Organization of Cytokeratin Bundles by Desmosomes in Rat Mammary Cells

Mauro Bologna,* Ross Allen,* and Renato Dulbecco*

*The Salk Institute, La Jolla, California 92037; †Department of Biological Sciences, University of L’Aquila Medical School, 67100 L’Aquila, Italy

Abstract. In a rat mammary epithelial cell line, LA-7, cytokeratin bundles recognized in immunofluorescence by a monoclonal antibody (24B42) disappear after trypsinization of cultures and are gradually reformed after replating.

We have followed the time course of cytokeratin filament reappearance by growing cells in low calcium medium (0.1 mM), which prevents desmosome formation, and then shifting to high calcium (1.8 mM) to start the process. By fixing the cells at various intervals and staining them in immunofluorescence for 24B42 cytokeratin and for desmosomal proteins, we found that cell to cell contact and desmosome formation are prerequisites for keratin filament formation in these cells. EGTA treatment, by disassembling desmosomes, causes the cytokeratin filaments to disappear and the 24B42 protein to pass into a soluble form in this cell line, as ascertained by 100,000 g fractionation and immunoenzymatic assay. Cycloheximide treatment also causes cytokeratin filaments to disappear, indicating that protein synthesis is needed for normal filament maintenance. In another related cell line (106A-10a) and in HeLa cells, trypsinization and EGTA exposure do not cause a complete loss of 24B42 immunofluorescence, although distinct filaments disappear, indicating the presence in these cells of different organizing centers, besides desmosomes, for cytokeratin bundle formation.

LA7 cells therefore seem to have a cytokeratin system strictly dependent on the presence of desmosomes, which act as an organizing center for filament assembly. 106A-10a cells (also rich in desmosomes) and HeLa cells (showing instead a reduced number of desmosomes) have a cytokeratin system partially or totally independent from that of desmosomes, with different organizing centers.

Intermediate size filaments have a molecular composition characteristic for the histological origin of the cell (27, 31). The major protein component of intermediate size filaments in both normal and neoplastic epithelial cells are cytokeratins, which form a family of related polypeptides present in various combinations in different tissues (12, 26, 29). These proteins have multiple antigenic determinants that can be useful for studies of cell differentiation (14, 35) and for diagnosing and classifying tumors histologically (31). Cytokeratin filaments occur in bundles containing two or more different cytokeratins, which are necessary for the formation of polymeric filaments (14, 28, 39).

Electron microscopy and immunofluorescence studies show that cytokeratin bundles contact the cell periphery at desmosomal plaques (13, 15, 30). These are the intracytoplasmic portions of desmosomes, which are highly conserved structures that probably perform a mechanical function. They are regarded as anchorage points between individual cells of a tissue, able to distribute the mechanical stress applied to one cell among nearby cells and thus to the entire tissue (4, 34, 37, 42). Other types of intermediate filaments have been described to be connected to desmosomes (22, 24).

Using a monoclonal antibody, 24B42, raised against bovine muzzle keratin (1), which cross-reacts with cytokeratins of rat and human mammary epithelial cells, we have followed by immunofluorescence the formation and disappearance of cytokeratin filaments in a line of rat mammary cells (9). Our observations show that the formation of keratin bundles in these cells is controlled by the desmosomes to which they are attached.

Materials and Methods

Cell Cultures

LA7 and 106A-10a cells, derived from a mammary carcinoma chemically induced in Sprague-Dawley rats (9), were maintained in Nunc dishes (Nunc, Roskilde, Denmark) using Dulbecco’s modified Eagle’s medium supplemented with 5% calf serum and 50 ng/ml each of hydrocortisone and insulin. NMuMG cells (33) were grown in the same medium supplemented with 10% fetal calf serum in place of calf serum. HeLa cells were grown in the same medium, without hormones. For special treatment, confluent cultures were exposed to the same medium supplemented by either 4 mM EGTA (Sigma Chemical Co., St. Louis, MO) for up to 4 h at 37°C or 1 μg/ml cycloheximide (Sigma Chemical Co.) for several days, also at 37°C. Low calcium medium was prepared using calcium-free Dulbecco’s modified Eagle’s medium supplemented with 5% Chelex-100 (Bio-Rad Laboratories, Richmond, CA)-treated calf serum (3) and adjusted to the desired calcium concentration with CaCl₂. For transfer, cells were detached from the dish by trypsin/EDTA (250 mg of trypsin and 90 mg
Antibody

The 24B42 mouse monoclonal has been described (1). The cytokeratin it recognizes in LA7 cells was characterized by labeling growing cultures with $[^{35}S]$methionine, solubilizing cells in 0.5% Nonidet P-40 in Tris buffer, and immunoprecipitating the supernatant with 24B42. The immunoprecipitate was suspended in buffer (50 mM Tris, pH 6.9, 10% glycerol, 2% SDS, 2% mercaptoethanol) and fractionated by 10% acrylamide gel electrophoresis. Autoradiographs of the gel showed two bands, 54 and 56 kD.

Immunofluorescence

Cells grown on glass coverslips were fixed for 1 min with a mixture of cold acetone/methanol (1:1) and stained with mouse monoclonal antibody 24B42 (IgG1, used as plain supernatant of the hybridoma culture, containing 106 μg/ml of immunoglobulin and supplemented with 0.1% sodium azide), followed by fluoresceinated goat antiserum to mouse immunoglobulins (Cappel Laboratories, West Chester, PA) at 1:100 dilution. Supernatant from a MOPC-21 myeloma culture (containing IgG2a) was used as negative control. In double immunofluorescence experiments 24B42 was used in conjunction with either a polyclonal guinea pig antiserum (gift of Dr. D. R. Garrod) raised against 205- and 230-kD bovine desmosomal plaque proteins (desmoplakins, see references 4, 6, and 30) at 1:200 dilution or a polyclonal rabbit antibody (DP 1/10, gift of Dr. M. S. Steinberg) raised against bovine desmplakin I and II (24) at 1:50 dilution. The second antibody system was either a mixture of rhodamine-conjugated goat antiserum to mouse immunoglobulins (1:100) or a mixture of fluoresceinated goat antiserum to mouse immunoglobulins (1:100) and rhodamine-conjugated goat antiserum to rabbit immunoglobulins (1:100). In other experiments LA7 cells were fixed in 3% formaldehyde for 5 min and then exposed to polyclonal rabbit antibodies raised against bovine desmocollins. Several antibodies were used: DG 11-1; DG 11-2, a gift of Dr. M. S. Steinberg (16); and HM-1, a gift of Dr. D. R. Garrod at 1:50 to 1:100 dilutions. Washes and dilutions were performed with PBS (pH 7.2) containing 0.1% sodium azide. The preparations were observed under a Zeiss photomicroscope equipped with phase-contrast and epi-ultraviolet-illuminator with selective filters for fluorescein and rhodamine.

Preparation of Cell Fractions Containing Assembled and Unassembled Cytokeratins

Total cytokeratin was estimated by enzyme immunoassay using monoclonal antibody 24B42. Confluent cultures of LA7 cells in 9-cm dishes, either untreated or treated with 4 mM EGTA for 1 h, were placed on ice, rinsed once with 10 ml Tris-buffered saline, and then scraped and homogenized in 10 mM Tris, pH 7.4, containing 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 100,000 g for 1 h and fractionated into pellets (P-100 or assembled) and supernatant (S-100 or unassembled). After protein concentration determinations were performed by the method of Bradford (2) each fraction was diluted in 60 mM sodium carbonate, pH 9.6, and plated in multiwell dishes (E.I.A. microtitration plates, Linbro, Flow Laboratories, Hamden, CT) at 5 μg/well. The proteins were allowed to bond hydrophobically to the dishes overnight at 4°C. Undiluted culture supernatants of 24B42 or a matched isotype control, MOPC-21, were added for 4 h at 22°C. Bound monoclonal antibody was detected by adding to each well 50 μl of goat antiserum to mouse immunoglobulins (1:1000) or a mixture of fluoresceinated goat antiserum to mouse immunoglobulins (1:100) and rhodamine-conjugated goat antiserum to rabbit immunoglobulins (1:100). In other experiments LA7 cells were fixed in 3% formaldehyde for 5 min and then exposed to polyclonal rabbit antibodies raised against bovine desmocollins. Several antibodies were used: DG 11-1; DG 11-2, a gift of Dr. M. S. Steinberg (16); and HM-1, a gift of Dr. D. R. Garrod at 1:50 to 1:100 dilutions. Washes and dilutions were performed with PBS (pH 7.2) containing 0.1% sodium azide. The preparations were observed under a Zeiss photomicroscope equipped with phase-contrast and epi-ultraviolet-illuminator with selective filters for fluorescein and rhodamine.

Results

Observations on LA7 Cells—Relation of Cytokeratin Bundles to Desmosomes

In confluent cultures of LA7 cells, 24B42 stains a meshwork of filaments that stretches from the surface to the nuclear membrane (Fig. 1, e and f). Many filaments have a general centripetal direction, whereas others are parallel to the nuclear membrane. At the cell surface each filament terminates with an expansion, or plaque.

Guinea pig and rabbit polyclonal antibodies against bovine desmoplakins stain desmosomal plaques at the borders between these cells, as they do in other epithelial cells (4, 6). Double immunofluorescence with mouse monoclonal antibody 24B42 and antisera to desmplakins shows that plaques stained by 24B42 are internal to those revealed by anti-desmoplakin antibodies and are always associated with them (Fig. 4, a and b). We will call them keratoplaques.

Formation of Bundles of Cytokeratin Filaments in Confluent Cultures of LA7 Cells

In confluent cultures almost all cells contain a cytoplasmic meshwork of keratin bundles stained by 24B42. Cells examined several hours after trypsinization and replating showed that most cells completely lack the meshwork. We studied the reappearance of the bundles during subsequent incubation. To this effect individual cells were assigned to several classes: (1) completely negative cells without bundles or keratoplaques. These cells are either single or in small groups (Fig. 1, a and b). (2) Cells with discrete keratoplaques at their edges, but lacking internal filaments. The plaques are always present at contacting edges, where a cell touches another cell; free edges are always devoid of plaques (Fig. 1 c). Not all contacting edges contain keratoplaques.

(3) Cells with short filaments anchored to keratoplaques at one end and with the other end radially directed toward the nucleus of the cell (Fig. 1, d and e) or cells with a complex meshwork of filaments (Fig. 1, f).

The proportions of cells positive only for keratoplaques (class 2) or for both keratoplaques and filaments (class 3) were determined during the reappearance of filaments. The results are shown in Fig. 2, plotted versus the time elapsed from trypsinization. With time, the population of cells is extensively characterized by a complex meshwork of filaments (Fig. 3, a and b).

Time Sequence of Desmoplakin and Cytokeratin Appearance in LA7 Cells

To prevent desmosomal formation, LA7 cells were grown in low calcium medium (0.1 mM calcium). In these conditions LA7 grow as single cells that lack linear reciprocal contacts and express neither desmoplakins nor keratoplaques and filaments. Only a small proportion of cells (<15%) shows a diffused or patchy perinuclear positivity for cytokeratins.

After being shifted to regular medium, containing 1.8 mM calcium, cells start making contacts, then expressing desmplakins, and finally expressing cytokeratin plaques and filaments, as illustrated in Figs. 4 and 5.

Desmosomal Glycoproteins in LA7 Cells

By the use of several rabbit antisera raised against different classes of desmosomal glycoproteins (desmocollins or desmogleins, references 5 and 16), LA7 cells showed a homogeneous distribution of granular positivity on the cell surface, both in single cells and in large colonies, without particular condensations of the positivity at cell mutual contacts. A slight condensation at this level became evident, however,
Figure 1. Appearance of cytokeratins in LA7 cells. 6 h after plating, cells are single or in small groups (a) and are 24B42 immunofluorescence negative (b). Later, keratoplaques and incipient filaments are stainable with 24B42 (c). Finally, most cells show a complete pattern of filaments, radially directed and aligned with corresponding filaments in the adjacent cells (d and e) or forming a complex meshwork (f). Bars, 10 μm.
after a brief EGTA treatment (4 μM for 15 min at room temperature).

Disappearance of Cytokeratin Bundles and Keratoplaques

Observations were made in cultures exposed to EGTA or cycloheximide.

EGTA Treatment

Immunofluorescence Studies. In confluent LA7 cells treated with 4 mM EGTA, an agent known to promote desmosomal breakdown (19, 21, 23, 40), the ability of 24B42 to stain filaments rapidly declines. After 4 h of treatment, only ~20% of the cells show filaments (class 3), as compared with ~95% in the untreated controls. In contrast, the proportion of cells showing 24B42-positive keratoplaques (class 2) undergoes a rapid and highly significant (P < 0.001) increase, followed by a plateau and then by a progressive decrease (Fig. 6).

Fractionation and Micro-Enzyme-linked Immunosorbent Assay. EGTA-induced loss of cytokeratin bundles, as judged by immunofluorescence of treated cells, could be the result of degradation of cytokeratin or merely a shift from assembled cytokeratin to unassembled. Following the approach used by Goud et al. (17) to show a shift in assembled versus unassembled clathrin, we fractionated LA7 cells into a 100,000 g × 1 h pellet (P-100) and a 100,000 g × 1 h supernatant (S-100). These two fractions, containing assembled (P-100) and soluble, unassembled cytokeratin (S-100), were assayed for cytokeratin recognized by 24B42 and total protein (Table I). In untreated LA7 cells the cytokeratin and protein are approximately evenly distributed between the two fractions. However, a major shift of cytokeratin to the unassembled form was seen in cells treated with EGTA.

Cycloheximide Treatment

It is known that protein synthesis is required for desmosome formation (32). Confluent LA7 cultures kept in the presence of cycloheximide (1 μg/ml) were therefore tested for 24B42 immunofluorescence at various intervals. The proportion of cells positive for both keratoplaques and filaments (class 3) dropped rapidly in the first 48 h and reached essentially zero in 7 d. The proportion of cells positive only for keratoplaques (class 2) showed initially a highly significant increase (P < 0.005), then a plateau, and finally a significant decrease (P < 0.02) (Fig. 7). In a small proportion of cells 24B42 immunofluorescence was localized in fine cytoplasmic granules, which are never observed in untreated growing cells. The disappearance of the filaments is not due to cell death caused by cycloheximide toxicity, because after addition of propidium iodide to the cultures we found very few stained nuclei up to 48 h after cycloheximide addition, when most cells have lost their cytokeratin network.

Relation of Cytokeratin Bundles to Desmosomes in 106A-10a Cells and HeLa Cells

106A-10a cells are derived from the same source as LA7 cells but do not have differentiation capabilities (9). They grow in monolayers with a polygonal shape, forming many desmosomes, and display an intense positivity for keratoplaques and filaments identified by 24B42. In contrast to findings with LA7 cells, trypsinization did not cause an extensive disappearance of cytokeratins detected by immunofluorescence: even single cells mostly retained a bright positivity, which, however, was either diffused or patchy. Distinct filaments could be seen only some hours after replating, when cells had spread out and reestablished mutual contacts and desmosomes. EGTA treatment on 106A-10a cells caused an analogous progressive disappearance of 24B42 filaments without the disappearance of 24B42 immunofluorescence. After 4 h of EGTA exposure, when the cells were largely rounded up...
Figure 4. LA7 cells grown in low calcium (0.1 mM) fixed 44 h after calcium reconstitution (1.8 mM). Double immunofluorescence for (a) desmoplakin I/II and (b) 24B42 cytokeratins. A clear correspondence is evident for desmoplakins and keratoplaques, frequently with a one-to-one ratio between plaques and filaments. All cells at this stage have desmoplakins, many have keratoplaques, and some have incipient or well-formed keratin filaments. Bar, 10 μm.
and desmosomes were disassembled, ~60% of 106A-10a cells retained a moderate patchy or diffused perinuclear positivity; 40% were completely negative.

HeLa cells of the line used here, although originally deriving from a stratified epithelial tissue, normally very rich in desmosomes, displayed a very small proportion of desmosomes, as ascertained by desmoplakin immunofluorescence. Most cells formed only occasional and nonlinear mutual contacts but had a very rich network of intermediate filaments stained by 24B42 monoclonal antibodies. Analogous to 106A-10a cells, EGTA treatment of HeLa cultures produced the disappearance of the filaments in most cells, which retained, however, a bright lumpy or diffused positivity; only a small proportion of the cells became negative.

To see whether keratin bundles behave similarly in non-neoplastic mammary cells, we have examined cells of the NMuGM line, which was obtained from normal mouse, as well as cryostat sections of normal mammary gland from a 3-wk-old rat. We found no keratoplaques in the NMuGM cells, which contain a rich cytokeleton network surrounding the nucleus. In the normal mammary gland desmosomes are present in all cells in endbuds, whereas in ducts they are mostly confined to the myoepithelial cells (8). Keratoplaques are much less frequent but can be recognized in sections of endbuds, often in association with desmoplaques.

**Discussion**

Using a mouse monoclonal antibody that recognizes a class of cytokeratins, we have studied the appearance and disappearance of bundles of cytokeratin filaments in rat mammary epithelial cells. In one line (LA7) the bundles are formed from the desmosomes in a series of steps as follows: keratin negative loose cells → contacts between cells → appearance of desmosomal glycoproteins → appearance of desmocollins and desmogloeins → appearance of desmoplakins → appearance of keratoplaque → appearance of plaque-attached keratin filaments → formation of meshwork.

The requirement for contacts between cells is supported by the observation that no filaments or keratoplaques are visible on the free borders of cells growing at the edge of colonies, on sparse cells after trypsinization, or in cells growing in low calcium medium. This observation conforms to previous observations in other systems (7, 19, 21, 32).

Once the desmoplakins are in place, cytokeratins recognized by 24B42 antibody accumulate in an area closely adjacent to them, forming the keratin component of the desmosomal plaque. This keratoplaque probably corresponds to a rather indistinct structure formed by convoluted or looping filaments previously observed by electron microscopy (the inner electron-dense plaque, reference 36). Bundles of cytokeratin-containing filaments assemble in continuity with the
keratoplques, radially directed toward the nuclear membrane. Subsequently the bundles reach that membrane. Finally, cross-crossing cytokeratin bundles appear. Some of them run parallel to the cell membrane, connecting several keratoplques. We assume that the presence of desmosomal glycoproteins (desmocollins or desmogleins) at contacts between cells initiate the process. Because the glycoproteins are not localized exclusively at desmosomes, they may play this role by local structural rearrangements.

A broadly reverse series of events takes place during the disappearance of cytokeratin bundles by exposing cultures to either EGTA or cycloheximide. In confluent cultures of LA7 cells the EGTA treatment leads to a rapid loss of cell-to-cell contacts. Then the number of cells positive for both keratoplques and filaments (class 3) rapidly decreases, whereas cells positivity only for keratoplques (class 2) increase. The rapid increase in the number of class 2 cells and their persistence and slow decline (Fig. 6) suggest that they are an intermediate stage in the evolution of the cells with both keratoplques and filaments (class 3) to negative cells (class 1). The frequency of cells in each of these classes during the formation of the bundles (Fig. 2) is also compatible with this conclusion.

Exposure of the cells to cycloheximide, which blocks protein synthesis, also causes the disappearance of cytokeratin filaments. Confluent cultures of LA7 cells displayed a significant decrease of stainable cytokeratin filaments in the presence of this drug. The decline, with a half-life of ~20 h, is much slower than after EGTA (half-life of ~50 min), but the success of events is similar; the proportion of cells in which only keratoplques were stained (class 2) also increased. Protein synthesis may be required for replacing either cytokeratin lost by normal turnover or other proteins needed for bundle polymerization, as is the case for several other cytoskeletal components (e.g., α-actinin and filamin stabilizing actin filaments [41] and filaggrin for intermediate filaments [38]).

The rapid disappearance of the cytokeratin bundles after trypsinization or EGTA treatment of confluent cultures is not accompanied by a disappearance of the cytokeratin molecules. After EGTA treatment the total amount of immunoreactive cytokeratin molecules is unchanged, but a conspicuous fraction of the membrane-bound molecules moves to the cytosol. Evidently the membrane-connected bundles break up, releasing smaller subunits that are no longer detectable by immunofluorescence. A breakdown of the cytokeratin bundle is noticeable also by immunofluorescence in cells exposed to cycloheximide (perhaps because it occurs slowly), which display rows of fine cytoplasmic granules stained by 24B42. These granules probably represent an intermediate stage of breakdown.

In conclusion, the formation of bundles of cytokeratin filaments in LA7 cells is strictly dependent upon the presence of desmosomes. No bundles are formed without desmosomes, and their polymerization starts at the periphery of the cell in a criss-cross pattern. Dissolution of desmosomes by EGTA (presumably by direct action on desmosoins) causes the rapid breakdown of existing bundles. Desmosomes are the principal organizing center of cytokeratin in these cells. In other cell types the control of formation of cytokeratin bundles may be different, as shown by the results with 106A-10a and HeLa cells.

In 106A-10a cells disruption of desmosomes by trypsinization or EGTA does not cause a complete depolymerization of cytokeratins, which remain detectable as diffused or patchy cytoplasmic immunofluorescence in most cells. In HeLa cells, which have very few desmosomes, EGTA also causes disruption of cytokeratin bundles but more slowly. EGTA seems therefore to disrupt the bundles by two mechanisms: a rapid and complete one, presumably through desmosome disruption, and a slower and less complete one independent of desmosomes. A direct Ca** effect on cytokeratin bundles is plausible because Ca** has been shown to be involved in the regulation of polymerization of cytoskeletal components (25). LA7 cells would have a cytokeratin system dependent exclusively on desmosomes; HeLa cells would have a desmosome-independent system, whereas 106A-10a cells would have both. The desmosome-independent system could be that identified by anchorage points on the cell membrane (10, 11, 20).

We do not know the relevance of the system we have described to the physiology of the mammary gland, from which LA7 cells were derived. We did not find it expressed in a line of normal mouse mammary cells. These cells, however, may be of a type different from LA7. In the rat mammary gland itself the association of keratoplques with desmosomes was observed in some endbud cells. This finding may be significant, because mammary cancers, such as that from which LA7 were derived, appear to originate in the endbuds (18).

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