁴Faculty of Biotechnology and Life Science, Sojo University, Ikeda 4-22-1, Nishi-ku, Kumamoto 860-0082, Japan

ABSTRACT. Multiple type I and II hair keratins are expressed in hair-forming cells but the role of each protein in hair fiber formation remains obscure. In this study, recombinant proteins of human type I hair keratins (K35, K36 and K38) and type II hair keratins (K81 and K85) were prepared using bacterial expression systems. The heterotypic subunit interactions between the type I and II hair keratins were characterized using two-dimensional gel electrophoresis and surface plasmon resonance (SPR). Gel electrophoresis showed that the heterotypic complex-forming urea concentrations differ depending on the combination of keratins. K35-K85 and K36-K81 formed relatively stable heterotypic complexes. SPR revealed that soluble K35 bound to immobilized K85 with a higher affinity than to immobilized K81. The in vitro intermediate filament (IF) assembly of the hair keratins was explored by negative-staining electron microscopy. While K35-K81, K36-K81 and K35-K36-K81 formed IFs, K35-K85 afforded tight bundles of short IFs and large paracrystalline assemblies, and K36-K85 formed IF tangles. K85 promotes lateral association rather than elongation of short IFs. The in vitro assembly properties of hair keratins depended on the combination of type I and II hair keratins. Our data suggest the functional significance of K35-K85 and K36-K81 with distinct assembly properties in the formation of macrofibrils.

Key words: cytoskeleton, hair keratins, intermediate filaments, protein-protein interaction, self-assembly

Introduction

Keratins are the most complex subgroup of intermediate filament (IF) proteins (Coulombe and Omary, 2002; Langbein et al., 1999, 2001; Langbein and Schweizer, 2005; Langbein et al., 2007; Rogers et al., 2004, 2005; Schweizer et al., 2007). In the human genome, IF proteins include at least 37 cytokeratin (epithelial keratin) genes expressed in the different types of epithelia and at least 17 hair keratin genes expressed in hair- or nail-forming cells (Coulombe and Omary, 2002; Langbein et al., 1999, 2001; Langbein and Schweizer, 2005; Langbein et al., 2007). The complex expression patterns of hair keratins as various isoforms. Like other IF proteins, keratins have a characteristic tripartite structure that includes a central α-helical rod domain flanked by non-α-helical N-terminal head and C-terminal tail domains (Fuchs and Weber, 1994). The rod domain, consisting of approximately 310 amino acids, has relatively conserved sequences at the N- and C-terminal ends, and displays long heptad repeat patterns (abcdefg) of hydrophobic residues (a and d). In contrast, the head and tail domains of IF proteins have variable length, sequences and chemical characteristics. From the extent of sequence homology of the rod domain, acidic keratins are classified into as type I, whereas neutral-basic keratins are type II. Both type I and II keratins are required to form IFs in vivo and in vitro (Coulombe and Omary, 2002; Fuchs and Weber, 1994; Herrmann and Aebl, 2004).

According to a recently introduced nomenclature for mammalian keratins, type I hair keratins are numbered from 31 to 40 (33a and 33b are isoforms), while type II hair keratins are from 81 to 86 (Schweizer et al., 2006). Here we describe these proteins as, for example, keratin 81 as K81. The complex expression patterns of hair keratins as
well as cytokeratins in human hair follicles have been determined (Langbein et al., 1999, 2001; Langbein and Schweizer, 2005; Langbein et al., 2007; Rogers et al., 2004, 2005; Schweizer et al., 2007). The tissue units outside the hair-forming compartments are the outer root sheath, companion layer and inner root sheath, each of which expresses specific sets of cytokeratins. By contrast, the hair-forming compartments such as the matrix (just above the germinative cells), cortex (differentiated from the matrix and constituting the bulk of hair fibers) and cuticle (surrounding the cortex) show complex expression patterns of hair keratins depending on hair differentiation. In the early stage of differentiation, the matrix/pre-cortex expresses K35 and K85, while the early cuticle expresses K32, K35 and K85. During the middle stage of differentiation, the lower cortex begins to express K31, and the cuticle begins to express K82. The lower cortex also weakly expresses K38 at this stage, and the K38-positive cells scatter heterogeneously in the cortex. At an advanced stage of differentiation, the mid- to upper cortex expresses almost simultaneously K33a, K33b, K36, K81, K83, K86, and finally K34 and K39. The cuticle layer expresses K39 and K40. The complex mechanism of hair keratin expression makes it difficult to clearly determine the specific type I and II hair keratin pairs used to form IFs, in contrast to the specific heterotypic pair expression of cytokeratins in various types of epithelia (Fuchs and Weber, 1994). It is also unknown why gene mutations have been reported only in type II hair keratins such as K81, K83, K85 and K86. These mutations are the underlying cause of inherited diseases including monilethrix and ectodermal dysplasia of hair and nail types (Schweizer et al., 2007; Shimomura et al., 2010). For cytokeratins, many point mutations have been discovered in both the type I and II cytokeratin genes in patients suffering from epithelial disorders such as epidermolysis bullosa simplex and epidermolytic hyperkeratosis (Fuchs and Weber, 1994).

IF formation in vivo and in vitro have been well documented for cytokeratins (Coulombe and Omary, 2002; Fuchs and Weber, 1994; Herrmann et al., 2002; Herrmann and Aebi, 2004). Both type I and II cytokeratins are needed to form heterotypic coiled-coil dimers that then polymerize into full-width short filaments, i.e., unit-length filaments (ULFs), with a diameter of approximately 15 nm and length of 50–70 nm (Herrmann et al., 2002). The ULFs anneal longitudinally and compact radially to form mature IFs with a diameter of approximately 10 nm and length of several micrometers (Herrmann et al., 2002). Few studies on IF assembly of hair keratins have been undertaken. Wang et al. (Wang et al., 2000) reported efficient in vitro IF assembly of a mouse hair keratin pair. Hofmann et al. (Hofmann et al., 2002) reported that in vitro IF formation of human hair keratins K31, K83 and K86 differs from that of human cytokeratins because of the requirement of a relatively high ionic strength. However, the specificity of the interactions between particular type I and II hair keratins has not been characterized. Furthermore, the fundamental significance of the multiplicity of hair keratins in hair-forming compartments remains unknown.

The formation of macrofibrils (MFs) is not well understood. MFs are the hair keratin IF bundles that are observed in the cortex of hair fibers and wool by transmission electron microscopy (TEM), and have maximum lengths of approximately 10 μm (Bryson et al., 2009; Chapman and Gemmell, 1971; Jones and Pope, 1985; Marshall et al., 1991; Morioka, 2009; Orwin, 1979; Orwin and Woods, 1982; Plowman et al., 2007). In MFs, hair keratin IFs are embedded in a matrix material composed of hair keratin-associated proteins (KAPs) that link each other and to IFs (Rogers et al., 2006). Three kinds of MFs have been observed and their distributions across hair fibers and wool may affect hair curvature or crimp (Bryson et al., 2009; Marshall et al., 1991; Plowman et al., 2007).

In this study, we prepared recombinant human type I hair keratins K35, K36, K38, and type II hair keratins K81 and K85, and examined their protein-protein interactions with an emphasis on the specificity of interactions between particular type I and II proteins. The proteins were also used to explore the characteristics of in vitro IF formation by changing the subunit combinations of the type I and II hair keratins. K35 and K85 are the first pairs expressed in the human hair matrix/pre-cortex in the early stages of differentiation, while K36 and K81 are expressed in the mid- to upper cortex at an advanced stage of differentiation (Langbein et al., 1999, 2001; Langbein and Schweizer, 2005; Schweizer et al., 2007). K38 is expressed in cells scattered randomly throughout the entire cortex (Langbein et al., 1999; Langbein and Schweizer, 2005; Yu et al., 2009), and is suggested to be related to the curvature of a hair fiber (Thibaut et al., 2007). We observed that the affinities in the heterotypic subunit interactions depend on the combination of type I and II hair keratins. In IF formation experiments, while K81 afforded IFs by copolymerization with type I hair keratins, the combination of K85 and K35 gave tight bundles of short IFs and their large paracrystalline assemblies. The importance of the different assembly properties of K81 and K85 in the formation of MFs in the cortex is discussed.

**Materials and Methods**

**Preparation of recombinant human hair keratins and cytokeratins**

The DNAs coding for complete sequences of human type I hair keratins (K35: NM_002280, 1.4 kb; K36: NM_003771, 1.4 kb; K38: NM_006771, 1.4 kb) and type II hair keratins (K81: NM_002281, 1.5 kb; K85: NM_002283, 1.5 kb), flanked by 5′ NdeI and 3′ BamHI restriction sites, were chemically synthesized.
The codon usage was adapted to the codon usage bias of *E. coli* genes. The DNAs sequences were cloned into appropriate plasmids (pBluescript, pMK or pMA) and then sequenced by restriction enzyme analysis and by expression in *E. coli* strain Rosetta(DE3) pLysS (Merck) using isopropyl-β-D-thiogalactopyranoside (1 mM) induction. Recombinant protein was enriched in the inclusion body, which was isolated by the method of Nagai and Thøgersen (Nagai and Thøgersen, 1987), as follows. After expression for 3–5 h at 37°C, approximately 4 g of bacteria were harvested and resuspended in 10 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 25% sucrose and 1 mM EDTA). The cells were lysed by addition of 25 mg of lysozyme (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 2.5 ml of the lysis buffer and then aging the mixture for 30 min on ice. MgCl₂, MnCl₂ and DNase I (Sigma-Aldrich) were then added to give final concentrations of 10 mM, 1 mM and 10 μg/ml, respectively. After 30 min, 25 ml of detergent buffer (20 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 1% deoxycholic acid, 1% Nonidet P-40, and 2 mM EDTA) was added to the lysate, which was then centrifuged at 5000×g for 10 min at 4°C. The resulting pellet was resuspended in 30 ml of washing buffer (20 mM Tris-HCl, pH 7.5, 0.5% Triton X-100, and 1 mM EDTA) and then centrifuged at 5000×g for 10 min at 4°C. The supernatant was discarded. This washing step was repeated until a tight pellet of inclusion body was obtained. Recombinant protein was extracted from the inclusion body using 30 ml of buffer 1 (10 mM Tris-HCl, pH 7.5, 8 M urea, 2 mM EDTA, 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), and an adequate amount of protease inhibitor cocktail) at 10°C. After centrifugation at 100,000×g at 10°C for 1 h, the supernatant was loaded on a Source 15Q (GE Healthcare, Buckinghamshire, UK) column (1×9 cm) equilibrated in buffer 2 (10 mM Tris-HCl, pH 8.0, 6 M urea, 1 mM EDTA, 5 mM DTT, 0.5 mM PMSF, and protease inhibitor cocktail). Proteins were eluted from the column by application of a linear concentration gradient of NaCl (0–0.3 M over 70 min) in buffer 2. The fractions containing desired proteins were collected, dialyzed against buffer 3 (10 mM Tris-HCl, pH 8.0, 8.5 M urea, 2 mM EDTA, 10 mM DTT, and protease inhibitor cocktail), cleared of aggregates by centrifugation at 100,000×g at 10°C for 1 h, and stored at −80°C. The authenticity of the protein was verified by SDS-PAGE, amino terminal sequence analysis using a PPSQ system (Shimadzu, Kyoto, Japan), and peptide mass fingerprinting using an Autoflex II matrix-assisted laser desorption/ionization mass spectrometer (Bruker Daltonics, Billerica, MA, USA). The concentration of each protein was determined spectrophotometrically using a Quant-iT Protein Assay kit (Life Technologies, Carlsbad, CA, USA) and Quant-iT Protein standard.

**Surface plasmon resonance measurements**

Homo- and heterotypic subunit interactions of the type I and II hair keratins were determined using surface plasmon resonance (SPR) technology on a Biacore 1000 (GE Healthcare). Activation of the carboxyl groups of a CM5 sensor chip was carried out according to manufacturer’s instructions. Type II hair keratin was immobilized on the sensor chip as a ligand through the amino group as follows. K81 or K85 was injected at a concentration of 165 or 172 μg/ml, respectively, in 10 mM sodium acetate, pH 4–5, 5 mM DTT and 6 M urea, at a flow rate of 5 μl/min. The amount of protein immobilized on the sensor chip was adjusted by controlling the contact time of the sample with the activated surface of the sensor chip. As a result, 7279 resonance units (RU) of K81 or 7260 RU of K85 was immobilized on the sensor chip (1000 RU=1 ng/mm²). Unreacted carboxyl groups of the sensor chip were blocked using 1 M ethanolamine (pH 8.5). Soluble type I or II hair keratin to be used as an analyte was diazylized against a buffer solution (2 mM Tris-HCl, pH 9.0, 5 mM DTT and protease inhibitor cocktail), and then diluted to a protein concentration of 125–500 nM in 10 mM HEPES-NaOH, pH 7.4–8.5, containing 50–200 mM NaCl, 3.4 mM EDTA, 5 mM DTT and 0.005% Tween 20. The analyte solution was injected over the sensor chip at a flow rate of 5 μl/min at 25°C. The surface of the sensor chip was regenerated by washing with a basic solution (10 mM HEPES-NaOH, pH 8.0, 50 mM NaCl, 5 mM DTT and 7 M urea). A control experiment was performed using a surface that did not contain immobilized protein. The blank sensorgram was subtracted from the assay curve using BIAevaluation 3.0 software (GE Healthcare).

**Two-dimensional gel electrophoresis**

Equimolar amounts of the purified type I and II hair keratins were mixed to give a total protein concentration of approximately 0.1 mg/ml in 400 μl of 10 mM Tris-HCl, pH 8.0, 9.5 M urea, 2 mM EDTA, 10 mM DTT, and then dialyzed against lower concentrations (4–7 M) of urea in 10 mM Tris-HCl, pH 7.5, 1% Triton X-100, 1% 3-(3-cholamidopropyl)dimethylammonio)propanesulfonate (CHAPS), 64 mM DTT and protease inhibitor cocktail. After addition of Pharmalyte (GE Healthcare) and Orange G (Sigma-Aldrich) to give final concentrations of 1% and 0.0025%, respectively, the sample solution was used to swell an Immobiline DryStrip (pH 4–7, 7 cm long, GE Healthcare) at 20°C for 16 h. Isoelectric focusing (IEF) in the first dimension was performed on a CoolPhorestar Type-P (Anatech, Tokyo, Japan) using the manufacturer’s program. After IEF, the gel strip was treated in 25 mM Tris-HCl, pH 6.8, 6 M urea, 30 mM DTT, 2% SDS, 25% glycerol and 0.0025% bromophenol blue for 30 min, and then treated with 10% SDS-PAGE in the second dimension. The separated proteins were visualized by staining with Coomassie Brilliant Blue.
Bovine serum albumin (BSA, Sigma-Aldrich) was used as a marker protein.

In vitro IF assembly

Equimolar amounts of purified type I and II hair keratins were mixed at protein concentrations of 0.05–0.1 mg/ml, in 10 mM Tris-HCl, pH 8.0, 9.5 M urea, 2.5 mM EDTA, 10 mM DTT, and an adequate amount of protease inhibitor cocktail at 25°C. The urea concentration was lowered by stepwise dialysis against 4 M and 2 M urea in the same buffer, and then against 25 mM Tris-HCl, pH 7.5, 10 mM DTT or 5 mM tris(2-carboxyethyl)phosphine (TCEP), 2.5 mM EDTA, and protease inhibitor cocktail at 25°C. Final dialysis was performed against “filament formation buffer” consisting of 25 mM Tris-HCl, pH 7.2, 1 mM MgCl₂, 180 mM NaCl, 10 mM DTT or 5 mM TCEP, and protease inhibitor cocktail, at 37°C. In these procedures, all buffers were degassed and then stored under N₂ atmosphere before use, and the dialysis was carried out in anaerobic bags filled with N₂ gas. For cytokeratins 14 and 5, IF assembly was carried out as follows. Equimolar amounts of both proteins were mixed at protein concentrations of 0.1–0.2 mg/ml, in 25 mM Tris-HCl, pH 7.5, 9.0 M urea, and 5 mM DTT. The urea concentration was lowered by dialysis against 2 M urea in the same buffer. Final dialysis was performed against 5 mM Tris-HCl, pH 7.5, 20 mM NaCl, and 5 mM DTT.

Electron microscopy

For negative staining, an aliquot (20 μl) of each sample was adsorbed for 3 min to a collodion film on a copper grid. Then the grid was exposed to 2% uranyl acetate for 30 s. After removal of excess liquid with filter paper, the sample was allowed to air dry. Samples were examined using a transmission electron microscope (TEM, JEM-1210, JEOL, Tokyo, Japan) that was operated at an accelerating voltage of 80 kV.

Results

Preparation of recombinant human hair keratins and cytokeratins

We expressed, in E. coli, the DNA sequences encoding human type I hair keratins 35 (K35; total 455 amino acid residues containing 23 cysteine residues, calculated pl 4.77), 36 (K36; total 467 residues containing 20 cysteine residues, pl 4.75) and 38 (K38; total 456 residues containing 28 cysteine residues, pl 4.64), type II hair keratins 81 (K81; total 505 residues containing 34 cysteine residues, pl 5.37) and 85 (K85; total 507 residues containing 27 cysteine residues, pl 6.42), human type I cytokeratin 14 (K14; total 472 residues containing 5 cysteine residues, pl 4.94), and type II cytokeratin 5 (K5; total 590 residues containing 4 cysteine residues, pl 7.77). The proteins were extracted from inclusion bodies using 8 M urea, and then purified by ion-exchange column chromatography as described under “Materials and Methods”. The authenticities of the proteins were verified by SDS-PAGE (Fig. 1), amino terminal sequence analysis, and mass spectrometry. Yields of the purified proteins were 6–56 mg/l of bacterial growth medium.

Binding measurements of human hair keratins using SPR

We used surface plasmon resonance (SPR) technique to examine the molecular interactions between the human hair keratins (Fig. 2). First, K81 or K85 was immobilized on the dextran surface of a sensor chip as described in the “Materials and Methods” section. The amount of K81 or K85 immobilized on a sensor chip was adjusted to approximately 7300 RU (Fig. 2b–d), by controlling the contact time of the sample with the activated surface of the sensor chip. When soluble K35 was injected as an analyte over the K81-immobilized sensor chip at a concentration in the range of 125–500 nM, sensorgrams were obtained that showed the concentration-dependent binding of K35 to the immobilized K81 (Fig. 2a). Conversely, when soluble K81 in the same concentration range was injected over the same sensor chip, concentration-dependent binding was observed with very weak resonance signals (Fig. 2a). Similarly, soluble K35 bound to the immobilized K85 with much more intense resonance signals compared with that for the binding of soluble K85 to the immobilized K85 (data not shown). Thus, the heterotypic interaction between the type I and II hair keratins was apparently superior to the homotypic interaction between the type II hair keratins, as expected from previous SPR measurements reported for cytokeratins (Hofmann and Franke, 1997). Unfortunately, because K36 and K38 readily aggregated in the running buffer used here, their binding to the immobilized K81 or...
K85 was unable to be determined.

The effects of pH and ionic strength on the interaction between soluble K35 and immobilized K81 or K85 were examined (Fig. 2b–d). Under the conditions used here, maximum binding of K35 to the immobilized K81 or K85 was obtained at pH 8.0 in the presence of 50 mM NaCl. Interestingly, the resonance signal obtained for the binding of K35 to the immobilized K85 at pH 8.0 was almost twice that observed for the binding of K35 to the immobilized K81 at the same pH (Fig. 2b and c). Because the amounts of K81 and K85 immobilized on the sensor chips were almost equal (Fig. 2b and c), these results suggest that K35 prefers K85 rather than K81 as a heterotypic partner.

**Heterotypic complex formation between human type I and II hair keratins**

To characterize the formation of heterotypic complexes between the type I and II hair keratins, two-dimensional gel electrophoresis was performed after dialysis of mixtures of both types of hair keratins from 9.5 M urea down to various lower urea concentrations as described in the “Materials and Methods” section. By lowering the urea concentration from 9.5 M, the type I and II hair keratins associated to form a heterotypic complex at a certain urea concentration. Complex formation and dissociation occur reversibly at the same urea concentration (Franke et al., 1983). Because the type I and II proteins have different isoelectric points, formation of a heterotypic complex is observed as a shift in isoelectric point in IEF measurements at the first dimension. SDS-PAGE at the second dimension dissociates the complex and hence allows identification of the proteins involved. This method has been used to characterize the interactions between type I and II cytokeratins in urea, and has revealed that cytokeratins obtained from various tissues and cultured cells form heterotypic complexes at 6.0–9.0 M urea depending on the combination of type I and II subunit proteins (Eichner et al., 1986; Franke et al., 1983). In this study, we observed that heterotypic complex formation occurred at different urea concentrations depending on the
combination of type I and II hair keratins (Fig. 3). K81 formed a complex with K35 at 5.0 M urea (Fig. 3a–c), with K36 at 5.5 M urea (Fig. 3d–f), and with K38 at 4.0 M urea (Fig. 3g–i). K85 formed a complex with K35 partly at 6.0 M urea and fully at 5.5 M urea (Fig. 3j–l), with K36 partly at 5.5 M urea and fully at 5.0 M urea (Fig. 3m–o), and with K38 partly at 5.0 M and fully at 4.0 M (Fig. 3p–r). In an equivalent experiment, human cytokeratins K14 and K5 formed a complex at 7.0 M urea (data not shown). Thus, the human hair keratins formed heterotypic complexes at urea concentrations lower than 6.0 M, suggesting lower compatibility between the type I and II hair keratins than between cytokeratin pairs. Among the combinations examined here, those of K35-K85 and K36-K81 formed relatively stable complexes compared with the other combinations. This suggests that the combinations that are expressed in the matrix or cortex at the same stage of differentiation have a higher affinity than other combinations. In contrast, K38 showed a relatively low affinity for K81 or K85.

**In vitro IF assembly conditions of human hair keratins**

In general, *in vitro* IF formation is influenced primarily by pH and ionic strength (Coulombe and Omary, 2002; Fuchs and Weber, 1994; Herrmann and Aebi, 2004). As a reference, cytokeratin K14 and K5 formed IFs with widths of 9±2 (mean±standard deviation) nm and lengths of several micrometers at neutral pH and low ionic strength such as 5 mM Tris-HCl, pH 7.5 and 20 mM NaCl (Fig. 4e), as reported previously (Herrmann et al., 2002; Herrmann and Aebi,
2004; Yano et al., 1991). In the current study, various conditions for IF assembly of the human hair keratins prepared in this study were examined. It was observed that IFs form at neutral pH and relatively high ionic strengths (25 mM Tris-HCl, pH 7.2 and 180 mM NaCl) compared with the conditions used for cytokeratins (e.g., 5 mM Tris-HCl, pH 7.5 and 0–20 mM NaCl) (Herrmann et al., 2002; Herrmann and Aebi, 2004; Yano et al., 1991) or type III and IV IF proteins (e.g., 20 mM Tris-HCl, pH 7.5 and 100–150 mM NaCl) (Ando et al., 2004; Gohara et al., 2008; Herrmann et al., 1992; Hisanaga and Hirokawa, 1990). To prevent unfavorable oxidation of cysteine residues contained in both types of human hair keratins, either 10 mM DTT or 5 mM TCEP was used as a reducing reagent. No obvious difference in the effects of DTT and TCEP on IF formation has been observed (Burns et al., 1991; Hofmann et al., 2002).

**In vitro assembly characteristics of combinations using K81**

Under the conditions used, the combination of K35-K81 formed IFs with widths of 7±1 nm and lengths of 400–800 nm (Fig. 4a), slightly narrower and shorter than IFs formed by K14 and K5 (Fig. 4e). The combination of K36-K81 gave IFs with widths of 7±1 nm and lengths of 200–500 nm (Fig. 4b). During IF assembly experiments, it was observed that at a low ionic strength (i.e., 25 mM Tris-HCl, pH 7.5) after removal of urea by dialysis, the combinations of type I and II hair keratins formed unit-length filaments (ULFs) with a diameter of 11±2 nm and lengths of 61±12 nm, as shown in the inset of Fig. 4a for the combination of K35-K81 and in Fig. 5a and b for the combination of K35-K85. To connect the ULFs longitudinally to form IFs, the filament formation buffer needed to contain 180 mM NaCl. Changing the NaCl concentration in the filament formation buffer from 180 to 135 or 200 mM resulted in formation of shorter IFs or an increased amount of aggregates, respectively. When K35, K36 and K81 were mixed with a molar ratio of 0.5:0.5:1.0 in 9.5 M urea, this combination gave IFs with widths of 7±1 nm and lengths of up to 1 μm (Fig. 4c). Because all three proteins were observed in the pellet fraction after centrifugation (20,000×g) of the reaction mixture followed by SDS-PAGE (data not shown), both K35 and K36 appeared to copolymerize with K81 to form IFs. The combination of three proteins always afforded longer IFs compared with those formed by the combination of K35-
K81 or K36-K81, so the type I proteins K35 and K36 seem to cooperate to elongate IFs, although the reason for this is unknown. Overall, IFs formed by the combination of K35-K81, K36-K81 or K35-K36-K81 seemed to be more linear (Fig. 4a–c) than the IFs containing K14 and K5 (Fig. 4e). The combination of K38-K81 failed to form IFs under the conditions examined here; only aggregates were observed (Fig. 4d).

**In vitro assembly characteristics of combinations using K85**

The combination of K35-K85 formed relatively short IFs with widths of 7±1 nm and lengths of 200–400 nm (Fig. 5c). Again, these shorter IFs appear to be more linear than those formed between K14 and K5 (Fig. 4e). The shorter IFs tended to associate laterally and tightly, and concatenated longitudinally with each other to form IF bundles with lengths of 300–400 nm. The dashed lines indicate two short IF bundles that are concatenating with each other (Fig. 5d). The IF bundles appeared to further associate longitudinally and laterally to form large paracrystalline assemblies. The dashed lines indicate two IF bundle units that are connected to each other (Fig. 5e, g). In the paracrystalline assemblies, periodic patterns of approximately 20 nm (square brackets) were sometimes observed. (i) The combination of K36-K85 formed loosely associated IF tangles. (j) The combination of K38-K85 gave only aggregates. Scale bars, 50 nm (a and b), 100 nm (f and g), 200 nm (c, d, i and j), 500 nm (e), and 5 μm (h).

Fig. 5. Electron microscopy of the assembly products formed by the combination of (a–h) K35-K85, (i) K36-K85 and (j) K38-K85. (a, b) The combination of K35-K85 formed ULFs at low ionic strength. (c) The combination of K35-K85 formed short IFs with lengths of 200–400 nm in 180 mM NaCl. (d) The short IFs tended to associate laterally and tightly, and concatenated with each other to form IF bundles with lengths of 300–400 nm. The dashed lines indicate two short IF bundles that are concatenating with each other. (e, h) The IF bundles appeared to further associate longitudinally and laterally to form large paracrystalline assemblies. The dashed lines indicate two IF bundle units that are connected to each other. (f, g) In the paracrystalline assemblies, periodic patterns of approximately 20 nm (square brackets) were sometimes observed. (i) The combination of K36-K85 formed loosely associated IF tangles. (j) The combination of K38-K85 gave only aggregates. Scale bars, 50 nm (a and b), 100 nm (f and g), 200 nm (c, d, i and j), 500 nm (e), and 5 μm (h).
In vitro assembly characteristics of combinations using both K81 and K85

When equimolar amounts K35, K36, K81 and K85 were mixed in 9.5 M urea for IF formation, mainly large tangles composed of many IFs with widths of 6±1 nm were observed, as shown in Fig. 6a and b. Compared with the IFs obtained by mixing K35, K36 and K81 (Fig. 4c), this result indicates that the addition of K85 promoted lateral association of IFs, although detailed knowledge of how each subunit protein is assembled into dimers, tetramers, ULFs and finally IFs is not known. We also preformed two kinds of ULFs composed of the combinations of K35-K85 and K36-K81 independently by dialysis, mixed both ULF samples, and then undertook the final dialysis against the filament formation buffer containing 180 mM NaCl. This method afforded primarily IF bundles with widths of 52±25 nm (Fig. 6c and d). Although both types of ULFs probably associate longitudinally with each other to form an IF, the ULFs composed of K35-K85 seemed to determine the assembly behavior of the reaction mixture.

In vitro assembly characteristics of K38

In contrast to the other type I human keratins, K38 appeared to lack an ability to form IFs de novo through polymerization with type II hair keratins (Fig. 4d and 5j). This might be partly caused by the low affinity of K38 for K81 or K85, as shown in the two-dimensional gel electrophoresis investigations (Fig. 3g–i and p–r). In the presence of K35 and K36, however, K38 appeared to be involved in IF formation. For example, when a mixture of K35, K36, K38, K81 and K85 in 9.5 M urea was prepared for IF polymerization, IF tangles similar to those shown in Fig. 6a and b were observed (data not shown). All of the proteins were recovered in the pellet fraction after centrifugation of the reaction mixture at 20,000×g followed by SDS-PAGE analysis, suggesting that K38 was incorporated into the IFs and IF tangles.

Discussion

In this study, recombinant human type I hair keratins (K35, K36 and K38), type II hair keratins (K81 and K85), human type I cytokeratin K14 and type II cytokeratin K5 were pre-
pared using the bacterial expression systems. K35 and K85 are the first pair expressed in the matrix/pre-cortex during the early stages of differentiation in human hair follicles, while K38 is produced in the lower cortex following the expression of K35 and K85 (Langbein et al., 1999, 2001; Langbein and Schweizer, 2005; Rogers et al., 2004, 2005; Schweizer et al., 2007). K36 and K81 as well as other human hair keratins including K33a, K33b, K83 and K86 are expressed in the mid- to upper cortex during the advanced stage of differentiation (Langbein et al., 1999, 2001; Langbein and Schweizer, 2005; Rogers et al., 2004, 2005; Schweizer et al., 2007). The recombinant human hair keratins prepared here were used to investigate protein-protein interactions with an emphasis on the specificity of interactions between particular type I and II hair keratins. The proteins were also used to explore the characteristics of in vitro IF formation by changing the subunit combinations of the type I and II hair keratins.

In the SPR measurements, the heterotypic binding of soluble K35 to immobilized K85 or K81 apparently predominated over the homotypic binding of either soluble K85 to immobilized K85, or soluble K81 to immobilized K81 (Fig. 2). These results support the idea that both type I and II hair keratins need to form coiled-coil dimers that then lead to IFs (Hofmann et al., 2002; Schweizer et al., 2007; Wang et al., 2000). Interestingly, soluble K35 displayed a signal upon binding to immobilized K85 that was twice as intense as that of binding to immobilized K81. This result suggests that there is a heterotypic binding specificity between the type I and II hair keratins. In the SPR measurements for non-epidermal cytokeratins, soluble type I cytokeratins such as K13 and K18 display much higher binding than K19 and K20 to the immobilized type II cytokeratin K8, and homotypic binding of soluble K8 to immobilized K8 was hardly detected (Hofmann and Franke, 1997). K8 and K18 are the only cytokeratin pair present in some simple epithelial cells such as early embryonic epithelia and hepatocytes (Moll et al., 1982). K8 and K13 are expressed together in tracheal epithelium and urothelium, while K8, K18, K19 and K20 are found in intestinal mucosa. Thus, some specific pairs of type I and II cytokeratins that are co-expressed in certain cell types and growth stages have intrinsic mutual high affinities. The SPR results obtained here suggest that K35 and K85 have mutually compatible structures as the first hair keratin pair expressed in the matrix/pre-cortex of human hair follicles.

The heterotypic interactions between the type I and II hair keratins were further characterized using two-dimensional gel electrophoresis in urea (Fig. 3). All combinations examined formed heterotypic complexes at lower urea concentrations (4–6 M) than that of the urea concentration (7 M) at which human cytokeratins K14 and K5 form a complex. The results were in good agreement with previous findings that the combinations of human hair keratins K31-K83 and K31-K86 afforded the corresponding heterotypic complexes at 5 M urea in two-dimensional gel electrophoresis (Hofmann et al., 2002). Similarly, hair keratins extracted from wool formed heterotypic complexes at urea concentrations of less than 6 M (Herrling and Sparrow, 1991). Combined, these results suggest that the heterotypic human hair keratin complexes are less stable in urea solutions than those of cytokeratins, although the biological significance of this is unknown.

Two-dimensional gel electrophoresis indicated that the stabilities of the heterotypic complexes in urea differ depending on the combination of type I and II hair keratins (Fig. 3), as observed for various cytokeratin combinations (Eichner et al., 1986; Franke et al., 1983). Among the combinations examined here, K35-K85 (Fig. 3j–l) and K36-K81 (Fig. 3d–f) formed relatively stable complexes compared with the other combinations. These results are consistent with the findings of the SPR measurements in which soluble K35 bound preferentially to immobilized K85 rather than to K81 (Fig. 2b and c), although the binding between K36 and K81 could not be characterized by SPR because of the insolubility of K36 in the buffer used. As described above, K35 and K85 are co-expressed in the matrix/pre-cortex during the initial stage of differentiation, while K36 and K81 are co-expressed in the mid- to upper cortex during the advanced stage of differentiation (Langbein et al., 1999, 2001; Langbein and Schweizer, 2005; Rogers et al., 2004, 2005; Schweizer et al., 2007). Although heterotypic complex formation for other hair keratin combinations remains to be analyzed, the relatively high affinity observed for the combinations of K35-K85 and K36-K81 might have biological importance when different hair keratin proteins exist in the same cell. These results provide useful information on the structural significance of type I and II hair keratins that are co-expressed in the matrix or cortex at the same stage of differentiation. Interestingly, K38 formed a heterotypic complex at a characteristically low urea concentration (4–5 M urea) (Fig. 3g–i and p–r), indicating a particularly low affinity of K38 for either K81 or K85. Because K38 is produced in the lower cortex following the expression of the first pair K35-K85 and simultaneously with K31 (Langbein et al., 1999; Langbein and Schweizer, 2005; Yu et al., 2009), the low affinity of K38 for K85 seems to be a disadvantage when competing with K35 and K31 for heterotypic complex formation with K85 in a cell.

IF assembly experiments carried out in this study revealed the assembly properties of human hair keratins. First, while the human hair keratins formed ULFs under low salt conditions (25 mM Tris-HCl and 0 M NaCl) (Fig. 4a inset, Fig. 5a and b), 180 mM NaCl was required to connect ULFs longitudinally into IFs (Fig. 4a–c and Fig. 5c). Because the head domains regulate IF assembly both in vitro and in vivo (Fuchs and Weber, 1994; Herrmann and Aebi, 2004; Inagaki et al., 1996), it is also likely that the head domain structure determines the optimal ionic strength for in vitro IF formation. In our previous study on
the type III IF protein vimentin, the aromatic residues in the motif (-Tyr-Arg-Arg-X-Phe-) near the amino terminus of the head domain were substituted with hydrophilic residues Asn and Ser (Gohara et al., 2008). The resulting vimentin mutants were very sensitive to ionic strength and formed mature IFs even at 50 mM NaCl, while the wild type required 150 mM NaCl to form IFs. The aromatic residues, especially the Tyr residue, in the motifs appeared to regulate the intermolecular interactions involved in IF assembly and determine an optimal ionic strength for IF assembly. Substitution of the two Arg residues with other amino acids resulted in the loss of the ability to form IFs (Gohara et al., 2008). These motifs can be found near the amino termini in the head domains of both type III and IV IF proteins (Herrmann et al., 1992), but not in the head domains of cytotkeratins. Although human type I and II hair keratins do not have the same motif in the head domains, type II hair keratins such as K81, K83, K85 and K86 all possess several -Tyr-Arg- sequences and a unique -Arg-X-Phe-X-Tyr-Arg-sequence in the head domain. It would be interesting to examine the effects of substitution of these Tyr residues on in vitro IF formation of hair keratins.

Second, the in vitro assembly properties of type II hair keratins K81 and K85 differed markedly. K81 afforded IFs by copolymerization with the type I hair keratin except K38 (Fig. 4a–d), although the IFs were slightly narrower and shorter than the IFs formed by cytotkeratin K14 and K5 (Fig. 4e). In contrast, K85 formed various types of assembly products, such as short straight IFs (Fig. 5c), IF bundles (Fig. 5d), large paracrystalline assemblies (Fig. 5e–h), and IF tangles (Fig. 5i), depending on the type I protein that was copolymerized with. In particular, the combination of K35-K85 resulted short IF bundles with widths of 79±37 nm and lengths of 300–400 nm (Fig. 5d). These IF bundles further polymerized into large paracrystalline assemblies (Fig. 5e–h). Thus, K85 promoted lateral association rather than longitudinal association of short IFs. The promoted lateral association is not caused by formation of disulfide bonds between K85 and the type I hair keratins because K85 has fewer cysteine residues (27 residues) than K81 (34 residues). Unfavorable oxidation of the cysteine residues during IF assembly experiments was suppressed sufficiently under N₂ atmosphere and by the use of an effective reducing reagent such as 10 mM DTT or 5 mM TCEP (Hofmann et al., 2002). Although the structural elements that control the assembly of K85 remain to be clarified, the terminal domains of K85 might be responsible, because the rod domain sequence of K85 did not contain any remarkable structural features when compared with those of other type II hair keratins. The head domain of K85 is relatively long (122 residues), and rich in both arginine (11 residues) and aliphatic residues (27 residues), compared with the head domains of K81, K83 and K86 (total of 105–110 residues; 8 arginine and 21 aliphatic residues). The tail domain (totally 73 residues) of K85 is relatively rich in arginine (5 residues) and aromatic residues (3 residues) but low in cysteine (6 residues) compared with the tail domains of K81, K83 and K86 (total of 69–88 residues; 3–4 arginine and no aromatic residue, and 8–14 cysteine residues).

Third, K38 appeared to lack the ability of de novo IF formation through copolymerization with the type II hair keratin K81 or K85 (Fig. 4d and Fig. 5j). Analysis of the molecular evolution based on the rod domain sequences of human type I hair keratins has revealed that K38 is positioned a relatively long distance from other type I hair keratins including K35 and K36, both of which are located close to each other (Rogers et al., 2004). In addition, K38, K35 and K36 lack homology in the head and tail domain sequences. It has been reported that the expression of K38 occurs in distinct cells that are randomly scattered throughout the entire cortex (Langbein et al., 1999). It has been also reported that K38 is asymmetrically expressed in the cortex of crimped human hair and wool (Thibaut et al., 2007; Yu et al., 2009). Further work is required to understand the biological significance of K38.

In this study, it is noteworthy that the combination of K35-K85 formed tightly packed, short IF bundles and large paracrystalline assemblies (Fig. 5d–h), rather than forming ordinary IFs in vitro. In human hair follicles, K35 and K85 are the first pair expressed in the matrix/pre-cortex during the initial stages of differentiation (Langbein et al., 1999, 2001; Langbein and Schweizer, 2005; Schweizer et al., 2007). They are expressed before hair keratin-associated proteins (KAPs), which are matrix proteins that embed hair keratin IFs in macrofibrils (MFs) (Rogers et al., 2006). Among the human KAPs, KAP8 and KAP11, which are high in glycine-tyrosine and sulfur, respectively, are expressed first in the lower cortex (just above the papilla) (Rogers et al., 2006). Many other KAPs as well as other type I and II hair keratins are expressed primarily in the mid- to upper cortex during the advanced stage of differentiation (Rogers et al., 2006). TEM studies of follicles have revealed the appearance of hair keratin IF bundles, i.e., proto-macrofibrils (proto-MFs), in the lower cortex (Jones and Pope, 1985; Marshall et al., 1991; Morioka, 2009; Orwin, 1979; Orwin and Woods, 1982). Small proto-MFs reported to date are around 80 nm wide and 300–700 nm long (Marshall et al., 1991; Orwin, 1979), so they are similar in size to the short IF bundles formed by the combination of K35-K85 in this study (Fig. 5d). Some TEM images of the cross-sections of early proto-MFs showed that distinctly separated IFs are arranged on a hexagonal basal lattice like a columnar hexagonal liquid crystal, with no matrix proteins interpolated between the filaments (McKinnon, 2006). Proto-MFs coalesce with each other into MFs that are around 400 nm wide and several micrometers long in the mid- to upper cortex (Jones and Pope, 1985; McKinnon, 2006; McKinnon and Harland, 2011; Orwin, 1979). It is more efficient to form proto-MFs composed of short IFs and then connect them longitudinally.
and laterally into MFs than to bundle long IFs into MFs. This study revealed that the pair K35-K85 formed short IF bundles even in the absence of KAPs (Fig. 5d). The intrinsic assembly property of K85 that promotes lateral association rather than longitudinal elongation of short IFs seems to be important for formation of proto-MFs. K85 is abundantly expressed in the matrix, pre-cortex, cortex and cuticle of the hair root, as well as in the nail matrix (Langbein et al., 2001; Langbein and Schweizer, 2005; Perrin et al., 2004; Schweizer et al., 2007). The characteristic assembly of K85 is probably crucial for hair and nail formation, because mutations of K85 result in severe phenotypes (Naem et al., 2006; Shimomura et al., 2010).

In this study, the combination of K36-K81 formed a relatively stable complex in the two-dimensional gel electrophoresis (Fig. 3d–f) and gave IFs with lengths of 200–500 nm in vitro (Fig. 4b). In hair follicles, the expression of both K36 and K81 starts in the mid- to upper cortex, where the expression of K35 has been completed (Langbein et al., 1999, 2001; Langbein and Schweizer, 2005; Schweizer et al., 2007). It may be speculated that IFs formed by the combination of K36-K81 play an important role in the growth of MFs, e.g., by connecting proto-MFs composed of K35-K85 laterally and longitudinally. It has been reported that the initiation of proto-MFs is limited to the lower cortex and that the increases in keratin content in the mid- to upper cortex result from increases in the size of existing proto-MFs, rather than through initiation of new ones (Marshall et al., 1991). Collectively, these results indicate that the controlled expression of hair keratins, especially type II proteins such as K85 and K81, is closely related to the efficient formation of MFs.

In TEM and electron tomographic studies of hair fiber and wool, three types of MFs with different IF arrangements have been identified (para, ortho, and meso), and their distribution across a fiber appears to relate to the crimp or waviness of a fiber (Bryson et al., 2009; Jones and Pope, 1985; Marshall et al., 1991; McKinnon, 2006; McKinnon and Harland, 2011; Morioka, 2009; Orwin, 1979). The molecular mechanism inducing the formation of these three types of MFs has not yet been clarified (Fraser et al., 2003; McKinnon, 2006; McKinnon and Harland, 2011). Differences in both the composition and content of KAPs in the three types of MFs suggest that they play an important role in determining the precise mode of MF packing (Fujikawa et al., 2011; Matsunaga et al., 2013; Plowman et al., 2007; Rogers et al., 2006). In addition, an unknown characteristic surface structure of hair keratin IFs, probably constructed by the head or tail domain, may also contribute to the IF-IF and IF-KAP interactions and the development of the three types of MFs. Further studies on the molecular interactions between hair keratins and KAPs are required to understand the development of MFs leading to the formation of hair fibers with variable waviness and physical strength, and also hereditary hair anomalies.

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References


Assembly of Hair Keratins


