Pair Formation and Promiscuity of Cytokeratins: Formation In Vitro of Heterotypic Complexes and Intermediate-sized Filaments by Homologous and Heterologous Recombinations of Purified Polypeptides

MECHTHILD HATZFELD and WERNER W. FRANKE
Division of Membrane Biology and Biochemistry, Institute of Cell and Tumor Biology, German Cancer Research Center, D-6900 Heidelberg, Federal Republic of Germany

ABSTRACT Cytokeratins are expressed in different types of epithelial cells in certain combinations of polypeptides of the acidic (type I) and basic (type II) subfamilies, showing “expression pairs.” We have examined in vitro the ability of purified and denatured cytokeratin polypeptides of human, bovine, and rat origin to form the characteristic heterotypic subunit complexes, as determined by various electrophoretic techniques and chemical cross-linking, and, subsequently, intermediate-sized filaments (IFs), as shown by electron microscopy. We have found that all of the diverse type I cytokeratin polypeptides examined can form complexes and IFs when allowed to react with equimolar amounts of any of the type II polypeptides. Examples of successful subunit complex and IF formation in vitro include combinations of polypeptides that have never been found to occur in the same cell type in vivo, such as between epidermal cytokeratins and those from simple epithelia, and also heterologous combinations between cytokeratins from different species. The reconstituted complexes and IFs show stability properties, as determined by gradual “melting” and reassociation, that are similar to those of comparable native combinations or characteristic for the specific new pair combination. The results show that cytokeratin complex and IF formation in vitro requires the pairing of one representative of each the type I and type II subfamilies into the heterotypic tetramer but that there is no structural incompatibility between any of the members of the two subfamilies. These findings suggest that the co-expression of specific pair combinations observed in vivo has other reasons than general structural requirements for IF formation and probably rather reflects the selection of certain regulatory programs of expression during cell differentiation. Moreover, the fact that certain cytokeratin polypeptide pairs that readily form complexes in vitro and coexist in the same cells in vivo nevertheless show preferential, if not exclusive, partner relationships in the living cell points to the importance of differences of stabilities among cytokeratin complexes and/or the existence of extracytokeratinous factors involved in the specific formation of certain cytokeratin pairs.

Intermediate-sized filaments (IFs) are characteristic structures present in the cytoplasm of most vertebrate cells which are formed by five different classes of proteins expressed in a cell type–specific manner: Vimentin is found in mesenchymally derived cells, desmin in most myogenic cells, glial filament protein in astrocytes, neurofilament proteins in neurons and some related cells, and cytokeratins in epithelial cells (for reviews see Franke et al., 1982c; Lazarides, 1982; Steinert et al., 1982; Osborn and Weber, 1983). Among these different IF protein classes the cytokeratins are particularly complex.
and ~20 different cytokeratin polypeptides, ranging in Mr, from 40,000 to 68,000 and in isoelectric pH under denaturing conditions from 5 to 8, have been distinguished and shown to be expressed in different sets in different epithelia of human (Moll et al., 1982a; Sun et al., 1984), bovine, and rodent (Schiller et al., 1982) tissues. All IF proteins are characterized not only by their unusually high resistance to treatments with various buffers and solvents, including high salt concentrations, but also by the tendency of the denatured polypeptides to reconstitute, in vitro, IFs, which by electron microscopy, x-ray diffraction analysis, and various biochemical parameters are indistinguishable from native IFs (Cooke, 1976; Lee and Baden, 1976; Steinert et al., 1976, 1979, 1980, 1982; Small and Sobieszek, 1977; Starger et al., 1978; Steinert, 1978; Sun and Green, 1978; Rueger et al., 1979; Geisler and Weber, 1980, 1981; Huiatt et al., 1980; Cabral et al., 1981; Franke et al., 1979, 1981a; Renner et al., 1981; Liem and Hutchinson, 1982; Zackroff et al., 1982; Aebi et al., 1983). In general, nonepithelial IF polypeptides can assemble into IFs alone whereas the formation of cytokeratin IFs requires at least two different cytokeratin polypeptides (Lee and Baden, 1976; Steinert et al., 1976, 1982; Steinert, 1978; Milstone, 1981). More specifically, it has been claimed by some authors that IFs would only form when the polypeptides are present at molar ratios of 2:1 or 1:1:1, in accord with the concept of a triple-chain α-helical core structure, which was the prevailing model until recently (Skerrrow et al., 1973; Lee and Baden, 1976; Steinert et al., 1976, 1979, 1980, 1982; Steinert, 1978). This model, however, is in contrast to other models of IF subunit organization, which are based on the existence of four-chain subunits, as suggested by analyses of proteolytically obtained fragments of sheep wool α-keratins (Ahmadi and Speakman, 1978; Ahmadi et al., 1980; Woods and Gruen, 1981; Crewhter et al., 1983; Gruen and Woods, 1983; Woods and Inglis, 1984). A tetrameric subunit organization has first been shown for desmin (Geisler and Weber, 1982; Geisler et al., 1982) and has been proposed to apply to IFs in general (Weber and Geisler, 1984).

In the last few years it has become apparent that the diverse cytokeratin epithelial cell types examined express at least one member of either subfamily (Moll et al., 1982a), and Sun and colleagues have emphasized the conspicious patterns of co-expression of certain cytokeratins that suggest the existence of distinct “expression pairs” (Sun et al., 1984; Cooper et al., 1985). In addition, it has been shown that pair combinations of type I and type II polypeptides (Fuchs et al., 1981, 1984; Crewhter et al., 1983) form, in vivo and in vitro, complexes that are remarkably stable to denaturing agents such as urea, and the dissociation (“melting”) point of the type I and II cytokeratins is a characteristic of a given pair (Franke et al., 1983, 1984).

Analyses of the free complexes obtained with moderate concentrations of certain denaturing agents (e.g., 4 M urea) have revealed the existence of distinct tetrameric subunits in cytokeratin IFs, containing two type I and two type II polypeptides arranged in two tightly associated coiled-coils (Quinlan et al., 1984a), in agreement with models proposed for sheep wool α-keratins (Ahmadi et al., 1980; Gruen and Woods, 1983; Woods and Inglis, 1984; for epidermal keratins see also Woods, 1983; Parry et al., 1985) and the homotypic four-chain subunit organization of desmin and vimentin IF (Geisler and Weber, 1982; Pang et al., 1983; Quinlan et al., 1984a; Geisler et al., 1985).

It is not clear, however, whether the co-expression of certain cytokeratin pairs merely reflects the coordinated regulation of these genes in the same cell differentiation program or whether there exist incompatibilities or selective structural requirements of cytokeratin “pairing” and IF formation. For example, Sun et al. (1984) and Cooper et al. (1985) have recently emphasized a systematic size difference of Mr ~8,000 between the specific type II and type I members of the cytokeratin pairs, which, if it were essential for IF formation, would exclude certain combinations of cytokeratin polypeptides. In the study described here we have systematically examined the compatibility of different purified cytokeratin polypeptides in the formation of complexes and IFs by combining in vitro different members of the type I subfamily with different members of the type II subfamily, including combinations never observed in vivo and heterologous combinations between different species. We show that (a) independent of the patterns of expression observed in cells and tissues, all examined type I cytokeratins can complex with all type II cytokeratins, which can then form IFs in vitro, and (b) heterologous recombinations of cytokeratins are readily obtained. The results indicate that cytokeratin IF formation does not appear to depend on the expression of certain pairs because of structural restrictions and incompatibilities of some cytokeratin polypeptides.

**MATERIALS AND METHODS**

**Cells and Tissues:** Cells of established cell lines (bovine MDBK and BMGE lines, human MCF-7 and Detroit 562) were grown as described (Franke et al., 1981c; Moll et al., 1982a; Schmid et al., 1983). Bovine muzzle epidermis and other bovine tissues were obtained from the local slaughterhouse (compare Franke et al., 1981c). Human epidermis from breast, abdominal skin, or foot soles, human tongue and oral mucosa, and intestinal mucosa were biopsy or autopsy materials (compare Franke et al., 1981c; Moll et al., 1982a, b). Rat tissues were obtained as described (Franke et al., 1981a-d).

**Preparation of Cytoskeletal Fractions:** Cultured cells were lysed and extracted with high salt buffers containing 1% Triton X-100 and 5 mM EDTA (Franke et al., 1981a-c). Cell residues were usually sheared briefly with a Dounce homogenizer in high salt buffer to reduce the amount of residual DNA (Quinlan et al., 1984a).

Cytoskeletal residues were prepared from small pieces of bovine and rat tissues by extraction in high salt Triton X-100 buffers as described (Franke et al., 1981a-d, 1982a; Quinlan et al., 1984a).

Cytokeratin-enriched fractions from human tissues were prepared as follows: The epithelial layers were scraped off, minced with scissors, homogenized briefly in detergent buffer (10 mM Tris-HCl, 150 mM NaCl, 0.5% Triton X-100, 5 mM EDTA, pH 7.6) using a Polytron homogenizer (Kinematica, Lucerne), and then extracted in high salt buffer containing 1% Triton X-100 and 5 mM EDTA.

**Purification of Cytokeratins:** Individual cytokeratins were electrophoretically separated, or were chromatographically purified using ion-exchange chromatography on DEAE-cellulose, or reverse-phase high-performance liquid chromatography (HPLC; Quinlan et al., 1984a). In some cases a combination of the two methods was necessary to obtain optimal purification. Gel electrophoresis-separated polypeptide bands were visualized by incubating the gels in 4 M sodium acetate solution (Higgins and Dahmus, 1979), excising, and then extracted in high salt buffer containing 1% Triton X-100 and 5 mM EDTA.

**DEAE-cellulose anion-exchange chromatography (DE52, Whatman Chemical Separation Inc., Clifton, N J; Millipore, Molsheim, France) was performed**: in 8 M urea in 30 mM Tris-HCl (pH 8) containing 5 mM dithiothreitol. Cytoskeletal proteins were dissolved in the 8 M urea buffer, bound to the column in the same buffer, and eluted with a linear gradient from 0-100 mM guanidinium-HCl (same buffer). Fractions were either used directly for reconstitution assays or applied to the HPLC column.

**For reverse-phase HPLC separations, a pump and control unit (LKB 2150 HPLC pump, 2152 HPLC controller, LKB Instruments Inc., Bromma, Sweden) and a reverse-phase column (Hi-Pore RP304 250 x 4.6 mm, Bio-Rad Labora-
tories, Richmond, CA) were used. The solvents applied were 0.1% trifluoroacetic acid (Fluka AG, Buchs, Switzerland) as aqueous phase and 0.07% TFA in acetonitrile (Liquistrol, chromatographic grade, Merck, Darmstadt, Federal Republic of Germany) as organic phase. Acetonitrile was removed by vacuum evaporation and the samples were then lyophilized. Protein determinations were performed according to the method described by Bradford (1976).

Renaturation of Proteins and Reconstitution of Complexes and IFS: Total cytoskeletal proteins or various combinations of purified cytokeratin polypeptides were dissolved at various ratios (adjusted to near-molar concentrations of 1:2, 1:1, 2:1) in 9.5 M urea in 5 mM Tris-HCl buffer (pH 8.0) containing 25 mM 2-mercaptoethanol and, for complex reconstitution, dialyzed for 3 h against 4 M urea in 5 mM Tris-HCl buffer (pH 8.0) containing 25 mM 2-mercaptoethanol. In experiments involving chemical cross-linking, the dialysis was against 4 M urea, 10 mM sodium phosphate buffer, 10 mM 2-mercaptoethanol (pH 8.0). Aliquots were analyzed by two-dimensional gel electrophoresis and electron microscopy. For analysis by differential "melting", polypeptide fractions (see above) containing 5 mM urea solution to 5 mM Tris-HCl buffer above) containing various concentrations of urea (4-9 M).

Reassembly of complexes into IFS was initiated by stepwise dialysis first to 10 mM Tris-HCl buffer (pH 7.6) and then to 30 or 50 mM Tris-HCl buffer (same pH), both containing 10 mM 2-mercaptoethanol. Aliquots were analyzed by electron microscopy and, after pelleting by centrifugation for 20 min at 100,000 x g (Airfuge, Beckman Instruments, Munich), by SDS PAGE. Amounts of pelleted and nonpelleted proteins were determined as described (Bradford, 1976).

Gel Electrophoresis: For SDS PAGE the system described by Laemmli (1970) was used. For two-dimensional gel electrophoresis isoelectric focusing (O'Farrell, 1975) or nonequilibrium pH gradient electrophoresis (O'Farrell et al., 1977) was used for separation in the first dimension (for some modifications see Franke et al., 1981c; Moll et al., 1982a). Reconstituted complexes were analyzed by isoelectric focusing or nonequilibrium pH gradient electrophoresis in the presence of the same concentrations of urea as in the sample (Franke et al., 1983). Gels were stained and densitometrically determined using the gel scanner system of the Gilford 2600 spectrophotometer (Gilford Instrument Laboratopries, Inc., Oberlin, OH).

Chemical Cross-linking: Combinations of two different cytokeratin polypeptides were mixed, at various ratios (see above), in 9.5 M urea, 5 mM Tris-HCl (pH 8), 25 mM 2-mercaptoethanol, and then dialyzed against 4 M urea in 10 mM sodium phosphate (pH 5.5) containing 10 mM 2-mercaptoethanol. Dimethyl suberimidate (DMS; Pierce Chemical Co., Rockford, IL) was dissolved in the same buffer, the pH was readjusted to 8.0, and the buffer was added to the samples so that 0.02-0.05 mg/ml protein was exposed to final concentrations of 0.5 and 1 mg/ml DMS, respectively. After incubation for 1-2 h at room temperature the reaction was stopped by precipitation with 10% trichloroacetic acid and two subsequent washes of the precipitate with acetone/water (1:1) for 24 h. For SDS PAGE, proteins were dissolved in sample buffer. For identification of cross-linked complexes, diagonal electrophoresis was performed as described by Packman and Perham (1982). Cross-linked samples were separated on SDS PAGE (7.5% acrylamide), and the gel track was excised and treated with 2 M methylvamine (analytical grade Merck) at pH 11.5 in 75% acetonitrile for 3 h at 37°C. After cleavage of the cross-links the gel tracks were re-equilibrated in SDS-containing sample buffer, and polypeptides were separated by the second dimension SDS PAGE.

Electron Microscopy: Preparation for electron microscopy, using the spraying-rotary shadowing or the negative staining technique, was as described (Franke et al., 1982a; Quinlan et al., 1984a).

RESULTS

Previous cytokeratin IF reconstitution studies, using polypeptide separated and/or analyzed by preparative gel electrophoresis (e.g., Lee and Baden, 1976; Steinert et al., 1976, 1982; Milstone, 1981), have documented the purity of their preparations by one-dimensional gel electrophoresis. Since certain cytokeratin polypeptides display very similar mobilities on SDS PAGE but differ in their isoelectric pH values (Franke et al., 1981c; Moll et al., 1982a), we found it necessary to monitor the purity and integrity of the preparations obtained by two-dimensional gel electrophoresis.

Purification of Cytokeratin Polypeptides

For this study it was necessary to purify individual cytokeratin polypeptides. To achieve this, different purification methods had to be used for different polypeptides. Whereas some cytokeratin polypeptides could be obtained in a form that appeared homogenous on two-dimensional gel electrophoresis by HPLC alone, by use of the technique of Quinlan et al. (1984a), other polypeptides could only be separated from each other by the combined use of DEAE-cellulose ion-exchange chromatography and subsequent HPLC. Still others could only be separated by dissection electrophoretically separated protein bands from polyacrylamide gels. Fig. 1, a–j, document the purity of several of the human cytokeratin polypeptides used in this study and also show that the procedures used for purification do not considerably alter the isoelectric pH value (compare with Moll et al., 1982a).

Formation of Heterotypic Cytokeratin Complexes from Purified Polypeptides

Individual cytokeratins were examined for their ability to reassociate into the heterotypic complexes of two type I and two type II cytokeratin polypeptides that have been shown to be identical to the tetrameric subunits of IFS (Geisler and Weber, 1982; Woods, 1983; Quinlan et al., 1984a, b; Parry et al., 1985). The individually purified human cytokeratin polypeptides A (No. 8) and D (No. 18), which are co-expressed in rat liver and human epithelial cells (Moll et al., 1982a; Cooper et al., 1985), were denatured (Fig. 2, a and b), mixed in 9.5 M urea containing buffer and dialyzed to buffer containing 4 M urea to induce complex formation. At molar ratios of 1:1 maximal recovery of both polypeptides in the isoelectric complex was obtained. The stability of this in vitro-formed complex to dissociation in increasing concentrations of urea ("melting") was compared with that of naturally occuring complexes of human components A and D as obtained from cultured hepatocellular carcinoma cells of the
FIGURE 1 Some examples demonstrating the purity of isolated human (a-j; numbers according to Moll et al., 1982a) and bovine (k-n; for numbering see Schiller et al., 1982) cytokeratin polypeptides as demonstrated by two-dimensional electrophoresis. Proteins were separated by reverse-phase HPLC (a, f), DEAE anion-exchange PLC line (our unpublished data), as determined by two-dimensional gel electrophoresis using different urea concentrations (see Franke et al., 1983). The “mating cross” summary of Table I lists the successful complex formations of purified human cytokeratins. Fig. 2, c–e presents some selected examples from such “melting series,” showing the intact complex at 5 M urea, the partly dissociated polypeptides at 7 M urea (Fig. 2 d), and the almost completely separated polypeptides at 7.5 M urea. The melting curve and the midpoint (at ~6.8 M urea; for definition of “melting points,” $U_m$, see Franke et al., 1983, 1984) were essentially the same as those observed for the complex occurring in vivo (see also Franke et al., 1984), indicating that the purification methods did not have any influence upon the association characteristics of the polypeptides. Similar observations were made with in vitro recombinations of purified bovine and rat cytokeratins A and D (data not shown).

To examine the compatibility of the corresponding cytokeratins from different species that are expressed in the same cell type, bovine cytokeratin A and human component D (No. 18) were mixed in nearly equimolar amounts in the presence of 9.5 M urea and allowed to reassociate into complexes by dialysis against lower concentrations of urea. Interspecies complexes were readily obtained (Fig. 2 f) and showed melting properties similar to those of the homologous complex of human cytokeratins A and D ($U_m$, ~6.7 M urea; Fig. 2, g–j). The reciprocal combination of human cytokeratin A (No. 8) and bovine cytokeratin D also resulted in stable interspecies complexes (Fig. 2, k–n) that revealed dissociation characteristics somewhat intermediate ($U_m$, ~6.4 M urea) between those of the native human and bovine complexes ($U_m$ of the latter, ~6.0 M urea; compare Franke et al., 1983, 1984).

Complex formation was also successful when we mixed cytokeratin polypeptides that in vivo are never co-expressed, such as epidermal cytokeratins with those of simple epithelia. Table I summarizes several examples of human cytokeratin complexes of this kind, and Fig. 3 presents in some detail two examples of bovine cytokeratin complexes, namely that formed by the simple epithelium-specific component A (No. 8) and the epidermis-specific component VI (No. 13), as well as that recombined from purified polypeptides A and VII (No. 16), the latter of which occurs in various stratified epithelia and certain cultured cells (Franke et al., 1981c; Schiller et al., 1982; Schmid et al., 1983; Schiller, 1985). The stability of the complex of cytokeratin polypeptides A and VI (Fig. 3, a–e) was comparable to that of the naturally occurring complexes containing component VI (Franke et al., 1983). The complex between polypeptides A and VII, however, appeared considerably less stable (Fig. 3, f–j) than the in vivo complexes between basic epidermal cytokeratins (I–IV) and VII (compare Franke et al., 1983).

Figures 2 and 3 indicate typical degradation products of cytokeratin 8.
FIGURE 2 Two-dimensional gel electrophoresis of homologous and heterologous combinations of cytokeratins A and D at different urea concentrations. Isoelectric focusing (IEF) was used in the first dimension, as indicated in some of the figures (a, f, k). BSA (B) and actin (a) were co-electrophoresed as marker proteins. All purified proteins were dissolved alone or in a mixture with the other polypeptide in 9.5 M urea and dialyzed against the different urea concentrations, as indicated in the upper right corner. Isoelectric positions of purified human cytokeratins A (a) and D (b) are not altered by the purification procedure. (c–e) Melting behavior of isolated and recombined human cytokeratins A and D: (c) at 5 M urea the complex is still intact, and both polypeptides focus together (A' and D' indicate positions of cytokeratins A and D in the complex); (d) at 7 M urea a portion of the polypeptides has dissociated and migrates as individual polypeptides (D, D'); (e) at 7.5 M urea the complex is completely dissociated (compare Franke et al., 1983, 1984). (f–j) Melting behavior of the heterologous complex of bovine component A (Ao and Ao') and human component D (Do and Do'): (f) the complex is stable at 5 M urea and partly dissociated at 6 M urea (g); (i) almost complete separation is achieved at 7.5 M urea; (j) complete dissociation occurs at 9.5 M urea. (k–o) Dissociation characteristics of the complex of human component A (Ah and Ah') and bovine component D (Db and Db'): (k, t) at 4 and 5 M urea the complex is still intact; (m) it partly dissociates at 6.5 M urea; complete separation is achieved at 7 M (n) and 8 M (o) urea.

**TABLE I. Human Cytokeratin Polypeptide Pairs Observed in Complexes and Filaments In Vitro and In Vivo**

| No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|-----|---|---|---|---|---|---|---|---|
| 9   | +/+| ND| +/o| +/o| +/o| +/o| +/o| +/o|
| 10/11 | +/+| ND| +/o| +/o| +/o| +/o| +/o| +/o|
| 12  | ND| ND| +/o| ND| ND| ND| ND| ND|
| 13  | +/o| ND| +/o| +/o| +/o| +/o| +/o| +/o|
| 14  | +/o| ND| ND| +/o| +/o| +/o| +/o| +/o|
| 15  | ND| ND| ND| ND| ND| ND| ND| ND|
| 16  | +/o| ND| +/o| +/o| +/o| ND| ND| +/o|
| 17  | +/o| ND| ND| +/o| +/o| +/o| +/o| +/o|
| 18  | +/o| ND| +/o| +/o| +/o| +/o| +/o| +/o|
| 19  | +/o| ND| ND| +/o| +/o| +/o| +/o| +/o|

Complex formation observed by two-dimensional gel electrophoresis, using different urea concentrations in the first dimension separation, and IFs observed by electron microscopy. In many experiments complexes have also been analyzed by "differential melting" in urea, and chemical cross-linking and proteins recovered in reconstituted IFs have been determined by gel electrophoresis. o, not observed in vivo (this does not exclude its possible existence in a specific cell type not yet identified). ND, not determined.

* Cytokeratin polypeptides are indicated by numbers according to Moll et al. (1982a).
* Component No. 2 is a minor polypeptide of epidermis and related epithelia and has not been included in this study.
* Components Nos. 10 and 11 are not well separated by the techniques used.
In contrast, combinations of two different cytokeratin polypeptides of the same subfamily never resulted in such complexes, not only with combinations of polypeptides from different tissues but also with cytokeratins that are co-expressed in certain cells (Fig. 3, k–o shows some examples).

We have previously shown (Franke et al., 1984) that certain cytokeratin polypeptides expressed in the same cell exist predominantly, if not exclusively, in different heterotypic complexes, which indicates that these polypeptides are not exchangeable in the living cell but display different affinities to the specific components of the other subfamily. We have therefore studied complex formation in vitro between cytokeratin polypeptides that are co-expressed but not complexed in vivo. Fig. 4, a–e shows that human epidermal cytokeratin polypeptide No. 1, which in vivo is complexed primarily with cytokeratins Nos. 10 and 11, in vitro can form complexes with epidermal cytokeratin Nos. 14, which in vivo is predominantly found in complexes with cytokeratins Nos. 5. However, the stability of the in vitro complex between cytokeratins Nos. 1 and 14 was found to be significantly lower ($U_m \sim 6.7$ M urea) than that of the corresponding in vivo complex of cytokeratins Nos. 5 and 14 ($U_m \sim 8.2$ M urea) but similar to that of the in vivo cytokeratin complex of Nos. 1 and 10/11. The reciprocal in vitro combination between polypeptides Nos. 5 and 10/11 also formed stable complexes (Fig. 4, f–j), which, interestingly, were similar in their stability to dissociation in urea to the natively occurring epidermal complexes between cytokeratins Nos. 5 and 14. For controls, we show in vitro reconstitutions of the complexes between polypeptides Nos. 5 and 14 (Fig. 4k) and 1 and 10/11 (Fig. 4e), which are known to occur in vivo (Franke et al., 1984).

Of special interest in this connection were certain epidermal keratins with unusual biochemical properties, which occur only in special body sites. The most prominent example is human cytokeratin No. 9, which is by far the largest type I cytokeratin ($M, 64,000$) with a rather low isoelectric pH value when denatured in urea (pH 5.4) and which has been found only in foot soles and palms (Moll et al., 1982a). Recently Sun et al. (1984) have expressed some doubts that this is a genuine type I cytokeratin at all. When we examined the ability of purified cytokeratin No. 9 to complex with various other purified type I and type II human cytokeratins we found that it formed stable complexes with all type II cytokeratins examined, including cytokeratin No. 1 (Fig. 4, m and n),
Figure 4 Two-dimensional gel electrophoresis of isolated and recombined human cytokeratin polypeptides at different urea concentrations. First dimension separation was NENPHGE (NE), as indicated in a, f, k, and p. Treatment of proteins and labels are as in previous figures. (a–e) Combinations of epidermal cytokeratins 1 and 14, which do not form complexes in vivo can complex in vitro: (a) at 4 M urea both polypeptides appear in an isoelectric position (1' and 14') due to their inclusion in a complex, which starts to dissociate at 6 M urea (b) and further at 7 M (c) and 8 M (d) urea. Total separation is achieved at 9.5 M urea (e). (f–j) Combinations of cytokeratins 5 and 10/11, which do not form complexes in vivo do so in vitro: (f) both polypeptides are in the position of a complex (5' + 10'/11') at 4 M urea. This complex is still stable in 6 M (g) and 7 M (h) urea, whereas at 8 M urea considerable amounts are dissociated (i). (j) Positions of fully separated cytokeratins Nos. 5 and 10/11 at 9.5 M urea. (k) Recombinations of complexes of purified epidermal cytokeratins Nos. 5 and 14 (a–d), which form a very stable naturally occurring complex (compare Franke et al., 1984). The complex (5'14') is stable at 7 M urea. (l) In vitro recombination of complexes from purified epidermal cytokeratins which form a complex in vivo (isoelectric complex positions denoted 1' and 10'/11' at 4 M urea). (m, n) Cytokeratin No. 1 from normal epidermis and No. 9 from human foot sole epidermis focus in the same position at 4 M urea due to complex formation (m) and are separated at 9.5 M urea (n). (o, p) Purified type II cytokeratin polypeptides Nos. 5/6 and 4/5 form complexes in vitro with the foot sole specific cytokeratin No. 9 (5'/6'9' and 4'/5'9'). (q) In vitro complex of cytokeratin No. 8 from simple epithelia and cytokeratin No. 9 from human foot sole epidermis (8'/9'). Note that in p and q, approximately equal amounts of the basic (type II) cytokeratins 4/5 and 8 co-migrate with cytokeratin No. 9 due to their inclusion in a complex (4'/5'9' in p, 8'/9' in q), whereas the excess amounts of components 4/5 and 8 are separated, indicating that they are excluded from complex formation. (r–t) Recombination of purified type II cytokeratins Nos. 5/6 and 4/5 with small type I cytokeratins Nos. 17 (5'/6' and 17' in r), 18 (5'/6' and 18' in s), and 19 (4'/5' and 19' in t) from simple epithelia.

which is its predominant partner in vivo, as well as cytokeratins Nos. 4, 5, and 6 (Fig. 4, o and p), with which it is not known to be complexed in vivo. Remarkably, the foot sole epidermal cytokeratin No. 9 could also form complexes with cytokeratin No. 8 from simple epithelial cells, both in homologous (Fig. 4q) and heterologous (with bovine and rat cytokeratin A; not shown) combinations. Similar observations were made with complexes formed in vitro between the corresponding bovine hoof-specific epidermal cytokeratin No. 9 (Schiller et al., 1982; Schiller, 1985) which formed complexes not only with its epidermal type II in vivo partners (bovine cytokeratins Nos. 1–6 of Schiller et al., 1982) but also with nonepidermal cytokeratin No. 8 (data not shown).

Table I summarizes our results of complex formations in vitro with all human cytokeratins, except cytokeratins Nos. 3 and 12, which are specific for cornea (Moll et al., 1982a;
dehydrogenase (M, 55,000), actin (Mr 42,000), and carbonic anhydrase complexes of polypeptides Nos. 17, 18, and 19 with type II that was in excess appeared to be excluded from the complexes component in most epithelial cells that contain this protein.

Electron Microscopy of Reconstituted Cytokeratin Complexes and Filaments

When individual purified cytokeratin polypeptides were first denatured in 9.5 M urea and then allowed to renature in low salt buffer solutions, we observed in no case the formation of 2- or 4-nm-diam protofilaments of considerable length. Using rotary metal shadowing and negative staining techniques, we noted that such preparations consisted primarily of rods 40–48 nm long (Fig. 6, a–c present examples of three different nonepidermal and epidermal cytokeratin polypeptides), which in negatively stained preparations revealed a diameter of 2–3 nm (not shown; compare Franke et al., 1982a, 1984). These rodlike complexes reconstituted from individual cytokeratin polypeptides were similar to the cytokeratin rods seen upon disintegration of hepatocytic IFs by 4 M urea or 2 M guanidine hydrochloride (Franke et al., 1984; Quinlan et al., 1984a) and reconstituted bovine epidermal keratin structures arrested in 4 M urea (Franke et al., 1982a). In some preparations, especially those made from type II cytokeratins, we also found sizable, but variable, proportions of 10–30-nm-diam granules or short cylinders (not shown), similar to those reported for renatured type I bovine keratin (Steinert et al., 1982) and bovine keratin mixtures (Franke et al., 1982a; for similar granules in preparations of vimentin IF see Renner et al., 1981).

When purified cytokeratin polypeptides were mixed in buffer containing 9.5 M urea so that the cytokeratin complement of the specific cytoskeletal IF was restored, and then allowed to renature and assemble in appropriate low salt buffer, protofilamentous structures and IFs readily formed. Fig. 7a presents such a control showing IFs formed from an ~2:1:1 (wt/wt/wt) mixture of human cytokeratins Nos. 8, 18, 19 as they occur in cultured human carcinoma cells of the MCF-7 line (Moll et al., 1982a). With prolonged incubation times the ratio of IFs to protofilament structures usually increased, but some residual rod complexes and protofilaments were always noticed (Figs. 7, a–d and 8a). That IF reconstitution was obtained independently of the specific method of polypeptide purification used (HPLC, DEAE-chro-
IFs from both homologically and heterologously combined cytokeratins showed the typical ~20-nm periodicity revealed upon spraying and rotary metal shadowing (e.g., Fig. 7c) typical for reconstituted IFs of various kinds of IF proteins, cytokeratins included (Ahmadi and Speakman, 1978; Franke et al., 1982a; Henderson et al., 1982; Milam and Erickson, 1982). In both homologous and heterologous recombinations we also observed, besides normal IFs, loosely fasciated protofilaments and residual 2-nm rods, 10–25-nm diam granules or annuli (e.g., Figs. 7, b–d, 8, a–d). We did not notice any systematic differences of frequencies of any of these structures with respect to the specific polypeptide combination examined. Therefore, we suspect that the variations in the relative frequencies of residual rods, granules, or protofilaments reflect variable minor differences of quantities (see also below) of polypeptides and/or marginal experimental influences during reconstitution.

An example of IF reconstitution from two purified epidermal polypeptides, i.e., a member of the bovine cytokeratin group la–c (for nomenclature see Franke et al., 1978, 1981c; these are Nos. 1–3 of the bovine catalog of Schiller et al., 1982), representative of the type II subfamily; and cytokeratin VI (No. 13), representative of type I cytokeratins, is shown in Fig. 8, a–c (see also Jorcano et al., 1984). Similar IF preparations were obtained in combinations of approximately equimolar amounts of other bovine epidermal cytokeratins such as la–c with VII, III/IV with VI, III/IV with VII (not shown). Moreover, when epidermal type I cytokeratins such as bovine epidermal components VI or VII were combined with type II cytokeratins from a different tissue that did not express cytokeratins VI and VII, reconstitution of IFs was also observed. For example, Figs. 8, d and e present IFs formed from purified bovine cytokeratin A (No. 8) from cultured kidney epithelial cells of the MDBK line and purified bovine cytokeratin VI (No. 13) purified from muzzle epidermis. This showed that in vitro a cytokeratin from a simple epithelium can properly recombine with a cytokeratin specific for epidermal differentiation and form normal IFs. Essentially identical results were obtained when bovine cytokeratin A was recombined with bovine cytokeratin VII (No. 16) or when epidermal cytokeratin polypeptides III and IV (Nos. 6 and 7) were allowed to react with cytokeratin D (No. 21).

Similarly, IFs were observed when various purified human type I cytokeratins were recombined with purified human type II cytokeratins, including combinations not found in vivo. The results of the various reconstitution experiments with human cytokeratins are summarized in Table I. Positive examples included recombinations of cytokeratin polypeptides that were not found to coexist in any of the cell lines and the tissues examined (compare Moll et al., 1982a; Schiller et al., 1982; Quinlan et al., 1984b; Schiller, 1985). We specifically examined whether certain human cytokeratins of unu-
FIGURE 8 Electron microscopy showing IFs formed in vitro from purified cytokeratin polypeptides. (a-c) IFs formed by polypeptides I and VI from bovine epidermis as visualized by rotary metal shadowing (a, b) or negative staining (c). Procedure was as in Fig. 8. Typical IFs but also some 40–45-nm rodlets (brackets in a) are seen. (d, e) IFs from purified bovine cytokeratin polypeptides A (No. 8) and VI (No. 13) using the rotary metal shadowing technique. Note IFs as well as a few annular structures (arrows in a, b, and d). Bars, 0.2 μm (a, b, c, e) and 0.5 μm (d).

Sial molecular properties and an unusually restricted occurrence could form IFs in vitro. For example, human cytokeratin No. 9 (see above) formed normal-looking IFs when combined in vitro with purified cytokeratin No. 1 (Fig. 9), with which it co-exists in vivo. However, it also formed IFs together with cytokeratin No. 8, with which it does not coexist in any of the cell types and tissues examined so far (Moll et al., 1982a). Similarly, human cytokeratins Nos. 10 and 11, two other relatively large acidic (type I) keratins of epidermis and some other related stratified epithelial (Moll et al., 1982a), could form IFs with cytokeratin No. 1 and other type II cytokeratins (data not shown).

Stoichiometry of Cytokeratin Polypeptides in Reconstituted Filaments

In several of our reconstitution experiments we examined
the polypeptide composition of the pelleted IFs (see Materials and Methods) by SDS PAGE and densitometry of the stained bands. In all cases the ratio of the type I and type II polypeptides in the IFs formed was close to equimolarity (Fig. 10). Moreover, in experiments in which one partner was added in excessive amounts this excess appeared to be excluded from the complexes (Fig. 4, p and q) and the resulting pelletable filament material (for two examples see Fig. 10, a, b and d). An experimental problem was encountered in such experiments with certain large epidermal cytokeratins (e.g., human and bovine components 1) which do not complex with each other into IFs but form unspecific aggregates pelletable in a non-IF form.

DISCUSSION

The results of our systematic study on the in vitro recombination of the diverse cytokeratins from different species allow us to draw several general conclusions about the interaction of polypeptides in cytokeratin complexes and IFs.

Our results confirm observations of other groups (Lee and Baden, 1976; Steinert et al., 1976, 1982; Moll et al., 1982a) that individual cytokeratin polypeptides of either subfamily cannot form IFs. Our present data further suggest that individual cytokeratin polypeptides cannot form protofilaments of appreciable length. On the other hand, we have found that several purified cytokeratin polypeptides can renature and reassemble into rodlike structures, which, in their shape and dimensions (~2 x 45 nm), are almost indistinguishable in the electron microscope from the rodlike heterotypic tetramer subunits that have been observed upon disintegration of cytokeratin IF denaturing agents (Franke et al., 1984; Quinlan et al., 1984a). We think, however, that the rodlets formed by individual cytokeratin polypeptides in the present study are predominantly homodimers, as judged from sedimentation equilibrium analysis, gel electrophoresis of the undenatured complex, and chemical cross-linking data (Hatzfeld, M., R. A. Quinlan, A. Lustig, and W. W. Franke, unpublished observations). We suggest that individual cytokeratin polypeptides can arrange, at least in vitro, into coiled-coil homodimers, and perhaps even a few homotetramers, but cannot tandemly associate to form protofilaments and IFs. This seems to be at odds with the recently published suggestion of Parry et al. (1985) which proposes that cytokeratin IFs formed are composed of heterodimer complexes (for discussion see also Steinert et al., 1984).

Probably the most important conclusion from our study is the finding that in vitro all of the examined cytokeratin polypeptides of either cytokeratin subfamily can form complexes and filaments with any member of the other subfamily. This does not hold only for co-expressed cytokeratins but also for polypeptides that obviously are not simultaneously synthesized in any cell type, i.e. proteins that never form complexes in vivo. Formation of heterotypic complexes and IFs is even observed in extreme combinations such as between cytokeratin A (No. 8 of the human and bovine catalogs), specific for various simple epithelia (Franke et al., 1981a–d, 1982c; Wu and Rheinwald, 1981; Moll et al., 1982a; Cooper et al., 1985), and cytokeratin No. 9, which is an epidermis-
specific keratin expressed only in certain body locations (in human foot soles and palms and in the posterior hoof pad of the cow; Moll et al., 1982a, b; Schiller et al., 1982; Sun et al., 1984; Cooper et al., 1985; Schiller, 1985).

This principle of pair requirement for cytokeratin IF formation is in agreement with the concept of "expression pairs" of Sun et al. (1984) and Cooper et al. (1985). The pair combinations successful in IF formation in vitro described in this study also provide an explanation for some earlier reports of in vitro formation of IFs, such as that describing filaments formed from two murine epidermal keratin polypeptides (Steinert et al., 1979) designated K1 (Mr 68,000, now known to belong to the type II subfamily) and K2 (Mr 59,000, now known to be type I) and closely related to bovine epidermal cytokeratin VI (compare Jorcano et al., 1984). Short (<0.2 μm) pieces of IFs have also been described (Whitman-Aynardi et al., 1984) in reconstitutions in vitro of Mr 54,000 HeLa cytokeratins (which is a mixture of cytokeratins Nos. 7 and 8; compare Franke et al., 1981c, 1982c; Moll et al., 1982a) and the low molecular weight range proteins of HeLa cells (which include cytokeratins Nos. 17 and 18; compare Franke et al., 1981c). The principle of type I-type II cytokeratin pairing is also compatible with most of the observations of

**Figure 10** Some examples demonstrating the stoichiometry of cytokeratin polypeptides in reconstituted, pelletable IFs as shown by SDS PAGE and densitometry of the Coomassie Blue-stained bands. In separate experiments, human cytokeratin No. 8 (A) was mixed with human cytokeratin No. 19 (denoted by the arrows in a, lane 6) or with bovine epidermal component VII (No. 16 of the bovine catalog of Schiller et al., 1982; denoted by the lower of the two arrows at the right margin in a) in low salt buffer containing 9.5 M urea in molar ratios of 1:1 (lanes 1 and 7), 1:1.9 (lane 4), and 1:1.6 (lane 10). The samples were dialyzed stepwise first into 10 mM Tris-HCl buffer (pH 8) containing 4 M urea, and then into 30 mM Tris-HCl buffer (pH 7.6). After centrifugation supernatant fractions (a, lanes 2, 5, 8, 11) and pelleted IFs (a, lanes 3, 6, 9, 12) were analyzed by SDS PAGE and densitometry. With near equimolar mixtures of type I and type II cytokeratins (a, lanes 1–3 and 7–9), supernatant fractions (lanes 2 and 8), as well as pelleted IFs (lanes 3 and 9) contained both polypeptides in a near-equimolar ratio (1:1.15 in lane 3, 1:0.8 in lane 9). Combinations in which one cytokeratin polypeptide exceeded that of cytokeratin No. 8 (e.g., human component No. 19 in lanes 4–6, and bovine component VII in lanes 10–12) showed near-equimolar ratios of the two specific cytokeratins in the pelletable IF material (a, lanes 6 and 12), whereas an enrichment of the excessive cytokeratin was noted in the supernatant fraction (component no. 19 in lane 5, component VII in lane 11). (b–e) Densitometric scans of gel tracks 5 and 6 (b, c) and 11 and 12 (d, e) showed for the supernatant material not recovered in reconstituted IFs, a molar ratio of 1:2.45 for the experiment using cytokeratins Nos. 8 and 19 (b) and 1:2.45 for the combination of human cytokeratin No. 8 and bovine component VII (d). In contrast, the corresponding pelleted IF contained polypeptides Nos. 8 and 19 as well as human No. 8 and bovine component VII in about equimolar amounts (1:1.2 in d and 1:1.01 in e, respectively). Numbers on top of each peak denote the integral peak areas. Molecular weight markers (lanes denoted R in a) are (dots, from top to bottom): myosin heavy chain (M, 200,000), β-galactosidase (M, 120,000), phosphorylase a (M, 94,000), bovine serum albumin (M, 68,000), and actin (M, 42,000). Arrowheads (a, lane 5, and b) denote a minor contaminating protein which is highly enriched in the supernatant fraction and not recovered in the pelleted IFs, thus demonstrating the specificity of IF formation.
Lee and Baden (1976) and Steinert et al. (1976) who, using bovine epidermal keratins, have reported successful IF formation in vitro from combinations of purified large cytokeratins (components 1a-3 of Steinert et al., 1976; components B and B' of Lee and Baden, 1976), which include the basic type II cytokeratins, with smaller cytokeratins (components 4-6 of Steinert et al., 1976; A and A' of Lee and Baden, 1976) representing the acidic type I cytokeratins. However, our findings are at odds with reports of Steinert et al. (1976, 1978, 1982) that IFs can also be formed from combinations of bovine epidermal keratins 1 and 2, 1 and 3, 4 and 5, and 4 and 6 (apparently these are components I and III, I and IV, VI and VII, and VI and VIII, respectively, in our nomenclature; Franke et al., 1978, 1981c). Combinations of the latter type have not been positive in our experiments and also do not meet the type I/type II pair-complex requirement. It may be, however, that the preparations of polypeptides designated 2 and 3 by Steinert et al. (1976) were contaminated with the unusually large, hoof-specific type I epidermal keratin of Mr ~64,000 (No. 9 of the catalog of Schiller et al., 1982).

In all combinations of cytokeratin polypeptides examined, using nearly equimolar amounts of type I and II polypeptides we have obtained good yields of pelletable material in the form of protofilaments and IFs. In experiments in which the concentration of one component has been chosen to exceed that of the other component we have observed that the excess amount is excluded from the heterotypic complexes formed (e.g., Fig. 4, p and q) and also from the IFs harvested (Fig. 10). This is in contrast with several reports in the literature such as those by Lee and Baden (1976) and Steinert et al. (1976, 1979, 1982) whose statements that molar ratios 1:2 or 2:1 are necessary for IF formation have been taken as support for the triple-chain helix model (Skarrow et al., 1973; Lee and Baden, 1976; Steinert et al., 1976, 1979, 1982; Steinert, 1978). It is obvious that the findings of our present study as well as our previous reports that IFs of certain epithelial cells such as early embryonic epithelia, hepatocytes, several cultured cell lines such as rat and human hepatoma cells, and bovine MDBK cells all contain only two cytokeratin polypeptides in nearly equal amounts (Jackson et al., 1980; Franke et al., 1981a-c; Moll et al., 1982a) are best explained by a 1:1 stoichiometry requirement for IF formation.

We have not identified any other requirement for successful pair formation than the principle that representatives of either subfamily react with each other. Sun and colleagues have recently proposed a model that requires that “within each keratin pair the basic member is always larger than the acidic member by approximately 8 KDa” (Sun et al., 1984; Cooper et al., 1985). Although we agree that such a size difference between the two partners is frequently observed with cytokeratin complexes in vivo, we also note that certain in vivo complexes do not comply with this rule, such as the Mr ~64,000 (No. 9) keratin of human foot soles and bovine hooves, which is complexed with cytokeratin No. 1 of Mr 68,000. From our in vitro experiments we conclude that size differences are not generally necessary for heterotypic complex formation and assembly into IFs. For example, the largest acidic (type I) cytokeratin (No. 9) of Mr 64,000 can readily complex with the smallest type II cytokeratin, i.e. No. 8 (Mr 52,500).

In all cases examined we have found that in vitro recombination of type I and type II polypeptides is possible not only between different cytokeratins of the same species but also between cytokeratins of different species such as man, cow, and rat. This indicates that there are no species restrictions in cytokeratin pairing and IF formation. Cross-species recombinations of cytokeratin polypeptides have also been reported, in the form of very short IF pieces, for certain mixtures of human HeLa and mouse epidermal cytokeratins (Whitman-Aynardi et al., 1984) and for several combinations of murine and bovine epidermal keratins (Steinert et al., 1982). The latter report, however, also claims successful IF formation between different members of the same subfamily, which disagrees with our findings (see also above).

Whereas our data show that there is no exclusiveness for a given cytokeratin to mate with any cytokeratin of the other subfamily, they also provide evidence for differences of stability between the diverse IFs and complexes formed in vitro. Analysis by melting in urea shows that the specific melting curve and U_m value of a given cytokeratin complex reconstituted in vitro is almost identical to that of the same cytokeratin pair complex formed in vivo. Systematic differences of melting characteristics can be observed for those in vitro combinations of cytokeratins that do not occur in vivo, and examples both for “dominance” of a given component and for “intermediate” properties have been found. The reasons for these differences of stability are at present not understood.

Whether the greater stabilities of certain cytokeratin complexes correlate with greater affinities and/or lower dissociation constants of the constituent polypeptides has to be examined. It will be particularly important to elucidate the forces involved in establishing and maintaining cytokeratin complexes and IFs as such studies might provide a clue to the understanding of some phenomena observed with cytokeratins of living cells. For example, in certain cells different, apparently mutually exclusive, cytokeratin pair complexes coexist, which indicates that polypeptide exchange and promiscuous recombinations occur rarely, if at all, in those cells (examples include keratinocytes of human and bovine epidermis and cells of the human Detroit 562 line; Franke et al., 1984 and this study). Whether this selectivity and “segregation” of certain cytokeratin pair in vivo is related to quantitative differences of affinity and/or dissociation rates or is effected by “factors” mediating the preferential formation of some complexes remains to be seen.

The finding that in vitro most, probably all, cytokeratins of one type (subfamily) can form pairs and IFs with most, probably all, cytokeratins of the other type has to be discussed in relation with the observation that in vivo only certain pairs are coordinately synthesized in a mode characteristic for a given cell type (e.g., see Franke et al., 1982c, 1984; Moll et al., 1982a; Schiller et al., 1982; Tseng et al., 1982; Cooper et al., 1985). Our present study shows that these selective pair combinations formed in vivo are not necessary for the formation of IF as such, and therefore the selectivity of expression observed in vivo is not explained by structural requirements of IF formation. It may be, however, that the regulatory elements of the different cytokeratin genes show pairwise similarities, that result in the coordinate transcription of only certain combinations related to the specific differentiation program of a given epithelial cell.

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