Abstract. The properties of keratin-containing intermediate filament (IF) networks in vivo were studied following the microinjection of biotinylated keratin. Keratin-IFs were biotinylated, disassembled, and separated into type I and type II proteins by ion exchange chromatography. Recombination of these derivatized type I and type II keratins resulted in the formation of 10-nm diameter IF. The type I keratins were microinjected into epithelial cells and observed by immunofluorescence microscopy. Biotin-rich spots were found throughout the cytoplasm at 15–20 min after injection. Short biotinylated fibrous structures were seen at 30–45 min after injection, most of which colocalized with the endogenous bundles of IF (tonofilaments). By 1 1/2 to 2 h after microinjection, extensive biotinylated keratin IF-like networks were evident. These were highly coincident with the endogenous tonofilaments throughout the cell, including those at desmosomal junctions. These results suggest the existence of a relatively rapid subunit incorporation mechanism using numerous sites along the length of the endogenous tonofilament bundles. These observations support the idea that keratin-IFs are dynamic cytoskeletal elements.

Keratin is the major component of epithelial cell intermediate filaments (IFs). Keratin-IFs typically are found in tightly packed bundles known as tonofilaments which are distributed throughout the cytoplasm. At the cell center they form an elaborate cage around the nucleus and appear to be attached to the nuclear surface. Tonofilaments extend from the nuclear region to the cell periphery where they associate with desmosomal plaques in regions of cell–cell contact. Keratin IFs contain types I (acidic) and II (neutral-basic) IF proteins. These two types have been characterized by their isoelectric points, sequence homologies, and immunoreactivity. In vitro polymerization studies have demonstrated that both type I and type II keratins are required for 10-nm filament formation at the heterodimer level and for this reason they are termed obligate copolymers.

In contrast to the dynamic properties of other cytoskeletal systems, keratin IFs, including keratin IF, are thought to be relatively stable components of the cytoskeleton of interphase cells. This latter notion can be attributed to the finding that IFs are insoluble in vitro under physiological conditions and that in general there is little evidence for the existence of pools of "soluble" subunit proteins in the cytosolic compartments of interphase cells. In contrast, there is evidence that IFs, including keratin-IF, are dynamic with respect to their disassembly and subsequent reassembly during mitosis in some cell types. Recently, cDNA transfection experiments have shed a great deal of light on the mechanisms of keratin IF assembly in vivo and have also provided useful clues regarding the dynamic properties of interphase keratin IF networks. It has been found that newly synthesized wild-type keratin is incorporated into the endogenous keratin IF network by 65 h after transfection. The results also have shown which domains of the keratin molecule are essential for the assembly of newly synthesized keratin into tonofilaments. Deletion constructs producing proteins that lack both the amino-terminal domain and the amino-terminal end of the central rod domain induce the disruption of the endogenous tonofilaments into perinuclear aggregates of keratin. Similar mutants lacking both the carboxy-terminal domain and the carboxy end of the central rod domain result in the formation of large cytoplasmic keratin-rich aggregates as well as a disrupted endogenous network. This effect is most likely due to an abortive incorporation of defective keratins into the intermediate filament network.
atin into the endogenous keratin network. By 92 h these transiently transfected cells begin to recover and reform an endogenous tonofilament system beginning in the juxtanuclear region, suggesting the presence of a keratin IF organizing center at or near the nuclear surface (2, 14, 16, 25). Other in vivo studies have employed the microinjection of keratin mRNA to induce the synthesis of keratin IF in living epithelial cells (21) and nonepithelial (37) cells. In contrast, the results of these experiments indicate that assembly of newly synthesized IF protein occurs at discrete locations throughout the cytoplasm of cells devoid of endogenous keratin IF networks.

Transfection experiments using other types of IF systems have been carried out. For example, a similar juxtanuclear accumulation is found 18-24 h after transfection with type IV IF cDNA constructs (neurofilament) in fibroblasts (11). The initial sites of incorporation of newly synthesized vimentin in 3T3 fibroblasts appear to occur throughout the endogenous vimentin IF network after induction of a transfected chicken vimentin gene (46). On the other hand, transfected mouse vimentin is first observed in a perinuclear region after its induction in human cells and is last observed in the juxtanuclear region after the cessation of the induction (53). In studies in which the vimentin network appears to be absent in cells, the assembly of newly synthesized vimentin appears to occur throughout the cytoplasm (53).

The dynamic properties of microtubules and microfilaments in situ and in vivo have been elucidated by following the distribution of microinjected tubulin or actin labeled with various "reporter" molecules such as fluorescein, rhodamine, and biotin (36, 41, 43, 44, 47, 52, 72). In general, experiments employing such methods have demonstrated that microinjected subunits exchange very rapidly with the appropriate endogenous polymer systems. Actin is associated with the microfilaments comprising stress fibers within 5-20 min (3); tubulin is incorporated into microtubules in 7 min (56); and the type III IF protein, vimentin, is incorporated into IF within 60 min (70).

To date, the direct injection of biotinylated keratin has not been used to study the properties of the keratin-rich tonofilament system in epithelial cells. This is due to the inherent difficulties in obtaining purified type I or type II keratins in an injectable form (21). We have been able to overcome this problem and have carried out studies aimed at determining the fate of microinjected vimentin type I keratin into primary mouse epidermal (PME) and into kangaroo rat kidney epithelial (PtK2) cells. Our results demonstrate that the microinjected keratin is incorporated into tonofibrils in vivo over relatively short time periods, indicating that the keratin IF system is more dynamic than previously indicated.

Materials and Methods

Cell Culture

PME cells were isolated by the trypsin flotation procedure of Yuspa and Harris (75) as described by Jones and Goldman (32). Cells were plated onto locator coverslips (Bello Glass Inc., Vineland, NJ) and observed in normal Ca2+-containing MEM (Gibco Laboratories, Grand Island, NY). Under these conditions, keratin IF networks extend to the cell surface and normal desmosome formation takes place in areas of cell-cell contact (32). PtK2 cells were grown in MEM (Gibco Laboratories, Grand Island, NY) and plated onto locator coverslips 24-48 h before microinjection. BHK-21 cells were cultured as described elsewhere (70).

Preparation of Biotinylated Type I Keratin

Fresh bovine tongues were obtained from the slaughterhouse and immediately immersed in crushed ice. Within 1-2 h the mucosa was removed; soaked in 20 mM EDTA/1 mM PMSF/PBSa, pH 7.4, at 4°C for 12 h; pulled away from the underlying dermis with forceps; and minced with scissors (35). Keratin IFs were prepared for extraction with urea buffer A (8 M urea, 50 mM Tris-HCl, pH 9.0, 20 mM b-mercaptoethanol, and 1 mM PMSF) for 45-60 min at 4°C with vigorous stirring (35, 57). Urea-insoluble material was removed by centrifugation at 200,000 g for 30 min. The protein concentration in the supernatant was determined by the Bradford method (10) and adjusted to 1.4 mg/ml with urea buffer A. This latter solution was dialyzed against 50 vol (2x) of assembly buffer (10 mM Tris-HCl, pH 7.4, 0.1 mM PMSF) overnight at room temperature to polymerize keratin IF. Polymerization was assayed by negative staining with 1% uranyl acetate on parlodion/carbon-coated copper grids, using a JEOL 1200EX electron microscope.

Polymerized IFs were biotinylated as follows: 200 mg of the labeling reagent, Succinimidyl-D-biotin (Molecular Probes, Eugene, OR), was dissolved in 55 ml of dimethylformamide (Fisher Scientific, Fair Lawn, NJ) immediately before use and was pipetted slowly into 500 ml of keratin IF in 10 mM Tris, pH 7.4, kept in suspension with continual stirring. This reaction mixture was incubated at room temperature for 30 min with occasional stirring. IFs were removed from the labeling reaction by centrifugation at 200,000 g for 30 min at 10°C. The resulting pellet was resuspended immediately in urea buffer B (8 M urea, 5 mM NaPO4, pH 7.4, 0.2% b-mercaptoethanol, 1 mM PMSF) in order to depolymerize IFs, which were subsequently repolymerized by dialysis against assembly buffer. This procedure was repeated once. To insure that IFs were forming, twice-cycled preparations were assayed by negative staining.

The 48/54-kD type I (acidic) and the 62/67-kD type II (neutral-basic) biotinylated bovine tongue keratins were separated by ion exchange chromatography (model DE-52; Whatman Chemical Separation, Inc., Clifton, NJ) (1.0 3 32 cm column), using a modification of the procedure of Steinert and Idler (59). Specifically, twice-cycled biotinylated IFs were collected by centrifugation at 200,000 g for 30 min and disassembled in column buffer (9.5 M urea, 20 mM Tris-HCl, pH 8.6, 1 mM EDTA, 1 mM DTT). The solubilized biotinylated keratins were applied to the column, washed, and eluted at 30 ml/h. Type II keratins were found in the flow through fraction. Type I keratins were eluted with a 200-ml linear salt gradient (0-100 mM NaCl) in column buffer. 1-ml fractions were collected and every third fraction was assayed by SDS-PAGE (39). Fractions containing the type I keratins were pooled and exhaustively dialyzed against 10 mM Tris, pH 7.4. These preparations were found to be suitable for microinjection and type I keratins remained in solution after centrifugation at 100,000 g for 30 min. No IFs were seen in this preparation by negative stain EM. Aliquots (200 μl) were frozen in liquid nitrogen and stored for microinjection experiments. In contrast, the type II proteins found in the flow through fraction were much more difficult to keep in solution. To date, despite numerous attempts using a variety of physiologically acceptable buffers, conditions compatible with microinjection into live cells have not been found for the 62/67 kD type II keratins.

Preparation of Biotinylated Vimentin

Biotinylated vimentin was prepared as described elsewhere (70, 71).

Western Blotting

Immunoblotting was carried out by the method of Towbin et al. (69). AEl and AE3 keratin antibodies were provided by Dr. T. T. Sun (New York University College of Medicine) and were used at a dilution of 1:50. A previously characterized rat monoclonal antikeratin (mAb anti-K) (30) was used undiluted. In order to detect biotinylated keratin, goat antiboitin antibody (Sigma Chemical Co., St. Louis, MO) was used at 1:1,000. Peroxidase-conjugated secondary antibodies included rabbit antigen (Amersham, Arlington Heights, IL), goat antirat (Jackson Immunoresearch Laboratories, Inc., West Grove, PA), and goat antimouse (Southern Biotechnologies Associates, Inc., Birmingham, AL), all used at a dilution of 1:1,000. Antikeratin antibody was previously characterized (70).

Keratin IF Isolation

IFs from cultured PME cells were isolated using the method of Zackroff and Goldman (76). The final pellet was washed three times in PBSa and frozen at -80°C for use in immunoblotting assays.
**Microinjection**

Microinjections were carried out as described in Vikstrom et al. (70, 71). Micropipettes were made with a vertical pipette puller (David Kopf Instruments, Tujunga, CA). Frozen aliquots of biotinylated keratin were thawed and the protein concentration in the sample to be injected was adjusted to 0.75-1 mg/ml (10). The samples were clarified in an Eppendorf microfuge for 2 min before microinjection. After microinjection, the dishes containing the cells on locator coverslips (see above) were returned to the incubator. At appropriate time intervals, cells were fixed and processed for immunofluorescence. Other researchers (3, 56) have estimated that 10% or less of the cell volume can be microinjected successfully into the cell.

**Indirect Immunofluorescence**

PME and PtK2 cells were processed for double-label indirect immunofluorescence as previously described (74). BHK-21 cells were fixed in 0.1% glutaraldehyde/0.15% Triton X-100/PBSa for 8 min and the free aldehyde groups reduced through three washes of 5 min each in a solution of 1 mg/ml sodium borohydride (55). Goat antibody (Sigma Chemical Co.) was used to detect biotinylated bovine keratin. In un.injected cells, a very low level of background mitochondrial staining (38) was detected using this antibody. However, in microinjected cells the biotin-keratin signal was so much greater than the mitochondrial staining that it was not obvious. mAb anti-K was used to detect the endogenous PME keratin IF network (34). mAb anti-K did not recognize the bovine tongue type I keratins by Western blotting (see Fig. 3). A rabbit polyclonal directed against the mouse keratin K2 was used to detect the endogenous keratin IF networks of PtK2 cells (33). This antibody also reacted with the bovine type I keratin by Western blotting, but the two distinct patterns obtained from indirect immunofluorescence staining at early times after microinjection (see Fig. 7, A and B) demonstrated that the antibiotin antibody clearly distinguished the microinjected keratin from the endogenous PtK2 keratin. Rhodamine-conjugated donkey antirat (Jackson Immunoresearch), fluorescein-conjugated donkey antigoat, and rhodamine-conjugated donkey antirabbit (Jackson Immunoresearch) were used as secondary antibodies. A Zeiss Axio phot microscope was used to observe and photograph the cells.

**Cycloheximide Treatment**

In some experiments, PtK2 cells were treated with 10 μg/ml cycloheximide (Sigma Chemical Co.) for 1 h or more before microinjection and maintained in the same culture medium during microinjection and subsequent incubations. This treatment inhibited 90-95% of protein synthesis as measured by

35S methionine (ICN Biomedicals, Inc., Irvine, CA) incorporation into PtK2 cells. Specifically, 60-mm dishes of subconfluent PtK2 cells were washed 3 x in 1 mM MgCl2/PBS and treated with either 0 or 10 μg/ml cycloheximide in methionine-free MEM (Northwestern University Cancer Center, Chicago, IL) containing 5 μCi/ml 35S methionine. After incubation at 37°C for 15, 30, 60, 120, and 240 min, cells were lysed in a solution of 0.5% SDS, 0.5 M NaCl, 5 mM EDTA and removed using a rubber policeman. Protein was then precipitated with TCA using BSA as a carrier and stored on ice for the duration of the experiment. Suspensions of precipitated protein were filtered through glass microfiber filters (model GF/G; Whatman Inc., Clifton, NJ), extensively washed with cold TCA, and counted in a liquid scintillation counter (model LS 6800; Beckman Instruments Inc., Palo Alto, CA).

**Results**

**Characterization of Biotinylated Keratin**

Keratin IFs prepared from bovine tongue epithelium, consist primarily of two major and two minor keratin bands of 67, 62, 54 and 48 kD (Fig. 1 A). This mixture of proteins has been biotinylated in their polymerized form so as to protect the molecular domains important for assembly (see Materials and Methods). After two cycles of assembly-disassembly, biotinylated keratin IFs appear normal as assayed by negative staining (Fig. 1 C). This demonstrates that biotinylation does not affect the overall polymerization of keratin. SDS-PAGE analyses of these twice-cycled biotinylated IF show that the overall profile of keratins is very similar to unbiotinylated preparations, showing only a slight upward shift in relative mobility (Fig. 1 B).

After the second cycle of in vitro polymerization, biotinylated keratin IFs were collected by centrifugation, disassembled in column buffer, applied to a DE-52 cellulose column, and the resulting fractions assayed by SDS-PAGE (Fig. 1 D; see Materials and Methods). The 67/62-kD higher molecular mass keratins are collected in the flow through fraction (Fig. 1 E), and the 54/48-kD lower molecular mass keratins

![Figure 1.](image_url)
begin to elute at \( \sim 45 \) mM NaCl using a 0-100-mM linear NaCl gradient (Fig. 1 F). Neither the higher molecular weight fraction nor the lower molecular weight fraction alone can be induced to polymerize into IF when dialyzed against assembly buffer. However, when the 62/67-kD fraction is recombined with the 54/48-kD fraction at approximately equal concentrations \((0.33 \text{ mg/ml})\) in column buffer, large numbers of 10-nm diameter IFs form rapidly upon dialysis against assembly buffer. These appear identical to those seen in Fig. 1 C. This demonstrates that the column purification procedure does not inhibit keratin polymerization.

Immunoblotting analyses with the AE1 and AE3 antibodies have been used to distinguish acidic and basic keratins (see Materials and Methods; 14). AE1 reacts mainly with the 48- and 54-kD keratins, indicating their acidic type I character (Fig. 2, lane 3), while AE3 reacts primarily with the 62- and 67-kD species, demonstrating their basic type II character (Fig. 2, lane 2). The type I fraction used for microinjection has been further analyzed by 2-D gel electrophoresis. The 48-kD protein focuses at pH 5.0-5.5 as a single spot, while the 54-kD keratin is only slightly more basic, further confirming their type I acidic nature (data not shown). The 48- and 54-kD species are present in an approximate ratio of 95 to 5\%, respectively, as shown by densitometric scanning of Coomassie blue-stained SDS-PAGE gels of the type I-enriched column fractions.

The antibodies to be used for immunofluorescence observations of microinjected mouse epidermal cells have been analyzed by immunoblotting. Antibiotin antibody reacts specifically with both biotinylated type I keratins (Fig. 3, lane 2). mAb anti-K is specific for the 48-kD keratin of PME IF (Fig. 3, lane 5), and does not react with the bovine tongue keratins (Fig. 3, lanes 3 and 8). These results demonstrate that we can clearly distinguish the biotinylated protein from the endogenous mouse keratin in PME cells.

**Microinjection Experiments**

On average, 45 cells on locator coverslips can be injected in 20 min. Each injected cell is observed by phase contrast and its position on the locator grid is noted along with the exact time of injection. The coverslips are then returned to the incubator. At time points after injection, cells on coverslips are fixed and double-label indirect immunofluorescence is carried out using goat antibiotin and mAb anti-K. At 20 min, antibiotin staining reveals a pattern of bright spots distributed in all areas of the cytoplasm (Fig. 4 A), while the endogenous keratin network retains its normal appearance (Fig. 4 B). By 30-45 min after injection, few spots remain obvious and short biotin-positive filamentous structures which tend to form a fine reticular pattern are seen throughout the cytoplasm (Fig. 4, C and D). Some of the obvious linear elements of this pattern appear to colocalize with the endogenous keratin IF tonofilaments (arrowheads), although the closely spaced tonofilaments and diffuse fluorescence seen in the thicker regions of the cytoplasm limit resolution at this stage. In a few peripheral regions of the cell, no obvious colocalization is seen (Fig. 4, C and D, arrows). From 40-90 min after microinjection an increase is seen in the

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**Figure 2.** Immunoblot analysis of bovine tongue keratin IF with AE1 and AE3 mAbs. AE3 has been shown to react primarily with type II keratins, and AE1 with type I keratins (14). Nonbiotinylated bovine tongue keratins (lane 1, SDS-PAGE, Coomassie blue) were transferred to nitrocellulose and probed with AE3 (lane 2) and AE1 (lane 3). (open circles) 67 and 62 kD; (asterisks) 54 and 48 kD.

**Figure 3.** Immunoblot analyses of antibiotin and mAb anti-K. Preparations of biotinylated type I keratins, PME cell IF, and unlabeled bovine tongue keratin IF (lanes 1, 4, and 7, respectively; SDS-PAGE, Coomassie blue) were transferred to nitrocellulose and probed with goat antibiotin antibody (lanes 2, 6, and 9). The 48- and 54-kD keratins of the biotinylated type I keratin preparation reacted (lane 2). The antibiotin antibody did not react with PME IF or unlabeled bovine tongue keratin (lanes 6 and 9). mAb anti-K was specific for the 48-kD keratin of the PME IF preparation (lane 5) and it did not react with either biotinylated or unlabeled bovine keratin (lanes 3 and 8).
clarity of the antibiotin pattern, as the fine reticular and diffuse fluorescence patterns disappear and tonofilament-like structures are readily distinguished (Fig. 4, E and F). By 90–120 min, most of the biotinylated type I keratin is found to be highly coincident with endogenous tonofilaments throughout the cell (Fig. 4, G and H). At the cell surface the antibiotin pattern appears to be superimposed on tonofilaments aligned at desmosomal junctions (Fig. 5, A and B).

Due to the overall thickness of cultured keratinocytes, it has been difficult to observe the morphological details of the transition from the biotinylated keratin spots (seen at short time intervals after microinjection) to the tonofilaments seen at later times. To gain additional insights into this process we have used PtK2 cells. These simple epithelial cells are larger, flatter, and possess fewer tonofilaments which are also well separated. These are all properties that facilitate detailed studies of individual tonofilaments by fluorescence microscopy. Using single-label immunofluorescence with antibiotin, spots are seen throughout the cell at 15–20 min after injection (data not shown). After 45–60 min, most of the antibiotin-containing spots are no longer visible and short wavy filamentous structures are seen throughout the cytoplasm from the nuclear region to the cell periphery (Fig. 6). Double-label immunofluorescence has been employed to determine whether or not the spots and short filaments of biotinylated keratin seen at early time points are associated with endogenous tonofilaments. At 15–20 min after injection, spots of biotinylated keratin appear to be distributed throughout the cell (Fig. 7, A and B). However, double labeling at this time period to reveal the endogenous network of tonofilaments shows an apparent absence of spots as seen in Fig. 7 B. This is somewhat surprising as the antikeratin polyclonal recognizes the injected bovine tongue type I keratin by immunoblotting (see Materials and Methods). One possible explanation for this observation is that the majority of spots are closely associated with the endogenous tonofilaments at this time interval after injection. In support of this possibility, preliminary morphometric analyses have involved the superimposition of double-label immunofluorescence micrographs (such as Fig. 7, A and B) taken at ~15–20 min after injection. In all cases, the vast majority of biotinylated keratin spots overlie endogenous tonofilaments (data not shown; similar results were also obtained in PME cells). By 60 min after injection most spots are no longer obvious in PtK2 cells and discontinuous fibrils containing biotinylated keratin appear coaligned with endogenous tonofilaments (Fig. 7, C and D, arrows). Between 2–4 h after injection extracellular overlap is seen between the biotinylated keratin pattern and endogenous tonofilaments (Fig. 7, E and F).

Moreover, PtK2 cells also possess a type III IF network, containing vimentin. We have used this latter property to determine whether or not keratin IF and vimentin IF exhibit different incorporation patterns within the same cell type as suspected from our previous studies employing biotinylated vimentin microinjected into fibroblasts (70). Indeed, when biotinylated vimentin is microinjected into PtK2 cells, a pattern distinct from that of biotinylated keratin is observed. Immediately after injection, spots of vimentin are distributed throughout the cytoplasm. At 60 min, the majority of spots are found in a large juxtanuclear region (Fig. 8, A and B). Within 2–4 h biotinylated vimentin–containing fibers are seen at the edge of the juxtanuclear region, and at later time intervals most of the biotinylated vimentin is found to be coincident with the endogenous vimentin network (Fig. 8, C and D). The endogenous vimentin network appears normal at all time points. Therefore, the overall pattern of incorporation of vimentin into the endogenous type III IF network appears to be different from the pattern seen for keratin in the same cell type.

Several additional controls have been carried out for these microinjection experiments. To determine whether any fluorescence in the cell could be due to background caused by unconjugated biotin, we have microinjected succinimidyl biotin alone which has been treated in a fashion identical to that used in the keratin labeling reaction. No detectable signal is found after the cells are processed for indirect immunofluorescence. In addition, after microinjection of biotinylated keratin, the overall pattern of other cytoskeletal elements such as microtubules appears normal for periods up to 2 1/2 h after injection (data not shown).

To demonstrate the importance of a type II keratin partner in the assembly process, the biotinylated type I keratin has been injected into BHK-21 cells which contain the type III IF proteins, vimentin and desmin, (76) without any obvious keratin. 4 h after microinjection, the biotinylated keratin is seen as nonfilamentous aggregates near the nucleus (Fig. 9, A and B).

We have also determined the fate of microinjected type I biotinylated keratin in PtK2 cells treated with cycloheximide. These experiments have allowed us to distinguish whether or not the type II partner proteins presumably needed for assembly into tonofilaments are derived from newly synthesized proteins or from existing endogenous proteins. Under these conditions 90–95% of protein synthesis is inhibited as assayed by the incorporation of 35S-methionine into total cell protein (see Materials and Methods). After injection, biotinylated keratin incorporates into extensive arrays of tonofilaments within 4 h (data not shown). No disruption of the endogenous keratin IF networks is observed in either injected or noninjected cells treated with cycloheximide for this same time period. Therefore, newly synthesized type II protein is not necessary for the incorporation of the injected keratin into tonofilaments.

**Discussion**

In this study, we have determined the fate of microinjected biotinylated keratin. To initiate these studies, we have developed a procedure for separating biotinylated keratin IF into type I and type II proteins. As a result we have been able to inject single cells with type I keratin alone. Our findings indicate that these keratins are rapidly incorporated into the endogenous tonofilament network by immunofluorescence criteria. This provides us with a simple approach for studying various aspects of the posttranslational assembly and dynamics of keratin-containing tonofilaments under different physiological conditions. It also provides the foundation for further investigations of the function and dynamic nature of keratin-IF in vivo using methods such as fluorescence recovery after photobleaching (68).
Figure 4. Time course of biotinylated type I keratin incorporation. Biotinylated type I keratin was microinjected into PME cells and processed for double-label indirect immunofluorescence using goat antibiotin (A, C, E, and G) and mAb anti-K (B, D, F, and H) at 20 min (A and B); 40 min (C and D); 60 min (E and F); and 90 min (G and H) after microinjection. Some linear arrays of biotinylated spots can be seen in areas of cell-cell contact in Fig. 4 A. A, E, and G, additional injected cells are denoted with i. Bar, 10 μM.
Figure 4.
After microinjection, the biotinylated type I keratin preparation assembles through a series of discrete, reproducible steps into a tonofilament-like network. Biotinylated keratin is seen first as bright spots dispersed throughout the cytoplasm. These spots disappear, as short and then longer tonofilaments appear. These observations suggest that the keratin aggregates give rise to the short tonofilaments that subsequently elongate to form a complete network by 1 1/2-2 h using light microscopic criteria in PME cells. Furthermore, the biotinylated keratin-tonofilament network is very similar to the endogenous network. These results suggest that biotinylated type I keratin is incorporated into the endogenous network at numerous sites. These observations add to the growing body of literature indicating that IFs are dynamic cytoskeletal elements with regard to protein subunit exchange (2, 12, 33, 51, 70).

There are numerous possible mechanisms by which the incorporation of microinjected subunits could take place. For example, if tonofilaments contain short IF with free ends at different points along their length, then individual IF could be lengthened by the addition of keratin subunits onto one or both ends. Alternatively, if individual IFs are continuous along the entire length of the tonofilament, then exchange of subunits could take place at certain locations along the length of IF. This latter possibility assumes that there are "hot spots" at which incorporation of subunits occurs at higher rates. Both of these possibilities could explain the formation of biotinylated spots, as well as the short discontinuous filaments seen in close association with tonofilaments at early time points after injection. Both of these mechanisms are consistent with the overall general model recently proposed by Coulombe and Fuchs (14). It should be emphasized that these alternatives cannot be distinguished at the light microscopic level of resolution. We may be able to gain more insight into these mechanisms in experiments now in progress using higher resolution avidin-gold labeling (7).

Microinjection of keratin mRNA also has been used to suggest a "multiple-site" mechanism for the assembly of newly synthesized keratin into tonofilament-like structures in nonepithelial cells. These structures are evident at 12-24 h after injection. In these studies the microinjection samples contain both types I and II keratin mRNA (36). When similar samples are injected into epithelial cells, the newly synthesized keratin is found assembled into the endogenous tonofilament network 6 h after microinjection (21). However, the number and distribution of assembly sites have not been determined in these cells (21), but the results do suggest that newly synthesized keratin can be incorporated into an established keratin IF network.

Therefore, all available information suggests that there are multiple mechanisms which exert their control at the transcriptional, cotranslational, and posttranslational levels to regulate keratin IF polymerization within epithelial cells. In addition, transient transfection studies have suggested that
Figure 7. PtK₂ cells were microinjected with biotinylated type I keratin. At 20 min (A and B), 1 h (C and D), and 4 h (E and F) after injection the cells were processed for double-label indirect immunofluorescence using goat antibiotin (A, C, and E) and a rabbit polyclonal against keratin (B, D, and F). Bar, 5 μm.
there is a nuclear-associated assembly mechanism involved in the incorporation of newly synthesized keratin into tonofilaments (1, 2). Our data suggest that there is a posttranslational exchange of keratin subunits into tonofilaments which permits incorporation throughout the keratin IF network; including the nuclear region, elsewhere throughout the cytoplasm, and at the cell surface in regions containing desmosomes.

In contrast to the results with biotinylated keratin, microinjected biotinylated vimentin shows a rapid accumulation in a large juxtanuclear region in cultured BHK-21 fibroblasts. Subsequently, vimentin IFs appear to assemble from this region toward the periphery of the cell until a normal appearing vimentin IF network is formed (70). The similar results reported in this study for the vimentin network of PtK₂ cells emphasize that separate mechanisms are utilized in the assembly and maintenance of the endogenous keratin and vimentin IF systems within a single cell type. On the other hand, incorporation of chicken vimentin in stably transfected mouse fibroblasts appears first as a discontinuous pattern throughout the entire vimentin IF network (46), suggesting multiple sites of cotranslational or posttranslational assembly along the endogenous system. After 24 h, newly synthesized vimentin colocalizes with the endogenous vimentin-IF network (46). Therefore, it appears that microinjected and newly synthesized IF proteins may follow different pathways for assembly into endogenous polymerized IF networks.

The results with the microinjected type I keratins are intriguing as numerous studies have demonstrated that type I and type II proteins are required for the formation of the heterodimers needed for IF assembly (14, 18, 30, 42, 58, 62). Our in vitro results support these findings; the purified type I bovine tongue keratin cannot assemble into IF in vitro unless it is mixed with its type II counterpart. The lack of any obvious keratin IF formation after microinjection of type I keratin into BHK-21 fibroblasts in this study provides further evidence that both type I and type II are required for IF formation in vivo.

Considering the rapid rate of incorporation of the microinjected type I keratin and in general the low level of biosynthesis of keratin (15), it is unlikely that the excess of type I protein complexes with a sufficient amount of newly synthesized type II protein to account for the formation of the heterodimers required for the IF/tonofilament assembly seen over the time course of our experiments. This is further supported by the finding that even when 90–95% of protein syn-

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**Figure 8.** PtK₂ were microinjected with biotinylated vimentin. At 60 min (A and B), and 16 h (C and D) after injection the cells were processed for double-label indirect immunofluorescence using goat antibiotin (A and C) and a polyclonal against vimentin (B and D). Bar, 10 μm.
thesis is inhibited by cycloheximide treatment, microinjected type I keratin still becomes incorporated into endogenous IF networks over short time periods. Therefore, it appears likely that a dynamic equilibrium exists in vivo which provides a small, most likely transient pool of monomeric keratin. This would allow for the formation of hybrid heterodimers containing biotinylated type I/unbiotinylated type II protein chains. Such heterodimers could then participate in IF formation and maintenance. This hypothesis could also account for the lack of colocalization between antibiotin and mAb anti-K seen at the thin edges of PME cells 30–45 min after injection (Fig. 4, C and D). We speculate that the tonofilament-like structures seen in this region with antibiotic may result from the pairing of microinjected biotinylated type I keratin with endogenous type II keratins not recognized by mAb anti-K (see Fig. 3). At this juncture it should be emphasized that we cannot formally rule out the possibility that biotinylated homodimers might also be assembling into IF in these peripheral regions devoid of detectable endogenous tonofilaments. In support of this latter possibility, Steinert (58) has shown that some homodimers may participate in IF formation when assembled from urea solubilized preparations.

As indicated above, biotinylated keratin is also found aligned with tonofilaments in regions of PME cell–cell contact containing desmosomes (see Fig. 5, A and B). Thus, it appears that microinjected keratin is also incorporated into tonofilaments at the cell surface (32, 33). However, there is no evidence from our work that desmosomes preferentially nucleate the assembly of keratin IF, a function which has been suggested for desmosomes of a rat mammary epithelial cell line (9). In fact, incorporation of biotinylated keratin into tonofilaments associated with desmosomes appears to occur at the same rate as seen throughout the entire keratin network.

In summary, biotinylated type I keratin incorporates rapidly into tonofilaments in epithelial cells as assayed by immunofluorescence microscopy. The rate of incorporation into tonofilaments suggests that subunit exchange occurs in vivo at the posttranslational level of regulation. The data presented in this study are consistent with the view that the keratin IFs comprising tonofilaments are dynamic structures in vivo. Furthermore, the use of biotinylated and/or fluorochrome-labeled keratin in future studies should yield important information regarding IF and their interactions with other cytoskeletal elements and organelles; and ultimately should shed light on the mechanisms underlying the changes in IF composition which take place during cellular differentiation.

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