Desmoplakin II expression is not restricted to stratified epithelia

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Summary

Desmosomes are major intercellular junctions found in association with intermediate filaments in epithelial, cardiac and arachnoidal tissue. Desmoplakins I and II (DPI and II) are highly related proteins localized in the innermost part of the desmosomal plaque and are candidates for linking intermediate filaments (IF) to the desmosomal complex. While investigators agree that DPI is present in all epithelia, they disagree on the distribution of DPII. Some have reported DPII to be restricted to stratified tissue and have furthermore suggested that the expression of DPII may be linked to stratification.

We have compared the expression of DPI and II at the mRNA and protein levels in cell lines derived from simple, transitional and stratified epithelia. Northern blot analysis revealed DPI and II mRNA to be present in all cell lines as well as simple and stratified epithelial tissues. However, DPII mRNA could not be detected in cardiac muscle tissue. Immunoblotting and immunoprecipitation demonstrated the presence of DPI and II in all cell lines at the whole-cell protein level as well as in association with cytoskeletal fractions. Immunofluorescence staining was used to correlate the biochemical findings with the localization of DPI and II. While most cell lines exhibited typical intercellular and in many cases cytoplasmic DP staining, T24 cells exhibited predominantly diffuse and dotted cytoplasmic staining. In addition, we investigated whether changes in DPI and II expression occurred following calcium-induced cell contact formation and stratification in the human pharyngeal cell line, FaDu. No significant changes in mRNA or whole-cell protein levels were observed during a period of 5 days following the calcium switch. However, immunoblotting revealed a significant increase in DPI and II levels in the insoluble protein pool during desmosome formation. These observations indicated a possible recruitment of soluble DPI/II into an insoluble pool after induction of desmosome assembly by the calcium switch, consistent with earlier reports for MDCK cells.

In summary, our results suggest that the expression of DPIII is not strictly linked to stratification or differentiation; however, the apparent absence of DPII mRNA from cardiac muscle suggests it may not be a constituent of all desmosomes.

Key words: desmoplakin, desmosome, epithelia, calcium.

Introduction

Desmosomes (spot desmosomes, macula adherens) are major intercellular junctions found in epithelial (Arnn and Staehelin, 1981; Cowin and Garrod, 1985; Franke et al. 1987) myocardial (Kartenbeck et al. 1983; Thornell et al. 1985) and arachnoidal (Kartenbeck et al. 1984; Cowin et al. 1985a; Moll et al. 1986) tissue. The symmetrical junction of 0.1-1.5 μm in diameter consists of a core portion, comprising the plasma membranes of adjacent cells separated by a 30 nm intercellular space (Cowin et al. 1985a; Steinberg et al. 1987). Subjected to the plasma membranes lie the multi-layered desmosomal plaques, which serve as attachment sites for intermediate filaments (Overton, 1962; Overton, 1975; Drochmans et al. 1978; Cowin et al. 1985a; Franke et al. 1987; Steinberg et al. 1987; Green and Jones, 1990). In order to understand the structure of desmosomes and their interaction with the cytoskeleton it is important to elucidate the occurrence, localization, function and regulation of its constituents.

The desmosomal membrane complex contains a unique set of proteins. The glycoproteins, desmogleins I-III, have been localized to the desmosomal core portion and are thought to contribute to the intercellular 'glue' of the desmosome (Steinberg et al. 1987; Miller et al. 1987; Skerrow et al. 1987). Desmoplakins I and II (DPI and II) are two highly related proteins of ~240 and ~210×10^{3} Mr, that have been localized to the innermost part of the desmosomal plaque (Jones and Goldman, 1985; Miller et al. 1987). Plakoglobin (desmoplakin III) has been localized to the desmosomal plaque but is also found in other symmetrical junctions in addition to desmosomes (Cowin et al. 1986; Gorbsky et al. 1985; Miller et al. 1987). Band 6 protein (desmoplakin IV) is found in the desmosomal plaque of complex and stratified tissue only (Kapprell et al. 1988). D1 antigen (Franke et al. 1987), desmocalmin (Tsukita and Tsukita, 1985) and more recently desmoyokin (Hieda et al. 1989) have also been identified as desmosomal proteins. For reviews see Garrod (1986), Franke et al. (1987), Steinberg et al. (1987) and Green and Jones (1990).

It is thought that DPI and II may play a role in linking
intermediate filaments with the desmosomal complex
(Green and Jones, 1990; Green et al. 1990). The cDNAs
encoding these two highly related proteins differ in a
1.8 kb (kilobase) sequence that is missing in DPII, most
likely due to differential splicing of a longer transcript.
(Green et al. 1988; Green et al. 1990). DPII has been
observed in all desmosomes and can therefore be regarded
as an obligate constituent of desmosomes. In contrast,
certain investigators have reported DPII to have a
restricted distribution among epithelia (Franke et al.
1986; Mueller and Franke, 1985; Giudice et al. 1984; Cowin
et al. 1985b; Moll et al. 1986). Cowin et al. (1986b) have
suggested that DPII may be differentially expressed in
stratified and pseudostratified tissues. As such DPIII might
play an important role in epithelial stratification and/or
differentiation. However, more recently other
investigators have reported DPIII to be present in cells of simple
epithelial origin, questioning the earlier findings (Suhbier
and Garrod, 1986; Pasdar and Nelson, 1988).
The switch from low (~0.04–0.15 mm) to normal calcium
(~1–2 mm) has been widely used to study desmosome
formation and cell differentiation in vitro (Hennings
et al. 1980; Hennings et al. 1983; Hennings and Holbrook, 1983;
Watt et al. 1984; Jones and Goldman, 1985; Mattey and
Garrod, 1986; Penn et al. 1987; Pasdar and Nelson, 1988;
Duden and Franke, 1986; Jones and Grelling, 1989).
Previous results have suggested that primary mouse keratinocytes lack DPIII in the insoluble, cytoskeleton-
associated protein pool of cells maintained in low calcium
(Jones and Goldman, 1985). Madine-Darby canine kidney
(MDCK) cells of simple epithelial origin were found to
express DPI II and II in the soluble and insoluble protein
pools of both low and normal calcium at a ratio of 3:1 to 4:1,
however, with increased stability of these proteins and
recruitment into the insoluble pool in normal calcium
(Pasdar and Nelson, 1988). More recently, normal human
epidermal keratinocytes were found to express only DPI
in culture (Jones and Grelling, 1989).

We have investigated the expression of desmoplakin I
and II on the mRNA and protein level for cells derived
from simple, transitional and stratified epithelia. In
addition, we have correlated these results with the
expression of DP mRNA in simple and stratified tissues.
Finally, we have studied possible changes in expression
of DPI and II on the mRNA and protein level during calcium-
induced desmosome formation and stratification in vitro.

Materials and methods

Cell cultures

FaDu cells (human pharyngeal squamous cell carcinoma, strati-
fied or pseudostratified epithelium) and A431 cells (human
epidermoid carcinoma, vulva) were obtained from Dr Jonathan
Jones and maintained in modified Eagle’s medium (MEM) and
10% fetal calf serum (HyClone Laboratories, Logan, UT) plus
100 units/ml penicillin plus 100 µg/ml streptomycin. SCC-13 cells
(human bladder carcinoma cells, transitional epithelium), ob-
tained from Dr Noel Bouch were maintained in Dulbecco’s
modified Eagle’s medium plus 10% fetal calf serum (HyClone)
plus 100 units/ml penicillin plus 100 µg/ml streptomycin. T24 cells
(human bladder carcinoma cells, transitional epithelium), ob-
tained from Dr Elaine Fuchs, were maintained in calcium-free Dulbecco’s modified
Eagle’s medium plus 10% fetal calf serum treated with Chelex-
100 according to the manufacturer’s directions (Biorad Chemical
Division, Richmond, CA) for 48 h. They were then switched to
Dulbecco’s modified Eagle’s medium plus 10% FCS to induce
desmosome formation and stratification.

Northern blot analysis

RNA was isolated from cell lines and tissues using the single step
method described by Chomczynski and Sacchi (1987). A 20 µg sample of total RNA per lane was then separated on a 1% agarose,
6% formaldehyde gel (Rave et al. 1979). Capillary transfer to
nitrocellulose (Schleicher and Schuell, Keene, NH) was per-
formed in 10× SSC (1× SSC is 0.15 M sodium chloride, 0.015 M
sodium citrate, pH 7.0) for 18–24 h. Alternatively, electrophoresis was performed at 80 °C for 10 min and subsequently at 200 V for 4 h. Blots were baked at 80 °C for 2 h and then probed with 32P-random primer labelled cDNA as described
(Green et al. 1988) using the following hybridization buffer at
42°C: 50% formamide, 5× SSC, 50 mM sodium phosphate, pH 6.5,
1% sodium dodecyl sulfate and 5× Denhardt’s (1× Den-
hardt’s: 0.02% each of Ficoll, bovine serum albumin and
polyvinyl pyrrolidone). After washing each in 2× SSC plus
0.1% sodium dodecyl sulfate and 0.2× SSC plus 0.2% sodium
dodecyl sulfate the filters were exposed to Kodak X-OMAT AR
film in the presence of Dupont Cronex intensifying screens.

Antisera

The mouse monoclonal anti-bovine desmoplakin antisera was obtained from Boehringer Mannheim, West Germany. The rabbit
anti-bovine desmoplakin antibody was kindly provided by Dr
Andrew Staehelin. The rabbit anti-human desmoplakin-trpE
fusion protein antibody was prepared as follows: A 1.8 kb HindIII fragment (3.45–5.19 kb on the map in Fig. 4) found in both human
DPI and II was cloned into pATH 3, a high-expression
vector (Dieckmann and Tzagaloff, 1985). The resulting fusion
protein was prepared as described (Klein et al. 1981) and gel-
purified on a 5% sodium dodecyl sulphate–polyacrylamide gel. Two
rabbits were immunized with the fusion protein gel slice and
subsequently boosted at 2-, 3- and 4-week intervals. The
immunization, boosts and bleedings were all performed by
Haselton Research Products (Denver, PA) and the antisera were
named NW4 and NW6.

Desmosome preparation

Desmosome-enriched fractions were prepared essentially as
described (Jones et al. 1988) with some modifications. In brief,
1 cm² pieces of epithelial and underlying connective tissue from
fresh bovine tongue were incubated in tongue buffer (120 mM
NaCl, 3 mM KCl, 20 mM EDTA, 2 mM dithiothreitol (DTT), 1 mM
phenylmethylsulfonyl fluoride (PMSF), 2 mM TAME (N-tosyl-
L-arginine methyl ester), 5 µg/ml leupeptin, 5 µg/ml pepstatin,
6 mM Na3PO4, pH 7.0) at 4°C overnight. The connective
tissue was pulled off and the finely minced pieces of epithelium
were processed in citric acid buffer (0.1 M citric acid, pH 2.6, plus
0.1% Triton X-100, 1 mM PMSF, 5 µg/ml pepstatin, 5 µg/ml}
Preparation of protein samples and immunoblotting
Whole cell protein and cytoskeletal fractions were prepared as described (Jones and Goldman, 1985). Protein content was determined by the method of Bradford (1976). A 20 µg sample of protein per lane was separated by 5% SDS-PAGE. Complete protein transfer to nitrocellulose (Schleicher and Schuell Inc., Keene NH) was performed in double glycine, double Tris buffer (50 mM Tris, 380 mM glycine, 0.1% sodium dodecyl sulfate, 20% methanol) at 30 V overnight. The blots were washed three times for 15 min in PBS (phosphate-buffered saline) plus 0.05% Tween 20 followed by eight brief rinses in PBS. Immunoblotting as described (Zackroff et al. 1984) was modified as follows: non-specific binding was blocked by incubation for 6–8 h in 5% non-fat dry milk in PBS and sonicated. Large pieces were removed by filtration through a cheesecloth and the supernatant was centrifuged at 10,000 g for 20 min at 4°C. The pellet was washed twice in citric acid buffer, resuspended in 40% sucrose in citric acid buffer, and layered over a sucrose step gradient (50%, 55%, 60% sucrose in citric acid buffer) (Skerraw and Matolsky, 1974). The 55%–60% interface was harvested, washed twice in PBS (phosphate-buffered saline without calcium or magnesium; 6 mM Na+ K+ phosphate, 171 mM NaCl, 3 mM KCl, pH 7.4) and the pellet was either stored at −70°C or resuspended in urea sample buffer (8 M urea, 65 mM Tris–HCl, pH 6.8, 1% sodium dodecyl sulfate, 10% glycerol, 5% 2-mercaptoethanol) and stored at −70°C.

Results

Desmoplakin mRNA expression
Cell lines derived from epithelia ranging from simple to stratified were selected for Northern analysis of DP mRNAs. Because previous reports regarding the presence of DPI in simple epithelia disagree, we were especially interested to determine whether DPI mRNA is present in these cells. Northern blot analysis was performed on RNA isolated from SCC-13 (stratified epithelium, facial epidermis), FaDu (stratified or pseudostratified epithelium, pharynx), A431 (epidermoid carcinoma, vulva), T24 (transitional epithelium, bladder) and HCT 116 (simple epithelium, colon) cells. The HindIII fragment encompassing 3.45–5.19 kb in Fig. 4 (below) was radiolabeled with [32P]dCTP and used as a probe. The DPI mRNA band of 9.5 kb as well as the DPII mRNA band of 7.5 kb (Fig. 1), previously reported for bovine tongue and human foreskin (Green et al. 1988; Green et al. 1990) were present in all cell lines, including A431. A431 cells have been reported to express only DPI at the protein level (Cowan et al. 1995b). Differences in mRNA levels among cell lines could be observed. DPI and II mRNA levels were found to be comparable for SCC-13 and FaDu. A431 cells showed a comparable amount of DPI message but a decreased level of DPII mRNA. Although minor differences in the DP mRNA levels among the HCT 116 cell lines are apparent in this Northern blot, they were reproducibly found to be similar among cell types.

Metabolic labeling and immunoprecipitation
Metabolic labeling and immunoprecipitation were performed as previously described (Nelson and Veshnock, 1986; Pasdar and Nelson, 1985, 1988; Pasdar and Nelson, 1989). Following solubilization and dilution, samples were precleared by incubation with 50 µg Staphyloccocus aureus suspension and 10 µl normal rabbit serum for 45 min. Subsequently the supernatant was incubated with 10 µl anti-desmoplakin fusion protein antibody NWS at 4°C overnight. Preimmune serum was used as a control. A 20 µl sample of a 1:1 suspension of protein A-Sepharose beads in Tris–PMSF was added and incubated for 2 h at room temperature. Samples were washed as described (Pasdar and Nelson, 1989). Antigen was released by boiling the Sepharose beads for 3 min in SDS sample buffer. Samples were then run on a 5% SDS–PAGE gel and the gel was soaked in 1 M sodium salicylate for 1 h. Kodak X-OMAT AR film was used for autoradiography.

Immunofluorescence
Cells on coverslips were washed 10 times in three changes of PBS, fixed for 2 min in −20°C methanol and allowed to air dry. The monoclonal mouse antibody to bovine desmoplakin antibody (Boehringer Mannheim) was diluted 1:10 and rabbit anti-human desmoplakin-trpE fusion protein antibody (NWS) was diluted 1:2000 in PBS and incubated at 37°C for 30 min. Goat anti-rabbit IgG–FITC (Sigma Chemicals, St Louis, MO) and goat anti-rabbit IgG–FITC (Sigma Chemicals, St Louis, MO) were diluted 1:15 in PBS and incubated for 30 min at 37°C. The coverslips were washed extensively in PBS and mounted in Gelvatol containing 100 µg ml−1 1,4-diabicycloc[2.2.2]octane (DABCO). The slides were observed under a Leitz Laborlux D microscope equipped with epifluorescence optics and a Leitz Vario-Orthomat Camera System for photography. Photography was performed with Kodak T-Max 100 film. Films were developed in T-Max developer (Eastman Kodak Company, Rochester NY).

Fig. 1. Northern blot analysis of cells derived from different epithelia: 20 µg total RNA per lane from SCC-13, FaDu, A431, T24 and HCT 116. D-, P and -U cells were separated on a 1% agarose/6% formaldehyde gel, transferred to nitrocellulose and hybridized with the 1.8 kb HindIII fragment specific for DPI and II (3.45–5.19 kb on the map in Fig. 4). On the left-hand side 9.5 kb and 7.5 kb indicate the positions of the DPI and DPII mRNAs. Note that DPI and DPII mRNA are seen in all cell lines.
comparable in three other blots. T24 cells showed the lowest levels of both DPI and II message of all cell lines in this study.

To ensure that DPII expression did not occur simply as a result of adaptation to tissue culture, total RNA was isolated from simple (colon) and stratified (skin) epithelial tissues, as well as from cardiac muscle. In addition, RNA isolated from early passage (second) normal human kidney cells was examined (Fig. 2). Consistent with the results for the cell lines, DPII mRNA was present in human foreskin, rat colon, and normal human kidney cells. However, only DPI mRNA could be detected in cardiac muscle (Fig. 2). The apparently faster migrating DPII mRNA in rat colon was probably not due to an actual difference in molecular weight, since the 28S and 18S ribosomal bands also migrated further on the Northern gel.

Desmoplakin protein expression

DPI and DPII expression at the protein level was studied in each of the cell lines described above, in order to determine whether translation of the DPII mRNA was restricted to cells of stratified origin. Total DP was assessed in whole cell extracts using several different antibodies. Samples of protein (20 μg) from SCC-13 (stratified), FaDu (stratified or pseudostratified), A431 (epitheloid), T24 (transitional) and HCT 116 (simple) cells were separated by 5% SDS–PAGE. First, immunoblotting was performed with a previously characterized rabbit anti-bovine desmoplakin antibody. This experiment revealed DPI to be present in all cell lines (Fig. 3). DPII was detected with certainty in SCC-13 and FaDu cells (stratified) only. However, in cells of simple epithelial origin we observed a faint band at a molecular weight of ~210×10^3 M_r that might be interpreted as DPII (Fig. 3). As described above, DPI and II mRNAs were observed in cells derived from all types of epithelia. Therefore, we wanted to investigate more carefully the possibility that DPII was actually present but not adequately detected by the anti-bovine DPI/II antibody.

Our approach was to generate a human desmoplakin-trpE fusion protein. For this we used a region of the cDNA found in both DPI and II encompassing 3.45–5.19 kb, as shown in Fig. 4. We then produced two rabbit antisera directed against the fusion protein. Immunoblotting analysis demonstrated that both antisera reacted with the 240×10^3 M_r and 210×10^3 M_r bands of DPI and II in a bovine tongue desmosome preparation.

The same whole-cell protein extracts seen in Fig. 3 were then separated by 5% SDS–PAGE and immunoblotted with the fusion protein antibodies (Fig. 5A and B). Again DPI was found in all cell lines but in addition DPII could clearly be detected in each case, with the exception of T24. NW4 was unable to detect DPII and NW6 was barely able to detect this protein, even though the DPII mRNA was clearly present. Other differences in DPI and DPII levels, and in the DPI:DPII ratio, were observed among the cell lines. In particular, A431 had a high DPI:DPII ratio, possibly explaining previous reports that DPII is not expressed in this cell line.

While no contaminating bands were observed with NW4, NW6 co-detected an intense band at a molecular weight of ~80×10^3 M_r in the protein samples of all cell lines. The ~80×10^3 M_r band was absent in the desmosome preparation from bovine tongue and in the cytoskeletal preparations of the human tumor cell lines and was not detected by NW4. It is therefore unlikely to be related to either DPI/II or the cytokeratins. We cannot rule out that its origin might be due to a unique autoimmune reaction of
NW6 or a reaction against an E. coli protein that shares common epitopes with a protein found in whole cell extracts of the human tumor cell lines. However, its equal detection among the different cell lines makes it useful as an internal standard for equal sample loading.

In order to confirm our immunoblotting results SCC-13, FaDu, A431 and HCT 116 P and U cells were subjected to metabolic labeling using \(^{35}\)S-methionine followed by immunoprecipitation with the desmoplakin fusion protein antibody (NW6) as described in Materials and methods. Samples were analyzed on a 5% SDS-polyacrylamide gel. Autoradiography revealed DPI and II to be present in all cell lines (Fig. 6), thus confirming the above immunoblotting results. The more intense DP bands for FaDu cells were due to 4-fold more protein in the original sample.

Are DPI and II both present in the insoluble protein pool?

Previously, DPII was reported to be absent in the insoluble protein pool of primary mouse keratinocytes maintained in low calcium (Jones and Goldman, 1985). In addition, rat bladder tumor cells have been reported to resemble normal cells in low calcium. That is, the more aggressive tumor cell lines exhibited a predominance of cytoplasmic rather than cell surface DP (Green et al. 1989). As discussed below, we also observed a reapportionment of DP to the cytoplasm in some of the human tumor cell lines. We were therefore interested to investigate whether DPII was present in the cytoskeleton-associated, insoluble protein pool. Samples (20 \(\mu\)g) of cytoskeletal protein extract from SCC-13, FaDu, A431, T24 and HCT 116 cells per lane were

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**Fig. 4.** Strategy used to generate the human DP-trpE fusion protein: the 1.8 kb HindIII fragment from base-pair 3450–5190 present in both human DPI and II, indicated by the filled box, was subcloned into pATH 3, a high-expression E. coli vector. The transcription start site and direction of trpE is indicated by the arrow. The region unique to DPI is shown by the open box. Note that this cDNA map represents only a portion of the entire DPI molecule (see Green et al. 1990). Restriction sites are: H, HindIII; P, PstI; B, BamHI; N, Ncol. The resulting trpE-human DP fusion protein was purified and used to immunize rabbits.

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**Fig. 5.** Immunoblotting assessing the presence of DPI and II with the anti-human DP-trpE fusion protein antibodies: 20 \(\mu\)g total cell protein per lane from SCC-13, FaDu, A431, T24, HCT 116 D, HCT 116 P and HCT 116 U cells were separated by 5% SDS-PAGE and transferred to nitrocellulose. (A) NW4; (B) NW6; 240 \(\times 10^3\) M, and 210 \(\times 10^3\) M mark the positions where DPI and DPII occur. Molecular weight markers, represented by dots are from top to bottom: 200 \(\times 10^3\) M, 116 \(\times 10^3\) M, 92 \(\times 10^3\) M, and 66.5 \(\times 10^3\) M.

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**Desmoplakin II expression** 251.
Fig. 6. Immunoprecipitation of DPI and II: one 35 mm culture dish of each SCC-13, FaDu, A431, HCT 116 U and HCT 116 P cells was solubilized and immunoprecipitation performed with the fusion protein antibody, NW6. 240×10^3 M_r and 210×10^3 M_r mark the position where DPI and DPII run. Markers, represented by dots are from top to bottom: 200×10^3 M_r, 116×10^3 M_r, 92×10^3 M_r and 66.5×10^3 M_r.

Fig. 7. Immunoblotting of cytoskeletal fractions: 20 μg cytoskeletal extract protein per lane from SCC-13, FaDu, A431, T24, HCT 116 D, HCT 116 P and HCT 116 U cells were separated by 5 % SDS–PAGE, transferred to nitrocellulose and reacted with NW6. 240×10^3 M_r and 210×10^3 M_r indicate the positions where DPI and DPII run. Markers, represented by dots are from top to bottom: 200×10^3 M_r, 116×10^3 M_r, 92×10^3 M_r and 66.5×10^3 M_r.

analyzed by 5 % SDS–PAGE; subsequently immunoblotting was performed with the anti-human DPI/II fusion protein antibody. As shown in Fig. 7, DPI and DPII were found in the cytoskeletal preparation of all cell lines. Differences in DPI and II levels among the cell lines were found. SCC-13, FaDu and A431 cells showed high levels of DPI and II. T24 cells exhibited somewhat enriched levels of DPI, as compared with total cell protein, but very low levels of DPII that were barely detectable by immunoblotting. In HCT 116 cells DPI and DPII levels were found to decrease in correlation with their differentiation state as designated by Nathan et al. (1990). That is, the cytoskeletal fraction of D cells contained the most DPI/II, whereas the U cells contained the least DPI/II. In most cell lines a minor additional band of ~190×10^3 M_r was co-detected, which we judge to be a breakdown product.

Desmoplakin distribution

While discrete desmoplakin staining at the cell–cell interfaces is thought to indicate integration of DPI and II into fully formed desmosomes in normal cells, cytoplasmic DP staining seen in normal cells in low calcium may represent desmoplakin that is associated with endocytosed vesicles or newly synthesized structures (Jones and Goldman, 1985; Duden and Franke, 1988; Green et al. 1989). As mentioned previously, the amount of cytoplasmic DP staining appeared to be greater in rat bladder tumor cells with invasive or metastatic potential (Green et al. 1989). In order to assess desmoplakin localization in the human tumor cell lines and correlate it to the biochemical findings, indirect immunofluorescence was performed on SCC-13, FaDu, A431, T24 and all three types of HCT 116 cells. As a primary antibody the anti-human DP fusion protein antibody or a mouse monoclonal anti-bovine desmoplakin antibody (Boehringer Mannheim, West Germany) was used. Both antibodies showed the same staining pattern. Various distributions of DPI and II were found (Fig. 8). SCC-13 (8A), FaDu (8B) and A431 cells (8C) showed typical intercellular, as well as punctate cytoplasmic staining. T24 cells (8D) showed sparse punctate and diffuse cytoplasmic staining and rare intercellular staining. HCT 116 D cells in contact (8E) showed discrete typical intercellular staining, but single cells displayed dotty cytoplasmic staining. The HCT 116 P (8F) cells showed intercellular as well as diffuse and dotty cytoplasmic staining, while HCT 116 U (8G) cells showed some intercellular and diffuse cytoplasmic staining.

Desmoplakin I and II expression following calcium-induced desmosome formation and cell differentiation in FaDu cells

A number of changes in the content and stability of the soluble versus the insoluble, cytoskeletal-associated DPI and -II protein pools have been reported to occur during calcium-induced desmosome formation (Jones and Goldman, 1985; Penn et al. 1987; Pasdar and Nelson, 1988). However, possible changes in DPI and II mRNA levels have never been studied or correlated with events occurring at the protein level during this process. Consequently, we studied cumulative levels of DPI and II mRNA, total cell protein and cytoskeletal-associated protein in FaDu cells maintained in low calcium (~0.1 mM) and 1, 3, 6, 24 h and 5 days following the switch to normal calcium levels (~2.0 mM) (Fig. 9). A 32P-random primer labelled cDNA fragment specific for DPI and II was used for Northern blot analysis. Using the 28S RNA band as a loading standard, it was determined that RNA from the 6-h time point represented at most 30 % of that loaded in the other lanes. Taking this into consideration, there did not appear to be a significant change in DPI or DPII mRNA levels following the calcium switch with the possible exception of a small increase after 5 days (Fig. 9A).

Whole-cell protein, as well as cytoskeletal extracts were assessed for DPI and II protein levels by immunoblotting using the human DP fusion protein antibody. Very little change in whole-cell protein was detected (Fig. 9B). In
contrast to previous reports for mouse keratinocytes (Jones and Goldman, 1985), but in accordance with the data for MDCK cells (Pasdar and Nelson, 1988), low levels of both DPI and II were found in FaDu cells maintained in low calcium. As early as one hour after the switch to normal calcium a dramatic increase in cumulative DPI

Fig. 8. Localization of DPI and II in cultured human tumor cell lines with indirect immunofluorescence: DPs were detected with the monoclonal mouse anti-bovine DP antibody. (A) SCC-13, (B) FaDu, (C) A431, (D) T24, (E) HCT 116 D, (F) HCT 116 P, (G) HCT 116 U. Note the sparse staining at cell-cell interface found for HCT 116 U cells as indicated by arrows in G. The boxed area for T24 cells in D furthermore clearly indicates DP staining in dotty structures in the cytoplasm (H). A–G, at the same magnification. Bar, 10 μm.

Desmoplakin II expression 253
and II levels was observed in the detergent-insoluble extracts (Fig. 9C).

Fig. 9. DPI and II mRNA, DPI and II total cell protein, and insoluble DPI and II protein levels, following calcium-induced desmosome formation in FaDu cells: (A) 20 μg total RNA from FaDu cells maintained either in low-calcium medium (LCM) or in normal calcium medium (NCM) for 1 h to 5 days were separated on a 1% agarose/6% formaldehyde gel, electrotransferred and hybridized with an HindIII fragment specific for DPI and DPII. 9.5 kb and 7.5 kb mark the positions where DPI and II occur. (B) 20 μg total cell protein per lane from FaDu cells in low- (LCM) and normal calcium medium (NCM) immunoblotted with NW6. 240×10^3 M_r and 210×10^3 M_r mark the positions where DPI and II occur. (C) 20 μg cytoskeletal extract protein from FaDu cells in low- (LCM) and normal calcium medium (NCM) immunoblotted with NW6. 240×10^3 M_r and 210×10^3 M_r mark the positions for DPI and II. Note the dramatic increase in DPI and DPII levels in the cytoskeletal fraction 1 h after the switch to normal calcium.

In order to ensure that desmosome assembly was occurring under these conditions, indirect immunofluorescence staining with the monoclonal anti-DPI/II antibody was performed. Striking changes in desmoplakin distribution were observed after the switch to normal calcium (Fig. 10). In low calcium medium diffuse and sparse dotty staining was detected. Within 24 h after the switch to normal calcium the typical desmosomal intercellular DP staining was observed.

Discussion

The proposed relationship between DPII expression and epithelial stratification is controversial. Reports of restricted localization (Franke et al. 1982; Giudice et al. 1984; Cowin et al. 1985b; Franke et al. 1987) have been questioned more recently by investigators reporting DPII in simple epithelia (Suhrbier and Garrod, 1986; Pasdar and Nelson, 1988). Furthermore, under culture conditions mimicking the basal cell phenotype, DPII was reported to be absent from the insoluble protein pool of mouse keratinocytes (Jones and Goldman, 1985), and DPII was found to be absent in whole cell protein extracts of human keratinocytes in low or normal calcium (Jones and Grelling, 1989).

A restricted distribution for DPII in cells derived from stratified tissues might reflect an important functional role for DPII during differentiation. For instance, DPII might specifically interact with keratin-containing intermediate filaments that are expressed suprabasally, generating a differentiation-specific desmosome-cytoskeleton link. It is of general importance, therefore, to clarify the expression patterns of DPII among different epithelia.

Because DP nucleic acid probes have recently become available we were able to compare DPII expression at the mRNA as well as protein levels. Northern blot analysis revealed both the 9.5 kb DPI and the 7.5 kb DPII message in all cell lines used in this study, as well as second passage normal human kidney cells. Furthermore, both simple and stratified tissues expressed the DPII mRNA, indicating that adaptation to culture did not result in aberrant expression of this DP gene product.

Whole cell DPI and II proteins were assessed by immunoblotting with a previously characterized anti-bovine desmoplakin and anti-human desmoplakin-fusion protein antibodies. Whereas DPIII was not readily detectable with the bovine antibody, the two anti-human DP-fusion protein antibodies clearly detected DPI and II in simple epithelial cell lines. Because whole-cell protein
samples were prepared by lysis of cells directly on the culture dish in 8 mM urea we feel confident that the lower band of 210×10^5 M_0, observed in our whole-cell extract immunoblots was not a breakdown product and can be regarded as the true DPII band described for bovine muzzle and tongue (Mueller and Franke, 1983; Jones et al. 1988).

The differing results with the polyclonal antibodies might be attributed to differences in antibody avidity (overall strength of an antibody--antigen interaction). The human DP-fusion protein is encoded by a 1.8 kb fragment present in the carboxy terminus of both DPI and II (see Fig. 4) and is therefore missing the DPI-specific portion of the molecule. The proportion of antibody molecules capable of binding to DPII is likely, therefore, to be greater in these antisera than in polyclonal antisera directed against the entire DPI/II proteins. Thus the sensitivity for DPII may be greater in the fusion protein antisera. However, we cannot rule out the possibility that species-specific differences in the anti-bovine and anti-human DP-fusion protein antibodies could be involved, resulting in decreased detectability of DPII. It should be noted that using the commercially available monoclonal antibody we were unable to detect DPII in every case. This result is consistent with a report by Jones and Grelling (1989), who were unable to detect DPII in cultured human keratinocytes using the mouse monoclonal antibody. One possible explanation for this might be that the epitope recognized by this antibody, although present in both DPI and II, is altered in DPII due to conformational differences resulting from the missing DPI-specific region.

Differences in DP levels among cell lines did not appear to be correlated with the complexity of the tissue from which the cells were derived. It did appear, however, that insoluble DPI and II were enriched in cells of stratified origin, that is SCC-13, FaDu and A431. Immunofluorescence indicated that DPs are localized predominantly at cell-cell interfaces in these cell lines, presumably in mature desmosomes. T24 cells exhibited very low levels of whole-cell DPI and II; the insoluble fraction was enriched somewhat in DPI, but DPII levels were barely detectable. It is interesting to note that T24 cells did not show significant intercellular DP staining but dotted and diffuse cytoplasmic staining, similar to normal cells maintained in low calcium. Pasdar and Nelson (1988) have shown for MDCK cells that the half-life of both the soluble and insoluble DPI/II pools in cells kept at low calcium to prohibit desmosome formation is only 8 h. In contrast the half-life of the insoluble DPI/II pool increases to 72 h following calcium-mediated cell contact formation. Therefore, the low levels of accumulated DPI and II in T24 cells may reflect the instability of cytoplasmic DP pools.

The HCT-116 cell series (HCT 116 D, P, U) exhibited a slight but reproducible decrease in whole-cell DPI and II protein levels and a more substantial decrease in insoluble DPI and II protein levels. It is interesting that these decreases correlate with an increase in tumorigenicity in nude mice and saturation density in culture (Brattain et al. 1981; Nathan et al. 1990). As in the case of the rat bladder tumor cells described by Green et al. (1989), an increase in cytoplasmic and a decrease in intercellular DP staining was found for the HCT cells in correlation with their tumorigenicity. This might lead to the speculation that cytoskeletal-associated DPI/II is turned over at a slower rate in the least tumorigenic HCT 116 D cell line, which displays an abundance of desmosomal cell surface DP staining. In contrast, the more tumorigenic HCT 116 U cells showed sparse intercellular immunofluorescence staining but prominent diffuse cytoplasmic staining. The decreased level of DPI/II protein found in HCT 116 U cells might then be a result of more rapid turnover of an unstable soluble pool of DPs. This observation suggests that in HCT 116 cells desmosome assembly or stability is increasingly disturbed with tumorigenicity.

In order to address the question of possible regulatory mechanisms during in vitro desmosome formation we have studied desmoplakin expression on the mRNA and protein level in FaDu cells. Northern and immunoblot analysis revealed no significant changes in the DPI and II mRNA or whole cell protein levels during the day following the switch to normal calcium and only a small increase, if any, after 5 days. In contrast to results reported for the mouse keratinocytes (Jones and Goldman, 1985), DPII was present in the insoluble fraction of FaDu cells maintained in low calcium, similar to what has been described for MDCK cells (Pasdar and Nelson, 1988). In addition an increase in cumulative levels of the detergent-insoluble DPI and II pool was observed as soon as one hour after the switch to normal calcium. Indirect immunofluorescence confirmed the redistribution of DP from a diffuse and dotted cytoplasmic to a typical intercellular desmosomal staining pattern that has been described for mouse and human keratinocytes (Jones and Goldman, 1985; Duden and Franke, 1988; Jones and Grelling, 1989). However, unlike mouse and human keratinocytes FaDu cells maintained in normal calcium exhibited dotted cytoplasmic staining in addition to intercellular staining. These dots may reflect DP associated with endocytosed vesicles or newly synthesized structures in the cytoplasm.

Taken together our results indicate that no major transcriptional or translational changes in DPI and II expression occur in response to a calcium signal alone in FaDu cells. However, pre-synthesized DPI and II are apparently recruited into an insoluble protein pool, as previously reported for MDCK cells (Pasdar and Nelson, 1988). We have observed similar results for adult normal human epithelial keratinocytes in a serum-free culture system (Angst and Green, unpublished data). We cannot rule out the possibility that conditions used in these studies were not optimal for inducing possible differentiation-dependent changes in DP expression. Yuspa et al. (1989) have demonstrated that induction of K1 and K10 mRNA is dependent on a narrow window of calcium concentration, intermediate in level to that used in this study. Likewise, normal human epithelial keratinocytes also seem to possess an optimal calcium concentration at which K1 or K10 show increased mRNA expression (Daniel Hohl, personal communication). A recent review (Watt, 1989) indicates that calcium may be an inadequate signal for terminal differentiation in rat and human keratinocytes, but that other signals like phorbol esters or the restriction of contact to the substratum can induce terminal differentiation. It is to date unknown how these different signals might affect the expression of desmosomal components and their assembly into intact desmosomes.

Our results clearly indicate that DPII is not restricted to stratified epithelia or cells derived from these epithelia. Calcium switch experiments indicate DPI and II to be present on the mRNA and protein level in similar amounts before and after calcium-induced desmosome formation and stratification in FaDu cells. Together, these findings indicate that DPII expression is unlikely to be linked to stratification. DPII, however, could have a functional

Desmoplakin II expression
significance that is yet unknown. DPI is missing a portion of the rod domain that is found in the DPP molecule, and is therefore likely to form less-stable dimers in vivo (Green et al. 1990). Although the functional significance of DPP structure is not yet understood, the possibility that it may play a unique role in desmosome assembly and maintenance may be revealed by further studies utilizing a molecular genetic approach.

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B. D. ANGOT ET AL.


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