Distribution of desmosomal proteins in F9 embryonal carcinoma cells and epithelial cell derivatives

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Summary

In diverse epithelia, cytoskeletal keratin intermediate filaments (IFs) associate with the cytoplasmic face of intercellular junctional desmosomes. The processes underlying desmosome formation and keratin IF interactions remain unclear. We have examined F9 embryonal carcinoma (EC) cell differentiation as a model for embryonic development of epithelial surface desmosomes. As determined by immunofluorescence microscopy and biochemical protein techniques, F9 EC cells, which lack surface desmosomes and keratin IFs, express the desmosomal proteins desmoplakins I and II (DP I/II), desmoglein I (DG I) and plakoglobin (PK). DP I/II are present at low levels and are relatively soluble in buffer containing Triton X-100. Immunofluorescence localizes DP I/II to the juxtanuclear, centrosomal region. Species of DG I and PK are detected in both the Triton X-100-soluble and -insoluble protein fractions. DG I appears dispersed throughout the cell while PK resides at cell-cell boundaries. In epithelial cell cultures induced by retinoic acid (RA) treatment, each of the desmosomal proteins is organized into punctate desmosome-like structures with the appearance of simple epithelial K8/K18 IFs. The steady-state levels of DP I/II and PK increase with a partitioning of the majority of the desmosomal components into the insoluble fraction. In epithelial cells which lack distinct surface desmosomes, an intracellular association of keratin bundles with DP I/II is observed, suggesting that keratin filaments may facilitate the translocation of these desmosomal components to the cell surface. Parietal endoderm-like cells, derived by treatment with RA and dibutyryl cAMP, are analogous to F9 EC cells in that the cells express desmosomal components and do not display surface desmosomes. Moreover, K8 and K18 do not form distinct filaments, and the protein and RNA levels of K8 are low relative to epithelial cells induced by RA alone. The F9 system appears to be a relevant model for studies of desmosome assembly and the potential interactions of desmosomal proteins and keratin IFs in embryonic epithelial cell types.

Key words: desmosomes, differentiation, embryonal carcinoma, keratins.

Introduction

The establishment of epithelial cell architecture is fundamental to embryo development and tissue morphogenesis and maintenance. Intercellular junctional desmosomes and cytoskeletal keratin intermediate filaments (IFs) are characteristic structural components of vertebrate epithelial cells (for review see Franke et al. 1987). Desmosomes consist of at least eight different proteins that are organized into two distinct plasma membrane domains: a membrane domain, involved in intercellular adhesion, and a cytoplasmic plaque, which serves as an anchorage site for keratin filaments (Steinberg et al. 1987; Franke et al. 1987; Green and Jones, 1990; Garrod et al. 1990). At the ultrastructural level, keratin filaments radiate from the nuclear region to the cell surface with attachment to the desmosome cytoplasmic face (for review see Goldman et al. 1990; Skalli and Goldman, 1991).

Several major desmosomal proteins and glycoproteins have been identified and localized by the use of specific antibodies. The adhesive, intercellular membrane domain of the desmosome is composed of glycoproteins, including desmoglein I (DG I; Mr, 165,000), the closely related desmocollins I and II (Mr, 130,000 and 115,000) and a Mr 22,000 component. Both DG I and desmocollin have been identified as members of the cadherin family of cellular adhesion molecules (Koch et al. 1990; Nilles et al. 1991; Mechanic et al. 1991). The cytoplasmic plaque consists of nonglycosylated proteins termed desmoplakins (DPs): DP I (Mr, 250,000), DP II (220,000), DP III or plakoglobin (PK; Mr, 83,000) and DP IV (Mr, 78,000). DP I and II are related and appear to be derived from a single gene
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In addition to these desmosomal proteins, minor components have been located in the desmosomes of diverse epithelia (Franke et al., 1987; Garrod et al., 1990). The primary method used to study the biochemical and morphological assembly and disassembly of desmosomes is to lower the external levels of calcium in epithelial cultures. Low calcium reduces intercellular adhesion and inhibits desmosome formation while surface desmosome assembly is reinitiated upon return to normal calcium levels (Green and Jones, 1990; Garrod et al. 1990). Conflicting results exist concerning the regulated assembly and disassembly of the desmosome and the potential role of keratin IFs in desmosome formation (Bologna et al. 1986; Duden and Franke, 1988; Jones and Goldman, 1985; Pasdar and Nelson, 1988a,b; Pasdar et al. 1991). Differing results may arise from the particular epithelial cell type used and its specific response to calcium levels.

Differentiation of murine F9 embryonal carcinoma (EC) cells provides a unique in vitro model to study processes of early embryonic epithelial cell biogenesis (for review see Hogan et al. 1983). The earliest stages of mammalian development involve a complex sequence of changes leading to the formation of extraembryonic epithelial cell types (Fleming and Johnson, 1988; Hogan et al. 1983). F9 EC cells, which undergo little spontaneous differentiation in vivo or in vitro, can be induced into distinct populations of extraembryonic endoderm by exposure to retinoic acid (RA) or RA and dibutyryl cAMP (Strickland and Mahdavi, 1978; Strickland et al. 1980; Hogan et al. 1983; Grover and Adamson, 1986).

Recently, we and others have used molecular genetic approaches to explore the interactions of desmosomal proteins and keratin IFs as well as the cellular function of keratin filaments. Our previous studies demonstrated a striking co-localization of DP I and II with collapsed K8/K18 filaments in RA-induced F9 cells expressing a dominant negative mutant of mouse K18 (Trevor, 1990). In addition, perturbation of K8/K18 IFs compromised the stable formation of visceral endoderm epithelium in differentiating F9 cell aggregates (Trevor, 1990). Similarly, a structural disruption of IFs is seen in epithelial cells expressing mutant domains of DP I which co-align with cytoplasmic IFs (Stappenbeck and Green, 1992), and biologically defective skin development is observed in mice expressing a dominant negative mutant of the epidermal keratin K14 (Vassar et al. 1991). It is interesting that embryonic stem (ES) cells carrying targeted, inactivated alleles of keratin K8 form visceral endoderm which appears to assemble surface desmosomes in the absence of simple epithelial K8/K18 IFs (Baribault and Oshima, 1991). These results raise questions concerning the potential role of keratin IFs in desmosome assembly and the structural requirements of keratin IFs for epithelial integrity.

We here report the expression and subcellular distribution of DP I and II, DG I and PK in F9 EC cells and differentiated derivatives. Although there is variance in the levels and localization of these proteins, the proteins are detected in the distinct F9 epithelial cell types, as well as undifferentiated F9 EC cells. In RA-induced epithelial cells, the desmosomal proteins appear to assemble into discrete, surface desmosome structures. Moreover, there is an obvious intracellular association between DP I and II and keratin IFs in differentiating cells which lack assembled desmosomes. This result suggests a role for keratin filaments in the formation of the desmosome. As shown in this study, the F9 system offers a unique, relevant model for studies of desmosome formation during the morphogenesis of extraembryonic epithelial cell types.

Materials and methods

Cell culture and differentiation

The F9.22 EC cell line has been described previously (Trevor and Oshima, 1985). Cells were cultivated in Dulbecco's modified Eagle's medium supplemented with pyruvate (110 mg/l), glutamine (0.04%), 10% fetal bovine serum, and gentamycin sulfate at 30 mg/ml.

To induce epithelial differentiation, cells were plated at a density of 6×10³ cells/cm² and RA (Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 1 μM for 24 hours to RA-induced epithelial cells, the desmosomal proteins appear to assemble into discrete, surface desmosome structures. Moreover, there is an obvious intracellular association between DP I and II and keratin IFs in differentiating cells which lack assembled desmosomes. This result suggests a role for keratin filaments in the formation of the desmosome. As shown in this study, the F9 system offers a unique, relevant model for studies of desmosome formation during the morphogenesis of extraembryonic epithelial cell types.

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To induce epithelial differentiation, cells were plated at a density of 6×10³ cells/cm² and RA (Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 1 μM for 24 hours. For differentiation to the PE phenotype, F9 EC cells were similarly plated and grown in the presence of 1 μM RA and 0.1 mM dbcAMP (Sigma). Cells were fed on day 3 with drug-containing medium and analyzed on day 5 of induction.

Immunofluorescent labeling and antisera

Immunofluorescent staining was performed on methanol-fixed cells as previously described (Oshima, 1981). A polyclonal rabbit antiserum recognizing DP I and II (DP I/II) and a second polyclonal rabbit antisera specific for DG I were provided by M. Pasdar and W. J. Nelson. Both antisera have been extensively characterized (Pasdar and Nelson, 1988a,b; Pasdar and Nelson, 1989). Trion 1, a rat monoclonal antisera to the mouse keratin K8, has been previously described (Trevor, 1990; Brulet et al. 1980). To verify the staining patterns of DP I/II in RA-induced cells, a mouse monoclonal antibody directed against DP I and II was used (Boehringer Mannheim Corp., Indianapolis, IN). A monoclonal mouse antibody against plakoglobin (PG5.1) and a polyclonal guinea pig antibody recognizing DG I were obtained from P. Cowin (Cowin et al. 1986; Cowin and Garrod, 1983). Mouse monoclonal antisera against vimentin was purchased from Sigma Chemical Co., St. Louis, MO. Primary antibodies were visualized by using the appropriate fluorescently labeled secondary antibodies: FITC- or rhodamine-conjugated goat antiserum to rabbit IgG, rhodamine-conjugated goat antiserum to mouse IgG, and FITC-conjugated goat antiserum to rabbit IgG (Sigma). Controls included incubation of fixed cells with only secondary antibodies and verification that secondary antibodies were species specific.

Wheat germ agglutinin was utilized to label the Golgi complex (Singh et al. 1988). Cells were preincubated with 2 μg/ml of unlabeled wheat germ agglutinin (Sigma) for 7 minutes at 4°C before fixation in cold methanol and were processed as for immunofluorescence staining using FITC-labeled wheat germ agglutinin (Sigma).

Cell fractionation

Separation of cellular proteins into soluble and insoluble
(cytoskeleton) fractions was performed using the procedure of Triton X-100/high-salt extraction (Fey et al. 1984; Pasdar and Nelson, 1988a). Cell monolayers were rinsed twice with cold phosphate-buffered saline, gently scraped from the dish and pelleted at 2,000 g for 3 min at 4°C. The cells were then extracted in buffer containing 50 mM NaCl, 300 mM sucrose, 10 mM PIPES (pH 6.8), 3 mM MgCl2, 0.5% w/v Triton X-100, 1.2 mM phenylmethylsulfonyl fluoride (PMSF), 0.02 mg/ml DNase I and 0.1 mg/ml RNase for 20 min at 4°C. A solution of 2.5 M (NH4)2SO4 was added to a final concentration of 250 mM and incubation was continued for another 5 min. Samples were centrifuged at 20,000 g for 15 min to yield a supernatant (soluble fraction) and pellet (insoluble, cytoskeletal fraction). The cytoskeletal pellet was resuspended in a buffer containing 1% w/v SDS, 5 mM EDTA, and 10 mM Tris-HCl, pH 7.5.

Immunoprecipitation and immunoblotting

The procedures used for total cell lysate preparation, immunoprecipitation and gel electrophoresis were described previously (Oshima, 1982). DP I and II proteins were detected by immunoprecipitation of metabolically labeled proteins using the polyclonal rabbit antisera against DP I and II. Cell monolayers were labeled for 3 h in medium deficient in methionine that was supplemented with 35S-methionine (150 μCi/ml) and 10% fetal bovine serum. For analysis of DP I and II in total cell lysates, the proteins were immunoprecipitated from 5 × 106 cts/min of [35S]methionine-labeled lysate. To determine the distribution of DP I and II proteins in the soluble and insoluble cell protein fractions, the proteins were immunoprecipitated from 5 × 106 cts/min of soluble, labeled protein and 5 × 105 cts/min of the insoluble cell fraction. As in previous reports (Cervera et al. 1981), approximately 15% of the cellular proteins was present in the pelleted, cytoskeletal protein pool as determined by radioactive counting of incorporated [35S]methionine in each cell fraction.

For immunoblotting, protein concentrations were determined by the BCA method (Pierce, Rockford, IL). Ten μg and 80 μg of the insoluble and soluble protein fractions, respectively, were separated on SDS-polyacrylamide gels (PAGE) and transferred electrophoretically to nitrocellulose filters. The filters were processed for protein analysis using the Amersham ECL Western blotting detection system (Amer- sham Inc., Amersham, UK). Primary antibody binding was detected by using the appropriate horseradish peroxidase-conjugated secondary antibodies followed by chemiluminescent fluorography. Each experiment was performed a total of three times. The relative amounts of respective proteins were determined from resulting fluorograms by laser scanning image analysis using a Bio Image Laser Scanner (Bio Image, Ann Arbor, MI).

RNA analysis

Total cellular RNA was isolated by the acid, single step procedure described by Chomczynski and Sacchi (1987). For Northern analysis, 20 μg/lane of total RNA was denatured in formaldehyde, electrophoresed through a 1.2% formalde- hyde/agarose gel, and transferred to nitrocellulose (Sambrook et al. 1989). 32P-labeled DNA probes were generated from cDNA fragment inserts by the random priming method (Feinberg and Vogelstein, 1984), employing the Multiprime DNA labeling system (Amersham, Amersham, UK). Filters were hybridized with 6 × 106 cts/min per ml of the respective probes and washed using standard conditions (Sambrook et al. 1989). The cDNA clones encoding human beta actin (pHBA-1), laminin B1 (pPE386) and mouse K8 (Endo A; clone alpha 2) have been previously described (Ponte et al. 1984; Kurkinen et al. 1983; Vassuer et al. 1985).

Results

Immuno-localization of DP I and II proteins in F9 EC cells and differentiated derivatives

As described previously (Hogan et al. 1983), F9 EC cells undergo dramatic morphological and biochemical changes when grown in the presence of inducing agents. Undifferentiated F9 EC cells form dense, tightly packed colonies of small rounded cells (Fig. 1A). After a 5 day exposure to RA, approximately 60-70% of the cells assume a flattened, epithelioid morphology with distinct, opposing cell boundaries (Fig. 1B). The cells are similar to primitive extraembryonic endoderm and possess numerous markers including simple epithelial keratin IFs composed of K8 and K18 (Trevor and Oshima, 1985; Oshima et al., 1988). Growth in the presence of RA and dbcAMP results in dispersed, rounded cells with long filopodia (Fig. 1C), which resemble primary cultures of embryonic parietal endo- derm (PE) (Strickland et al. 1980; Hogan et al. 1983). Less than 20% of induced PE cells show a more flattened, epithelial morphology.

Indirect immunofluorescent staining using an anti-serum which recognizes DP I and II (DP I/II) localized these proteins to a perinuclear region in both undifferen- tiated F9 EC cells and the rounded PE cells (Fig. 1D,F). In RA-treated cultures, areas of flattened cells displayed a punctate alignment of DP I and II at cell-cell boundaries (Fig. 1E), characteristic of surface desmosomes.

The juxtanuclear localization of DP I/II in F9 EC cells and PE cells suggested that the proteins might reside in the vicinity of the centrosome. As Golgi stacks are known to be grouped near the centrosomal region (Bershadsy and Vasiliev, 1988), cells were stained simultaneously with DP I/II antibodies followed by rhodamine-conjugated secondary antibodies and FITC-labeled wheat germ agglutinin, which detects the Golgi complex (Singh et al. 1988). Double fluorescence detection revealed very similar perinuclear staining patterns (Fig. 2), suggesting that DP I/II are present in this subcellular region.

Solubility properties of DP I and DP II

Investigations of the subcellular state of surface desmo- somal components in epithelial cell cultures have demonstrated that desmosomal proteins, including DP I and II, are notoriously insoluble in Triton X-100/high-salt buffer (Jones and Goldman, 1985; Bologna et al. 1986; Kapprell et al. 1987; Pasdar and Nelson, 1988a). The solubilities of DP I and II are enhanced when epithelial desmosomes are disrupted by decreasing the external level of calcium (Jones and Goldman, 1985; Duden and Franke, 1988; Pasdar and Nelson, 1988a).

Prior to analyzing the subcellular distributions of DP I and II in the F9 cell types, the steady-state levels of DP I and II were examined by immunoprecipitation of total,
Fig. 1. Morphological features of F9 cell types and immunolocalization of DP I/II. (A) Morphology of undifferentiated F9 cells; (B) RA-derived epithelial cells; (C) PE cells after treatment with RA and dbcAMP. (D) Indirect immuno-localization of DP I/II in F9 EC cells; (E) RA-induced epithelial cells; (F) PE cells. Cells were methanol-fixed and preincubated with a polyclonal rabbit antibody against DP I/II followed by rhodamine-labeled goat anti-rabbit IgG. Note that areas of cells shown in (A-C) are different from those shown in (D-F). Differentiated cell cultures were analyzed after 5 days growth in the presence of inducing agents. Bar, 10 μm.

Labeled cellular proteins (Fig. 3A). Densitometric scanning of the autoradiogram indicated that DP I and II were present at approximately an 8-fold lower level in undifferentiated F9 cells relative to RA-derived epithelial cells. In PE cells, the amounts of DP I and II were about 50% less than detected in RA-treated cells. Analysis of the solubilities of the proteins, as characterized by their extraction in Triton X-100/high-salt buffer, showed that in both undifferentiated F9 cells and PE cells, the majority of DP I and II existed in the soluble protein fraction (Fig. 3B). Moreover, smaller protein bands were consistently observed in the soluble fraction of the PE cells suggestive of a degradation or modification of soluble DP I and/or II proteins in this cell type. In RA-induced cells, an enrichment of DP I and II was detected in the pelleted, cytoskeletal fraction with approximately 70% of the proteins partitioned into this fraction.

Detection of DG I and PK in F9 EC cells and differentiated derivatives
Because DP I and II were detected in undifferentiated F9 cells, we investigated the nature of two other desmosomal components, DG I and PK, in F9 EC cells and the differentiated cell types. The transmembrane glycoprotein DG I is found in all desmosomes and has been implicated in the adhesive mechanism of surface desmosomes (Franke et al. 1987; Garrod et al. 1990). Although originally described as a desmosomal component of the cytoplasmic plaque, PK is detected not only in desmosomal junctions but also in other types of adhering intercellular junctions, including intermediate junctions (Cowin et al. 1986; Kapprell et al. 1987).

Immuno-staining of F9 EC cells localized PK to cell-cell boundaries in a pattern outlining the periphery of the cells (Fig. 4), indicative of its presence in intercellular adhering junctions (Cowin et al. 1986). In RA-
induced epithelial cells, a faint linear staining along cell boundaries was discerned, underlying discrete spots. Characteristic of epithelial cell types, the distribution of PK in punctate, surface spots most likely corresponds to its association with desmosomes while the more linear pattern of staining is representative of its distribution in other types of intercellular adhering junctions (Cowin et al. 1986). In PE cells, which lack intimate cell-cell contacts, a very faint staining for PK was observed in the cell body.

Indirect immunofluorescence of DG I showed a diffuse, grainy staining in undifferentiated F9 cells while RA-induced epithelial cells displayed a punctate, surface pattern typical of desmosomes formation with some intracellular staining (Fig. 4). In nearby epithelial cells, which appeared to lack a distinct surface localization for DG I, a scattered, granular staining was observed with more intense grains in the vicinity of the nucleus. As for F9 EC cells, cells differentiated to the PE cell phenotype displayed a scattered pattern of staining over the cell body and faint fluorescent grains extending into the long cytoplasmic filopodia.

Immunoblotting identified PK in both the soluble and pelleted, insoluble fractions of F9 EC cells (Fig. 5). Protein bands smaller than PK (M_r 83,000) were also detected which probably represent degradation products. Accounting for these smaller species, the amount of PK in the pelleted, insoluble fraction of F9 EC cells was only slightly higher than that partitioned into the soluble pool. In RA-derived epithelial cells, the relative level of insoluble PK was increased with approximately twice as much PK partitioning into this fraction. Estimations of the total amount of PK in each cell type indicated that RA epithelial cells possessed a 3-fold higher level of PK relative to F9 EC cells. Barely detectable amounts of PK were identified in the fractionated protein pools of PE cells, confirming the observed faint immunostaining of this cell type.

Analysis of DG I (M_r 165,000) revealed that each cell type had similar levels of the protein present in the insoluble fraction (Fig. 5). In addition, a distinct lower M_r protein estimated at 125,000 was detected in the soluble protein pools of both undifferentiated F9 cells and PE cells. This species was virtually undetectable in RA epithelial cells.

**Intracellular association of DP I/II and keratins**

In RA-treated cultures, approximately 50% of the epithelial cells displayed punctate staining for DP I/II, DG I and PK at opposing cell-cell membranes, indicative of desmosome formation. The remaining cells of epithelial morphology displayed a linear surface staining for PK (not shown), as might be expected because PK localized to cell boundaries in undifferentiated F9 cells. As mentioned, DG I was distributed in a...
highly diffuse, granular pattern. Double immunofluorescence of DP I/II and keratin IFs revealed some cells with filamentous-like speckles of DP I/II decorating keratin filaments while other cells displayed distinct, larger spots of DP I/II along the filaments (Fig. 6A-D). This intracellular association was most apparent in cells lacking a punctate surface staining for DP I/II. As shown in Fig. 6 (E and F), little if any DP I/II staining co-aligned with keratin IFs in those cells displaying DP I/II at the cell surface. Moreover, similar patterns of DP I/II distribution were observed when only DP I/II staining was performed using this particular antibody as well as when employing a monoclonal antibody recognizing DP I/II (data not shown).

Unlike epithelial cells derived by RA treatment, the rounded PE cells did not possess distinct keratin filaments as determined by immuno-staining using an antibody recognizing K8 (Fig. 7A). Some cells completely lacked keratin staining while others displayed a weak co-localization of aggregated keratin material to the perinuclear region. Double immunofluorescence showed that this staining coincided with that of DP I/II (Fig. 7A,B). Similar staining was observed when employing an antibody recognizing K18 (data not shown). Although extended keratin filaments appeared to be absent in PE cells, immunofluorescent staining for vimentin revealed elongated vimentin IFs traversing the cytoplasmic filopodia (Fig. 7C).

Expression of keratin K8 in PE cells and RA-induced epithelial cells

Western analysis of K8 confirmed that the amount of keratin protein present in PE cultures was low relative to RA-induced cells (Fig. 8A). Densitometric scanning of immunoblots revealed that PE cells possessed a 5-
of steady-state K8 RNA was about 4-fold less than that detected after RA induction, similar to the differences of laminin Bl while there is a more modest increase (Cooper et al. 1983). F9 EC cells express only low levels of encoding K8 RNA (Fig. 8B). The amount of K8 RNA in PE cells correlated with a lower level of K8 protein than that detected in RA-induced epithelial cells. As approximately 10-20% of the cells assumed a more flattened, epithelioid morphology possessing K8/K18 IFs, a portion of the detected K8 protein is accounted for by these cells. In RA-treated cells, K8 partitioned into the insoluble, cytoskeletal fraction as expected for a filamentous keratin protein. PE cells also exhibited a segregation of K8 to the insoluble pool, indicating that the aggregated nature of K8 and K18 proteins observed by immunostaining did not influence the solubility properties of the proteins.

Northern analysis demonstrated that the amount of K8 protein synthesized in PE cells correlated with a lower level of encoding K8 RNA (Fig. 8B). The amount of steady-state K8 RNA was about 4-fold less than that detected in RNA isolated from RA epithelial cells. Characteristic of the PE phenotype, the steady-state level of RNA encoding the extracellular matrix protein laminin B1 was dramatically increased in PE cells. As described by others, high amounts of laminin B1 RNA parallel the increased amount of laminin B1 protein synthesized upon F9 EC cell differentiation in the presence of RA and dbcAMP (Durkin et al. 1986; Cooper et al. 1983). F9 EC cells express only low levels of laminin B1 while there is a more modest increase after RA treatment. In undifferentiated F9 cell RNA, the amount of actin RNA was slightly lower than that observed in RA-induced epithelial cells. Perhaps, in F9 EC cells there is a lower expression of the single gene which is thought to encode both DP I and II (Green et al. 1988; Green et al. 1990). The steady-state amounts of DP I and II detected in undifferentiated F9 cells are 8-fold less than that observed in RA-induced epithelial cells. Perhaps, the proteins are more rapidly degraded when soluble, as demonstrated in studies of desmosome assembly in MDCK epithelial cells exposed to low calcium medium (Pasdar and Nelson, 1988a).

In contrast to the more soluble states of DP I and II in F9 EC cells, the major glycosylated form of DG I is detected in the insoluble protein fraction. The steady-state level is comparable to that in RA-induced epithelial cells which are undergoing desmosome formation, suggesting that the protein is relatively stable in the undifferentiated cells. In addition, a soluble species of about 125,000 Mr, is detected, which is larger than the estimated Mr of 107,703 of unglycosylated human DG I. (Nilles et al. 1991). This protein species may be a form of DG I which has yet to undergo complete glycolytic processing or may represent a degradation product of the fully processed DG I. Previous investigations of MDCK cells have indicated that DG I is glycosylated in the Golgi complex and rapidly titrated into an insoluble pool with subsequent transport via microtubule “tracks” to the plasma membrane (Pasdar and Nelson, 1989a; Pasdar et al. 1991). In undifferentiated F9 cells, DG I appears dispersed in a granular pattern rather than entirely localized to the Golgi region or distributed in a filamentous pattern as might be expected for an association with microtubules. Further biochemical and cytological analyses will reveal aspects of DG I processing and its subcellular location(s).

In F9 EC cells, PK occurs at cell-cell boundaries, consistently about 30% less than that observed in RNA from RA-treated cells.

**Discussion**

We have determined that undifferentiated F9 cells, which lack surface desmosomes and keratin IFs, possess pre-existing pools of at least four known constituents of the desmosome: DP I, DP II, PK and DG I. Like F9 EC cells, cells of the PE phenotype do not assemble surface desmosomes, although the cells express desmosomal components. Upon RA-induced epithelial cell differentiation, the proteins assemble at cell-cell boundaries as discrete structures, characteristic of desmosomes.

In undifferentiated F9 cells, DP I/II appear to exist in the juxtanuclear, centrosomal region of the cell as relatively soluble components. Although DP I/II immunolocalization coincides with the Golgi complex, as determined by wheat germ agglutinin labeling, a more detailed microscopic examination is required to determine their exact subcellular location. Also, recent analysis of P19 EC cells has demonstrated low levels of soluble DP I/II localized near the nucleus (Schmidt et al. 1992). The steady-state amounts of DP I and II detected in undifferentiated F9 cells are 8-fold less than that observed in RA-induced epithelial cells. Perhaps, in F9 EC cells there is a lower expression of the single gene which is thought to encode both DP I and II (Green et al. 1988; Green et al. 1990). Gene expression may be increased following RA exposure. Alternatively, the proteins are more rapidly degraded when soluble, as demonstrated in studies of desmosome assembly in MDCK epithelial cells exposed to low calcium medium (Pasdar and Nelson, 1988a).
Fig. 6. Intracellular association of DP I/II and keratin IFs in RA-induced epithelial cells. Cells were fixed and co-stained with a rabbit monoclonal against DP I/II (A, C, E) and a monoclonal rat antibody (Troma I) recognizing K8 (B, D, F). A and B, C and D, E and F represent different patterns of DP I/II staining seen relative to keratin IFs. Primary antibodies were visualized using the appropriate rhodamine- and FITC-labeled antisera as secondary antibodies. Bar, 5 μm.

indicating an association with intercellular junctions. PK exists not only in epithelial cell desmosomes but in other types of intercellular adhering junctions, including intermediate junctions, where it is associated with microfilaments, vinculin and alpha-actinin (Cowin et al. 1986; Kapprell et al. 1987). A slightly higher portion of PK partitions into the insoluble protein pool relative to the soluble fraction. Identification of PK in both the soluble and insoluble protein fractions might be expected, as these different forms have been observed in both desmosome and non-desmosome-bearing cells and tissues (Cowin et al. 1986; Kapprell et al. 1987).

Analogous to undifferentiated F9 cells, PE cells exhibit similar immunolocalization characteristics and biochemical distributions of both DP I/II and DG I. However, unlike F9 EC cells, the level of PK is barely detectable, as might be expected for a cell type which lacks intimate cell-cell contacts. Either PK is rapidly degraded or the level of PK gene expression is relatively low. Moreover, immunofluorescence reveals that F9-
derived PE cells do not possess extensive keratin IFs, as suggested previously (Boiler and Kemler, 1983). When present, aggregated keratin proteins co-localize with DP I/II to the juxtanuclear region while vimentin exists as elongated filamentous structures. As compared to RA-induced epithelial cells, PE cells express lower amounts of keratin proteins, which correspond to reduced levels of keratin RNA.

In contrast to PE cells derived in vitro, PE cells of the mouse embryo appear to possess both K8/K18 and vimentin IFs (Lane et al. 1983; Lehtonen et al. 1983). This discrepancy in IF content between PE cells in vivo and those differentiated in vitro may relate to the represented developmental stage. Embryonic PE cells were examined in later post-implantation embryos, which possess a well-established parietal endoderm. F9-derived PE cells possibly represent an earlier, pre-implantation stage of PE differentiation. The features of PE cells derived in vitro are highly reminiscent of that observed for fibroblastoid cells derived from the conversion of epithelial cells: intercellular junctions, including desmosomes, are absent; DP I and II are more soluble; keratin expression is decreased; and vimentin IFs are predominant (Boyer et al. 1989). In addition, this cell conversion is dependent on protein phosphorylation, which is similarly implicated in the F9 system. Generation of the PE phenotype requires dbcAMP or other compounds which elevate cAMP levels in combination with RA (Strickland et al. 1980). It is of significance that the levels of the mRNAs encoding retinoic acid receptors (RARs) are greatly reduced in the presence of RA and cAMP analogs relative to RA treatment alone (Hu and Gudas, 1990). A modulation of the effects of RA on gene expression by cAMP analogs may account for the reduced expression of keratins detected in PE cells.

In RA-induced epithelial cell cultures, DP I/II, DG I and PK assemble into desmosomes, on the basis of their characteristic punctate, surface staining. Relative to F9 EC cells, the levels of DP I/II and PK increase, and the majority of the proteins are transferred to the insoluble protein fraction, indicating of desmosomal biogenesis. The majority of DG I appears as a fully processed protein with only low levels of the soluble M, 125,000 species observed. In differentiating cells lacking discrete, surface desmosomal structures, dual immunofluorescence reveals a distinct co-alignment of DP I/II with

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**Fig. 7.** Immuno-localization of keratin protein and DP I/II in PE cells. (A and B) Double indirect immunofluorescence for keratin protein and DP I/II, respectively. Keratin was detected using a rat monoclonal antibody (Troma 1) against K8. DP I/II was localized employing a polyclonal rabbit antiserum against DP I/II. Appropriate FITC- and rhodamine-labeled antisera were used as secondary antibodies. (C) Detection of vimentin filaments using a primary monoclonal mouse antibody followed by rhodamine-labeled goat anti-mouse secondary antiserum. Bar, 10 μm.

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**Fig. 8.** Expression of keratin K8 in F9 cells. (A) Immunoblot of mouse K8 (mK8). Ten μg and 80 μg of the pellet, insoluble (P) and soluble (S) protein fractions, respectively, were separated by SDS-PAGE (12.5% gel), transferred to nitrocellulose and immunoblotted using the rat monoclonal Troma 1 antiserum. Protein bands were developed using a horseradish peroxidase-labeled anti-rat antiserum followed by chemiluminescent fluorography. Undifferentiated F9 cells (UD); RA-derived epithelial cells (R); PE cells (R/D). Bars indicate relative molecular mass standards: upper bar, bovine serum albumin (77 x 10^3); lower bar, ovalbumin (46.5 x 10^3). Lower bands detected in the RA-treated sample most likely represent degradation, which can occur during protein isolation. (B) Northern analysis. Following isolation of total cellular RNA, 20 μg/lane was denatured in formaldehyde, electrophoresed through a 1.2% formaldehyde/agarose gel, and transferred to nitrocellulose. RNA species were detected by hybridization with the respective [32P]-labeled DNA probes followed by fluorography. mK8, mouse K8 RNA; LN B1, laminin B1 RNA.
K8/K18 filaments. Individual cells display faint speckles of DP I/II or distinct bead-like dots which appear to decorate keratin IFs. As RA-induced differentiation is not a fully synchronous phenomenon, one interpretation is that these patterns of DP I/II staining represent stages of DP I/II assembly prior to their appearance at the cytoplasmic membrane. Further transport of DP I/II to the cell surface may be facilitated by translocation along keratin IFs.

Studies of developing embryos and cultured epithelial cells have resulted in opposing views concerning desmosome assembly and keratin IF interactions. One opinion is that the desmosome serves as an organizing center for IFs following desmosome assembly (Overton, 1962; Dembiter et al. 1980; Jackson et al. 1981; Bologna et al. 1986; Duden and Franke, 1988). Other results suggest that keratin IFs may play a regulatory role in desmosome assembly (Jones and Goldman, 1985; Jones and Grelling, 1989; Pasdar and Nelson, 1988a,b; Pasdar et al. 1991). Recent investigations of MDCK epithelial cell cultures maintained in low calcium-containing medium and switched to a normal calcium level have demonstrated that a soluble complex of DP I and II is recruited to the insoluble cell fraction in association with keratin IFs upon initiation of desmosome assembly (Pasdar and Nelson 1988a,b; Pasdar et al. 1991). Moreover, a colocalization of DP I/II with disrupted K8/K18 IFs is observed in RA-induced F9 cells expressing a dominant negative mutant of K18 (Trevor, 1990). Transient expression of mutant domain regions of DP I in epithelial cells demonstrates a colignment of the proteins along keratin IFs (Stappenbeck and Green, 1992). This result supports a previous notion that DP I/II might interact with IFs, on the basis of a common periodicity of charged residues (Green et al. 1990). In this report, we demonstrate the occurrence of a spatial interaction of DP I/II with K8/K18 filaments during F9 epithelial cell differentiation without direct manipulation of normal cellular physiology or cytoarchitecture.

A recent study on desmosome biogenesis in pre-implantation mouse embryos has demonstrated that surface desmosome formation first appears in the trophectoderm epithelium (Fleming et al. 1991) with their subsequent occurrence in the later-stage visceral endoderm surrounding the egg cylinder (Hogan et al. 1983). As in F9 EC cells, a linear pattern of PK membrane association is seen in the 16 to 32-cell stage morula (Fleming et al. 1991). K8 and K18 protein synthesis and filament polymerization precedes the appearance of surface desmosomes, occurring at the 4- to 8-cell stage of mouse development (Oshima et al. 1983; Chisholm and Houliston, 1987). Taken together, these reports are provocative when considering a role for keratin filaments in the facilitation of desmosome assembly.

Of significance, embryonic stem (ES) cells, which lack keratin IFs due to targeted inactivation of both K8 alleles, show an apparently normal differentiation to visceral endoderm, including the plasma membrane appearance of DP I/II in desmosome-like structures (Baribault and Oshima, 1991). Yet, dominant negative mutants of keratin proteins have been shown to prevent visceral endoderm formation in F9 cells (Trevor, 1990) or result in defective skin epithelium as in the human genetic disorder epidermolysis bullosa simplex (Vassar et al. 1991; Coulombe et al. 1991). These two latter results suggested that keratins have an important structural function. Considering that visceral endoderm can form in the absence of keratin IFs, the effects of mutant keratin proteins may reflect secondary structural alterations arising from the induced collapse of keratin filaments. In F9 epithelial cells, one such perturbation appears to be a physical pulling of associated DP I/II with their subsequent localization to K8/K18 aggregates (Trevor, 1990).

Although differentiated K8(−) ES cells lack keratin IFs, the K8 partners, K18 and K19, are detected at low levels and are observed to associate with surface DP I/II in a subpopulation of the cells. How these keratin proteins arrive at the plasma membrane is unclear. Detailed comparison of DP I/II localization during differentiation of normal and K8(−) ES cells will help to clarify the potential role of keratin IFs in DP I/II assembly and translocation. Such analysis may reveal differences in desmosome assembly and/or structure. At present, there is no evidence supporting a direct role for IFs in intracellular transport, as is well known for cytoskeletal microtubules (Vale, 1987; Kreis, 1990). However, a mechanism for a kinesin-dependent interaction of vimentin IFs with microtubules and transport vesicles has been proposed (Gyoeva and Geldard, 1991).

As explored in this study, the F9 differentiation system provides an opportune model for addressing numerous questions regarding desmosome assembly under conditions which mimic the normal differentiation of early embryonic epithelial cell types. At present, we are in the process of evaluating the expression and distribution of other known desmosomal components. Further investigations will elucidate our understanding of the establishment and maintenance of simple epithelial cell, as well as tissue, architecture.

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