

Structure and Assembly of Desmosome Junctions: Biosynthesis, Processing, and Transport of the Major Protein and Glycoprotein Components in Cultured Epithelial Cells

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Abstract. Extracts of metabolically labeled cultured epithelial cells have been analyzed by immunoprecipitation followed by SDS-PAGE, using antisera to the major high molecular mass proteins and glycoproteins (>100 kD) from desmosomes of bovine muzzle epidermis. For nonstratifying cells (Madin-Darby canine kidney [MDCK] and Madin-Darby bovine kidney), and A431 cells that have lost the ability to stratify through transformation, and a stratifying cell type (primary human keratinocytes) apparently similar polypeptides were immunoprecipitated with our antisera. These comprised three glycoproteins (DGI, DGII, and DGIII) and one major nonglycosylated protein (DPI). DPPII, which has already been characterized by others in stratifying tissues, appeared to be absent or present in greatly reduced amounts in the nonstratifying cell types.

The desmosome glycoproteins were further charac-

terized in MDCK cells. Pulse-chase studies showed all three DGs were separate translation products. The two major glycoprotein families (DGI and DGII/III) were both found to be synthesized with co-translational addition of 2-4 high mannose cores later processed into complex type chains. However, they became endo- β -*N*-acetylglucosaminidase H resistant at different times (DGII/III being slower). None of the DGs were found to have O-linked oligosaccharides unlike bovine muzzle DGI. Transport to the cell surface was rapid for all glycoproteins (60-120 min) as demonstrated by the rate at which they became sensitive to trypsin in intact cells. This also indicated that they were exposed at the outer cell surface. DGII/III, but not DGI, underwent a proteolytic processing step, losing 10 kD of carbohydrate-free peptide, during transport to the cell surface suggesting a possible regulatory mechanism in desmosome assembly.

DESMOSOME junctions are found in epithelial, myocardial, and arachnoidal tissues where their morphology is now well characterized, especially in epithelia (McNutt and Weinstein, 1973; Staehelin, 1974). Adhesion is believed to be mediated by extracellular cross-bridging structures that span the 30-nm space between the parallel plasma membranes. Tonofilament bundles terminate near or pass through electron dense submembranous plaques.

Desmosomes are most abundant in tissues prone to mechanical stress; for example the epithelium of skin, uterus, cervix, and bladder (McNutt and Weinstein, 1973) and would seem to assemble during development when strong and stable intercellular adhesion is required (Lentz and Trinkaus, 1971; Ducibella et al., 1975). Desmosome structures from some tissues remain intact during harsh isolation conditions (Skerrow and Matoltsy, 1974; Drochmans et al., 1978; Gorbosky and Steinberg, 1981; Blaschuk et al., 1986), treatment with buffers containing low and high salt, broad pH ranges, denaturing reagents, and detergents (Mueller and Franke, 1983; Franke et al., 1983a).

At present most information on the biochemistry of desmosome components is available for the junctions from bovine muzzle, where desmosomes are particularly abundant. A method has been developed for desmosome isolation from this source in quantity and good purity. This involves use of a pH 2.3 citric acid buffer to disrupt and solubilize the tonofilament network and a nonionic detergent to remove contaminating nonjunctional membrane (Skerrow and Matoltsy, 1974). Such a preparation, when analyzed by SDS-PAGE, shows enrichment of seven major, nonkeratin polypeptides, six of which have now been localized to the desmosome by immunoelectron microscopy (Franke et al., 1983c; Gorbosky et al., 1985; Steinberg et al., 1987). Different systems of nomenclature have been proposed. Here we shall refer to the nonglycosylated desmosomal proteins (DP)¹ in order of descending molecular mass as DPI, DPPII, and DPPIII, and similarly for the desmosome glycoproteins (DG) DGI, DGII, and DGIII. The nonglycosylated proteins have

1. *Abbreviations used in this paper:* endo H, endo- β -*N*-acetylglucosaminidase H; DG, desmosome glycoprotein; DP, desmosome protein; MDBK, Madin Darby bovine kidney.

approximate molecular masses: DPI, 250; DPII, 215; DPIII, 83 kD; and the glycoproteins are likewise: DGI, 150; DGII, 120; and DGIII, 100 kD. In addition a minor calmodulin-binding protein (240 kD), DPIV (75 kD), and a smaller glycosylated component DGIV (22 kD) are reported to copurify with desmosomes (Gorbsky and Steinberg, 1981; Gorbsky et al., 1985; Tsukita and Tsukita, 1985). DPI and DPII show immunological cross-reactivity and have similar isoelectric points, peptide maps, and amino acid composition (Mueller and Franke, 1983; Kapprell et al., 1985). DGI is distinct from DGII and III, which themselves have similar amino acid composition and peptide maps (Cohen et al., 1983; Kapprell et al., 1985).

Although desmosomes from different vertebrate sources are similar in morphology, their biochemical components cannot be assumed to be identical. Antibodies raised against the desmosome components of bovine muzzle epidermis provide powerful reagents to locate desmosomes in a wide variety of cell types and species by immunofluorescence microscopy due to their cross-reactivity (Franke et al., 1981, 1982; Cowin and Garrod, 1983; Franke et al., 1983b; Cowin et al., 1984a; Osborn and Weber, 1985). These studies indicate at least some conservation of epitopes, more extensively with DPs and DGI than with DGII/III. Furthermore some conservation of adhesive mechanism is indicated by mutual desmosome formation between five diverse cell types (Mattey and Garrod, 1985).

Studies on the biochemical composition of desmosomes other than those from epidermis and other stratified epithelia are few due to the problems of detecting such minor cell components. Recent studies on desmosome-enriched preparations from tissues and cultured cells from several species by gel electrophoresis and immunoblotting indicate variations in the spectrum of DPs expressed and in the molecular mass of DGs (Franke et al., 1982; Giudice et al., 1984; Cowin et al., 1985a; Suhrbier and Garrod, 1986).

We have used antisera raised against the major bovine epidermal desmosome components in a sensitive assay, combining metabolic labeling of cell monolayers and immunoprecipitation of labeled components, to detect immunologically cross-reactive components in cultured epithelial cells. We examined Madin Darby canine kidney (MDCK) cells, Madin Darby bovine kidney (MDBK) cells, A431 cells (derived from a human adenocarcinoma of the vulva), and primary human keratinocytes. These cells exemplify simple epithelia (MDCK and MDBK cells), a cell line that grows in culture as a monolayer but was derived from a stratified epithelium (A431 cells), and a cell type that stratifies in culture (keratinocytes). They also encompass three different tissues and species.

Several major desmosome components were found to be common to all four cell types, but the nonstratifying cell types probably lack an additional component, DPII. The components common to all cell types were very similar from one cell type to another in terms of mobility on SDS-PAGE except for some variation in DGII/III. An advantage of our immunoprecipitation assay was that it could be combined with pulse-chase studies to investigate the biosynthesis of the desmosome components in MDCK cells, and to characterize various co- and posttranslational modifications that might contribute to the adhesive mechanism. Preliminary informa-

tion on topology within the plasma membrane of DGI is also reported.

Materials and Methods

Materials

[³⁵S]Methionine (specific activity >800 Ci/mmol), ¹²⁵I-protein A (>30 mCi/mg), D-[6-³H]glucosamine hydrochloride (20–40 Ci/mmol), and horseradish peroxidase-conjugated streptavidin were from Amersham International, Aylesbury, Bucks (UK). Tunicamycin, monensin, protease inhibitors, and biotinylated lectins were from Sigma Chemical Co. Ltd., Poole, Dorset (UK). Endo-β-N-acetyl-glucosaminidase H (endo H) was from Du Pont (UK) Ltd., Stevenage, Herts. Castanospermine was a kind gift of Dr. B. Winchester (Queen Elizabeth College, London).

Preparation of Desmosome Antisera

Desmosomes were prepared from frozen bovine muzzle as described by Mueller and Franke (1983). The major desmosome components (DPI/DPII, DGI, DGII/III) were separated on 3-mm thick SDS-polyacrylamide slab gels for 1,200 Vh (Laemmli, 1970). The desmosome components were located by rapid staining/destaining of a narrow strip of gel removed from each edge with Coomassie Blue and appropriate regions excised from the remaining unfixed gel. Protein was eluted from the gel electrophoretically for 16 h at 50 V into 1 ml of 25 mM Tris, 0.2 M glycine, 0.1% SDS, and the purity confirmed by further analytical SDS-PAGE, and silver staining of the gel (Morrissey, 1981). Each antigen was emulsified with an equal volume of Freund's complete adjuvant and guinea pigs received 100–200 μg of protein subcutaneously. Three booster injections in incomplete Freund's adjuvant were administered; the first after 6–8 wk, then at monthly intervals. The animals were bled 7 d after the final injection.

Peroxidase Blotting Method Using Antisera or Biotinylated Lectins

Desmosome components, resolved on 7.5% acrylamide gels, were transferred to nitrocellulose as in Towbin et al. (1979) for 18 h at 30 V at 4°C, but including 0.05% SDS in the transfer buffer. The efficiency of transfer was checked by staining with Ponceau S (BDH Chemicals Ltd., Poole, Dorset, UK). Nonspecific binding was reduced by a 16-h incubation of the nitrocellulose in Tris-buffered saline (TBS: 150 mM NaCl, 10 mM Tris-HCl pH 7.4) containing 3% (wt/vol) BSA at 4°C. The nitrocellulose was reacted with antisera diluted 1:5,000 or biotinylated lectins at 10 μg/ml in TBS, 0.25% (wt/vol) gelatin, 1% (wt/vol) NP-40, 0.1% (wt/vol) SDS (buffer A) for 2 h at room temperature. Four 20-min washes in buffer A were given. ¹²⁵I-protein A or horseradish peroxidase-conjugated streptavidin were applied at 0.02 μCi/ml or 1:300 respectively in buffer A for 1 h at 20°C. Nitrocellulose was washed again as above. Specifically bound ¹²⁵I-protein A was detected by autoradiography. Specifically bound lectin was visualized by transfer to 50 mM sodium acetate pH 5.0, and addition of 3-amino-9-ethyl carbazole (0.2 mg/ml) and hydrogen peroxide (0.01%) as substrate (Clegg, 1982). The reaction was terminated by washing in TBS.

Cell Cultures

Human keratinocytes prepared from newborn foreskin were kindly provided by Dr. Fiona Watt (Kennedy Institute of Rheumatology, London); MDBK and MDCK cells were obtained from Gibco Ltd., Uxbridge, Middlesex, UK (the latter being the high passage, low resistance type [Richardson et al., 1981]) and A431 cells from Dr. P. Goodfellow (Imperial Cancer Research Fund, London).

Keratinocytes were maintained in three parts Dulbecco's modified MEM to one part Ham's F12 medium supplemented with 10% (vol/vol) FCS (Sera-Lab Ltd., West Sussex, UK), 10 ng/ml epidermal growth factor, 0.5 μg/ml hydrocortisone, 5 μg/ml insulin, 10⁻¹² M cholera toxin, and 1.8 × 10⁻⁴ M adenine. MDCK, MDBK, and A431 cells were passaged twice a week using trypsin-EDTA into MEM containing 10% (vol/vol) FCS, 0.006% (wt/vol) penicillin, 0.01% (wt/vol) streptomycin, and incubated at 37°C in a 5% CO₂ atmosphere. For experiments confluent flasks were divided into 35- or 50-mm plastic dishes in medium described to give just confluent monolayers after 16-h growth to allow recovery from trypsinization.

Immunofluorescence

MDCK cells, grown on glass coverslips, were fixed and permeabilized by treatment with acetone at -20°C for 5 min and stained with guinea pig antiserum at 1:50 for 40 min. Cells were washed extensively between incubations with TBS containing 1% (wt/vol) BSA. Dilutions were made in the same buffer. Cells were mounted in TBS containing 50% (wt/vol) glycerol and 1 mg/ml paraphenylenediamine (Sigma Chemical Co., Ltd.).

Pulse-Chase Experiments

Confluent monolayers of cells were prepared as above in 35- or 50-mm plastic dishes. Before labeling cells were rinsed twice with 2 ml/dish PBS then preincubated for 30 min in MEM lacking methionine. Cells were either labeled for 16 h with medium containing one-tenth the normal level of methionine and 25 $\mu\text{Ci/ml}$ [^{35}S]methionine or pulse labeled with methionine-free medium containing 500-1,000 $\mu\text{Ci/ml}$ for 10 min (1 ml/35-mm or 2 ml/50-mm dishes). After the labeling period the cells were rinsed with PBS as before and chase medium (MEM plus 10 times the normal level of unlabeled methionine) added to dilute out the isotope. Cells were harvested after washing with ice-cold PBS and scraped off the dish into sample buffer (Laemmli, 1970) containing 62.5 mM Tris-HCl, 2% (wt/vol) SDS, 10% (wt/vol) glycerol (pH 6.8) to solubilize them.

When cells were to be trypsinized, the last PBS wash was aspirated and 1 ml of trypsin (0.125% (wt/vol) in 0.01% (wt/vol) EDTA) added at 20°C . This was left until cells began to loosen from the plastic, when they were diluted to 10 ml with MEM containing serum and then scraped off using a pipette tip. Cells were recovered by centrifugation at 200 g for 5 min at 4°C and lysed as above.

In experiments using drugs during labeling, preincubations were performed as follows: tunicamycin 5 $\mu\text{g/ml}$ for 2 h; monensin 10 μM for 15 min; castanospermine 100 $\mu\text{g/ml}$ for 2 h; and leupeptin 500 $\mu\text{g/ml}$ for 30 min. The same drug concentrations were added to pulse and chase media.

Immunoprecipitation

Cell monolayers were either solubilized directly in 200 μl /dish of sample buffer (Laemmli, 1970) or scraped off in 1 ml of ice-cold PBS using a pipette tip, recovered by centrifugation at 8,000 g for 1 min in an MSE Microcentaur microfuge (Scientific Instruments, Crawley, Sussex, UK), and solubilized as above. The viscous solution was repeatedly pipetted up to shear DNA and boiled for 5 min. All subsequent steps were performed at 4°C using ice-cold buffers. Residual insoluble cell material was removed by centrifugation at 10,000 g for 10 min in a microfuge. Aliquots of solubilized cells were diluted to 0.1% (wt/vol) SDS with RIP buffer (20 mM Tris-HCl, 0.15 M NaCl, 1 mM EDTA, 1% NP-40, pH 7.5). Each antiserum or normal guinea pig serum (5-10 μl to give saturating conditions) was incubated with the solubilized cell extract (equivalent to $\sim 5\text{ cm}^2$ of cells) for 1 h with occasional agitation. The antigen-antibody complex was collected by addition of 20 μl of a 1:1 suspension of protein A-Sepharose CL-4B (Pharmacia Ltd., Pharmacia House, Milton, Keynes, UK) in RIP buffer and incubation continued for 2 h with frequent agitation. The Sepharose beads were washed as follows: 1 ml RIP buffer; 1 ml RIP buffer containing 0.5 M NaCl; 1 ml RIP buffer containing 0.5% deoxycholate, 0.1% SDS; 1 ml 100 mM Tris-HCl, pH 6.8. Immunoprecipitated proteins were released by boiling for 5 min in sample buffer containing 100 mM dithiothreitol and analyzed by SDS-PAGE using an acrylamide concentration of 7.5%. Gels were treated for fluorography, dried, and exposed to preflashed Kodak XAR5 film at -70°C for 3 d to 3 wk (Bonner and Laskey, 1974).

For endo H treatment the immunoprecipitated sample was diluted to 0.1% SDS with 0.3 M sodium citrate (pH 5.5). A cocktail of protease inhibitors (1% [vol/vol] Trasylol, 1 mM phenylmethylsulphonyl fluoride, 0.2 mM *N*- α -tosyl-L-lysine chloromethyl ketone) was added together with 10 ng endo H. Samples were incubated for 18 h at 37°C , pH adjusted to 6.8 with Tris, the SDS concentration increased to 2%, and analyzed by SDS-PAGE. Parallel controls were incubated at 37°C with inhibitors only.

Results

Comparison of Desmosome Components in Different Epithelial Cells

The antisera used in this study were raised in guinea pigs to

the major desmosome components from bovine muzzle epidermis. The specificity of each polyclonal antiserum was determined by immunofluorescence microscopy on densely grown monolayers of MDCK cells (Fig. 1). Antiserum raised to DPI/II, DGI, and DGII/III stained cell-cell boundaries in a punctate fashion as reported by others (Franke et al., 1981, 1983b; Cowin and Garrod, 1983; Cowin et al., 1984a, b). Furthermore the specificity was confirmed by immunoblotting on a cytoskeletal preparation from MDCK cells (Fey et al., 1984; Fig. 2, lanes 1 and 3-5) and compared with that seen on isolated desmosomes (Fig. 2, lanes 6-8). The three antisera recognized predominantly DPI and DPII (DPI only in MDCK cells), DGI, and DGII/III, respectively. The molecular masses in bovine muzzle desmosomes and MDCK cells corresponded exactly.

The major protein components of each of the four epithelial cell types examined has been compared with bovine muzzle desmosome proteins in Fig. 3. None of the major proteins were seen to correspond in molecular mass. The proteins and glycoproteins immunoprecipitated by the desmosome antisera from extracts of metabolically labeled epithelial cells are shown in Fig. 4. From all four epithelial cells, anti-DGI antiserum immunoprecipitated a major polypeptide (molecular mass 140 kD) of equivalent electrophoretic mobility (Fig. 4 A, lanes 1-4). This major polypeptide band also comigrated with the Coomassie Blue-stained band of DGI from bovine muzzle epidermis when the two were mixed and analyzed on the same gel (see Fig. 4). In the case of the bovine epidermal DGI however, the band is more diffuse and thought to be a triplet (Cohen et al., 1983; Mueller and Franke, 1983).

Using the anti-DGII/III antiserum we were able to identify polypeptides cross-reactive with bovine muzzle epidermal DGII/III (Fig. 4 A, lanes 5-8). MDCK and MDBK DGII/III were found to have the same electrophoretic mobility as each other (Fig. 4 A, lanes 5 and 7) and to co-migrate with bovine muzzle epidermal DGII/III when mixed and analyzed on the same gel. However, the two human cell types had the same electrophoretic mobility as each other (Fig. 4 A, lanes 6 and 8), but migrated slightly slower than the bovine and canine cell lines.

From human keratinocytes, anti-DPI/II antiserum immunoprecipitated two polypeptides in approximately equimolar amounts and of the same molecular mass as the two polypeptides observed in bovine muzzle epidermis, as shown by mixing the two samples and analyzing them on the same gel (Fig. 4 B, lane 2). From MDCK, MDBK, and A431 cells however, only one major polypeptide equivalent in mobility to DPI was immunoprecipitated (Fig. 4 B, lanes 1, 3, and 4). Several minor polypeptides migrating faster than DPI were observed in variable amounts from experiment to experiment, possibly indicating degradation products of DPI, a small amount of DPII, or other related plaque components. Results of Cowin et al. (1985a) using monoclonal antibodies suggest the first interpretation to be correct.

Carbohydrate Addition and Processing of DGI

Pulse-chase studies and treatment of cells with tunicamycin were used to obtain further information on the carbohydrate chains of the DGs from MDCK cells. In a 10-min pulse, DGI was observed as a single polypeptide (Fig. 5, lane 2) that

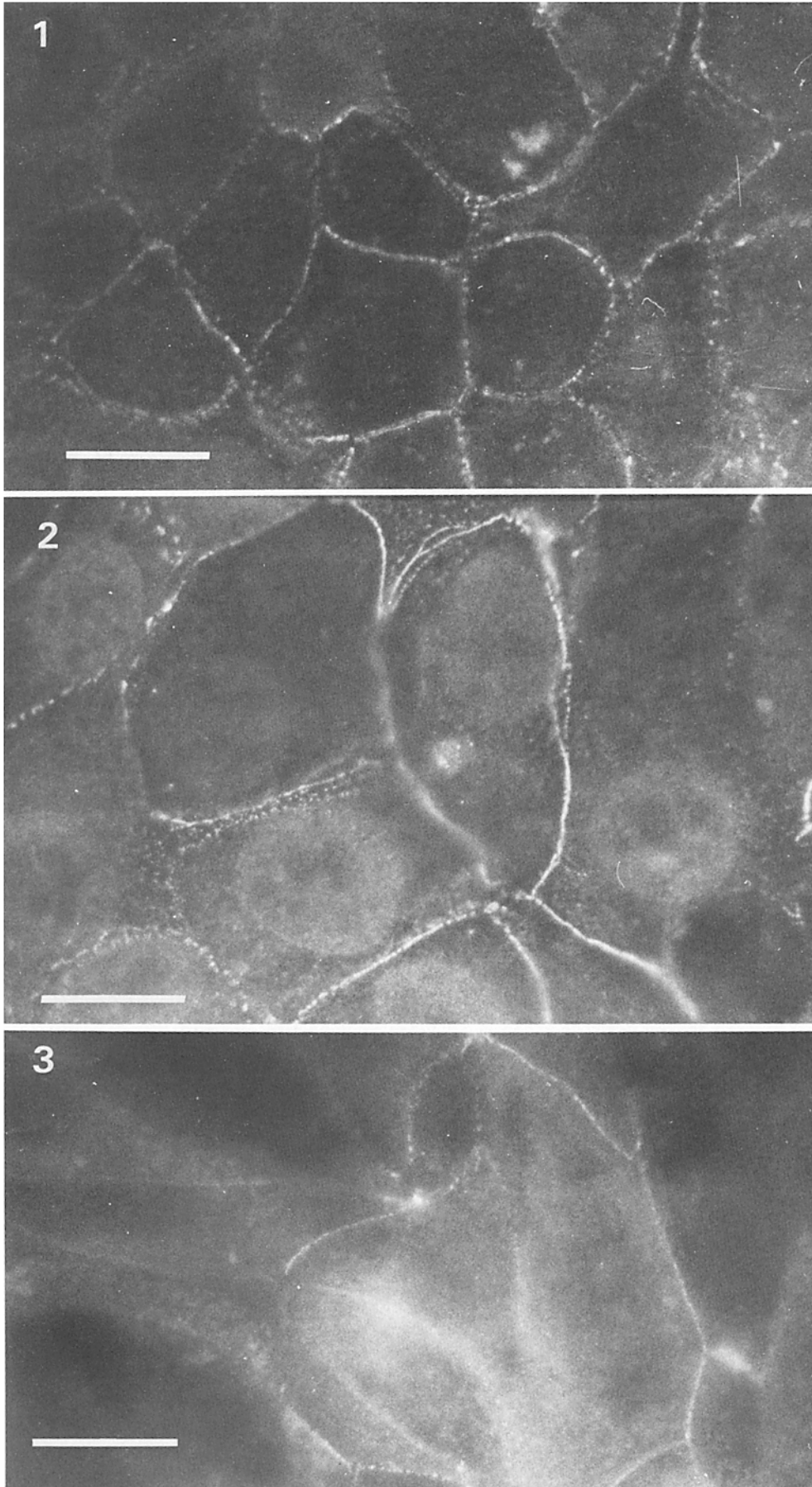


Figure 1. Specificity of desmosome antisera determined by immunofluorescence on MDCK cells. Confluent monolayers of MDCK cells were stained with (1) anti-DP, (2) anti-DGI, and (3) anti-DGII/III antisera as described in Materials and Methods. Bars: (1 and 3) 100 μm ; (2) 50 μm .

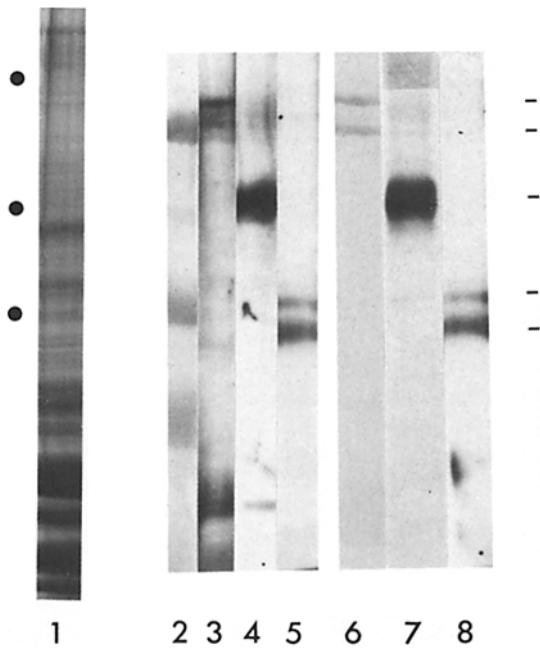


Figure 2. Specificity of desmosome antisera determined by immunoblotting on a cytoskeletal preparation from MDCK cells and bovine epidermal desmosomes. A cytoskeletal fraction was prepared from MDCK cells by removing the soluble material from a confluent monolayer by a 10-min treatment with RIP (20 mM Tris-HCl, 0.15 M NaCl, 1 mM EDTA, 1% NP-40, pH 7.5) buffer (see Materials and Methods) at 4°C, then removing the resultant cytoskeletal fraction with Laemmli sample buffer. Lane 1 shows 100 µg of such a fraction stained with Coomassie Blue (molecular mass markers in descending size are: 200; 116; 92; 68 kD). The cytoskeletal fraction (100 µg; lanes 3–5) and bovine muzzle desmosomes (10 µg; lanes 6–8) were separated by SDS-PAGE and transferred to nitrocellulose as described in Materials and Methods. The antisera used were as follows: anti-DPI/II (lanes 3 and 6); anti-DGI (4 and 7); anti-DGII/III (5 and 8). The position of the desmosome components, determined by staining with Ponceau S, is indicated in descending order: DPI; DPII; DGI; DGII; DGIII. Lane 2 shows molecular mass markers: 200; 92; 68 kD. Occasional reaction of some antisera with keratin polypeptides is seen with immune serum (lane 3) and normal serum (not shown) due to the endogenous anti-keratin activity (Franke et al., 1981).

chased into a slower migrating, more diffuse species by 30 min (Fig. 5, lane 3) equivalent to the band observed in the 16-h continuous label (Fig. 4, lane 1). The slight decrease in mobility of DGI observed in Fig. 5, lanes 3 and 4 probably corresponds to the processing of the high mannose cores, which are believed to be added co-translationally, to complex oligosaccharide chains. This processing appeared to be complete by 30 min.

To examine the glycosylation of DGI the cells were treated with tunicamycin, a specific inhibitor of N-linked glycosylation of asparagine residues, by blocking formation of the high mannose-dolichol phosphate intermediate (Tkacz and Lampen, 1975). Synthesis of DGI in the presence of tunicamycin (Fig. 5, lanes 5–8), reduced the polypeptide molecular mass by 5–10 kD. This shift is consistent with the removal of 2–4 N-linked carbohydrate chains (Owen et al., 1980, 1981; Schwartz and Rup, 1983). The identical mobility of newly synthesized and mature DGI polypeptide chains without N-linked sugars (shown in Fig. 5, lanes 7 and 8) would

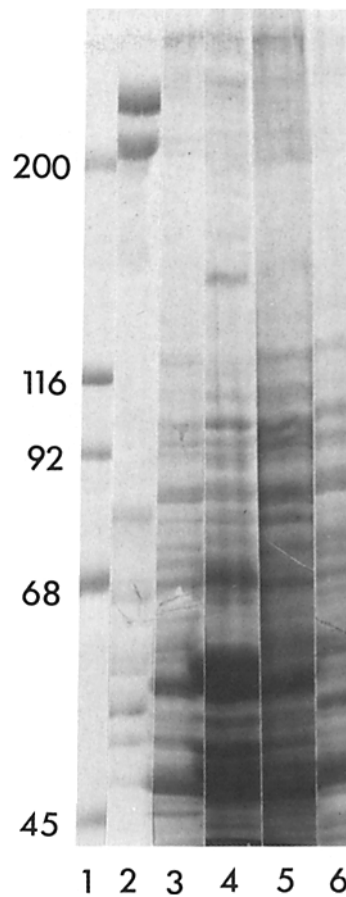


Figure 3. Protein composition of MDCK, MDBK cells, keratinocytes, and A431 cells. Confluent monolayers of MDCK cells (lane 3), keratinocytes (4), MDBK cells (5), and A431 cells (6) were disrupted in Laemmli sample buffer and 100 µg of protein separated by SDS-PAGE and stained with Coomassie Blue. The protein composition is compared with that of bovine muzzle desmosomes (lane 2).

suggest that late processing by addition of O-linked carbohydrate chains probably did not occur, possibly since N-linked chain addition was a prerequisite to such late processing. Identical shifts in the mobility of DGI were observed when MDBK cells, A431 cells, and human keratinocytes were treated with tunicamycin (data not shown), suggesting a similar amount of N-linked glycosylation on each of the DGI polypeptides.

Carbohydrate and Proteolytic Processing of DGII/III

The DGII and III polypeptides synthesized during a 10-min pulse were compared with those detected after a 2-h chase period (Fig. 6 A, lanes 3 and 4). The DGII/III antiserum was often found to immunoprecipitate the DGI polypeptide also (cf. Fig. 6 A, lanes 3 and 4, and Fig. 6 A, lanes 1 and 2, which were immunoprecipitated with the DGI antiserum). This is thought to be due to the guinea pig having been immunized with DGII/III possibly contaminated with breakdown products of DGI that co-migrated with DGII/III (see Suhrbier and Garrod, 1986). This contamination was not detected by the less sensitive immunoblotting technique (Fig. 2) unless a higher concentration of antiserum was used.

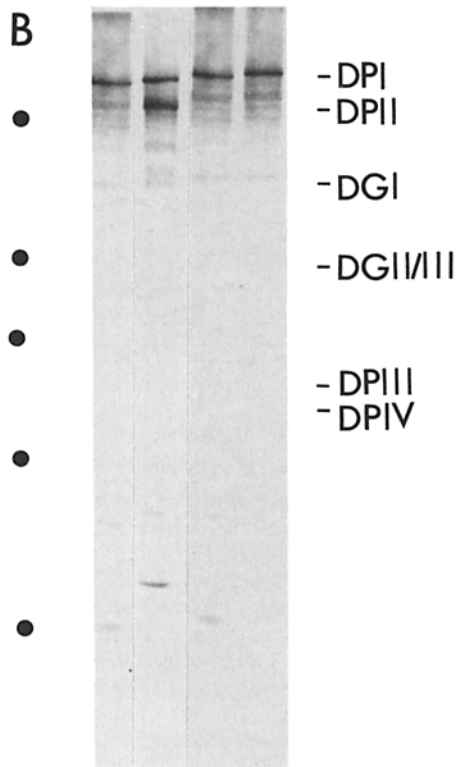
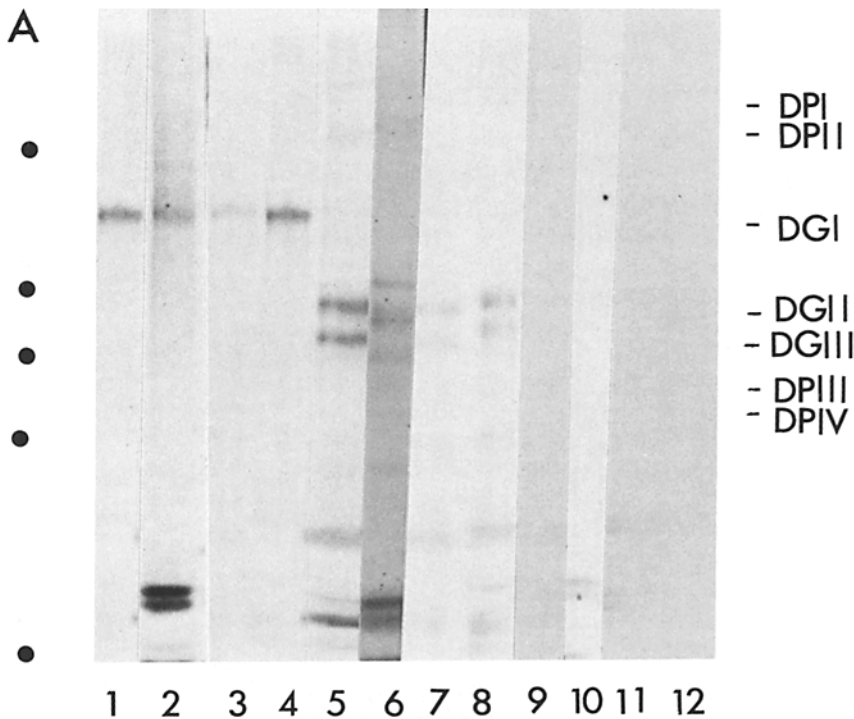


Figure 4. Detection of DPs and DGs in MDCK, MDBK cells, keratinocytes, and A431 cells. (A) A 35-mm dish of each cell type MDCK cells (lanes 1, 5, and 9), human keratinocytes (lanes 2, 6, and 10), MDBK cells (lanes 3, 7, and 11), and A431 cells (lanes 4, 8, and 12) was labeled for 16 h with [³⁵S]methionine as described in Materials and Methods. Immunoprecipitations were performed on cell lysates using anti-DGI (lanes 1-4), anti-DGII/III (lanes 5-8), or normal guinea pig serum (lanes 9-12). Before analysis by SDS-PAGE, 100 μg of a bovine muzzle desmosome preparation was added to the immunoprecipitate and the position of migration of each component was determined by Coomassie Blue staining of the gel before fluorography and is marked. B shows DPI and II components of each cell line in the same order as in A obtained using anti-DPI/II antiserum. Experimental details were as in A. Bands present in the lower portion of the gel are keratin polypeptides nonspecifically precipitated in variable amounts especially from keratinocytes that express large amounts of these proteins. Normal serum also precipitates these bands due to endogenous anti-keratin activity (see Fig. 3).

DGII and III were found to be synthesized concurrently as two discrete polypeptides differing in molecular mass by ~6 kD; thus they are most likely the translation products of two separate mRNA species. As the anti-DGII/III antiserum cannot distinguish DGII and DGIII, we have named the slower and faster migrating band DGII and DGIII, respectively. After a 2-h chase period these two polypeptides had disappeared and two new polypeptides were observed that

most likely arose by removal of 10 kD from each polypeptide, but could have been due to removal of 20 kD from DGII. Antibodies that can distinguish DGII from DGIII would be required to resolve these possibilities. Other post-translational modifications, including processing of the N-linked carbohydrate chains, would also have occurred during this chase period and indeed the processed DGII/III bands were more diffuse. The same proteolytic processing

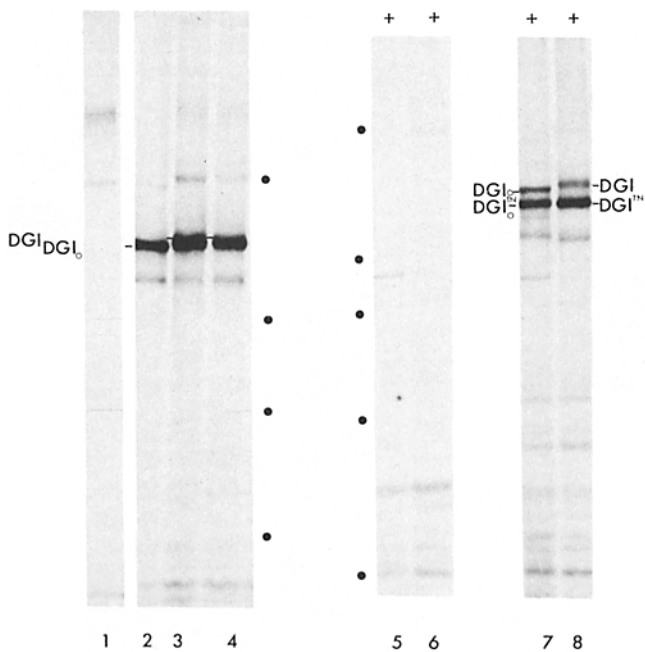


Figure 5. Carbohydrate processing of DGI in MDCK cells. Immunoprecipitates were prepared using anti-DGI antiserum (lanes 2-4, 7, and 8) or normal guinea pig serum (1, 5, and 6) from cells pulse labeled for 10 min then chased for 0 (lanes 1, 2, 5, and 7), 30 (3, 6, and 8), and 60 min (4). Cells were treated with tunicamycin (+) at 5 μ g/ml both in a 2-h preincubation period and during labeling (see Materials and Methods). The precursor form of DGI is marked by the subscript O and the superscript TN refers to bands observed with tunicamycin. In lanes 7 and 8 the tunicamycin effect is only partial. The molecular masses are indicated in descending order: 200; 116; 92; 68; 45 kD.

of DGII/III was observed for MDBK cells, A431 cells, and human keratinocytes (data not shown).

To date we have been unable to inhibit the proteolytic processing of DGII and DGIII. Monensin, an ionophore that blocks protein transport through the Golgi complex (Tartakoff and Vassalli, 1977) was without effect (data not shown) suggesting the event could occur before this point or the enzyme responsible was co-transported with DGII/III through the cell. Leupeptin, an inhibitor of thiol proteases that can enter cells, was also ineffective (data not shown). Neither tunicamycin (see Fig. 6 A, lanes 8 and 9) nor castanospermine (not shown), an inhibitor of glucosidase I, (Sasak et al., 1985) had any effect on proteolytic processing. DGII and III synthesized in the presence of tunicamycin, either in the newly synthesized (Fig. 6 A, lane 8) or proteolytically processed form (Fig. 6 A, lane 9), showed a shift in mobility on SDS-PAGE corresponding to loss of \sim 3 N-linked carbohydrate chains from each. The cleaved polypeptide fragments clearly did not contain the carbohydrate moieties as tunicamycin effected shifts in newly synthesized and mature DGII/III polypeptides in an identical manner. Tunicamycin effected the same shift in electrophoretic mobility of DGII/III from MDBK cells, A431 cells, and human keratinocytes (data not shown).

To determine the rate of proteolytic processing of DGII/III polypeptides in MDCK cells the pulse-chase experiment shown in Fig. 6 B was performed. After a 15-min chase most of the DGII/III remained in the precursor form whereas by

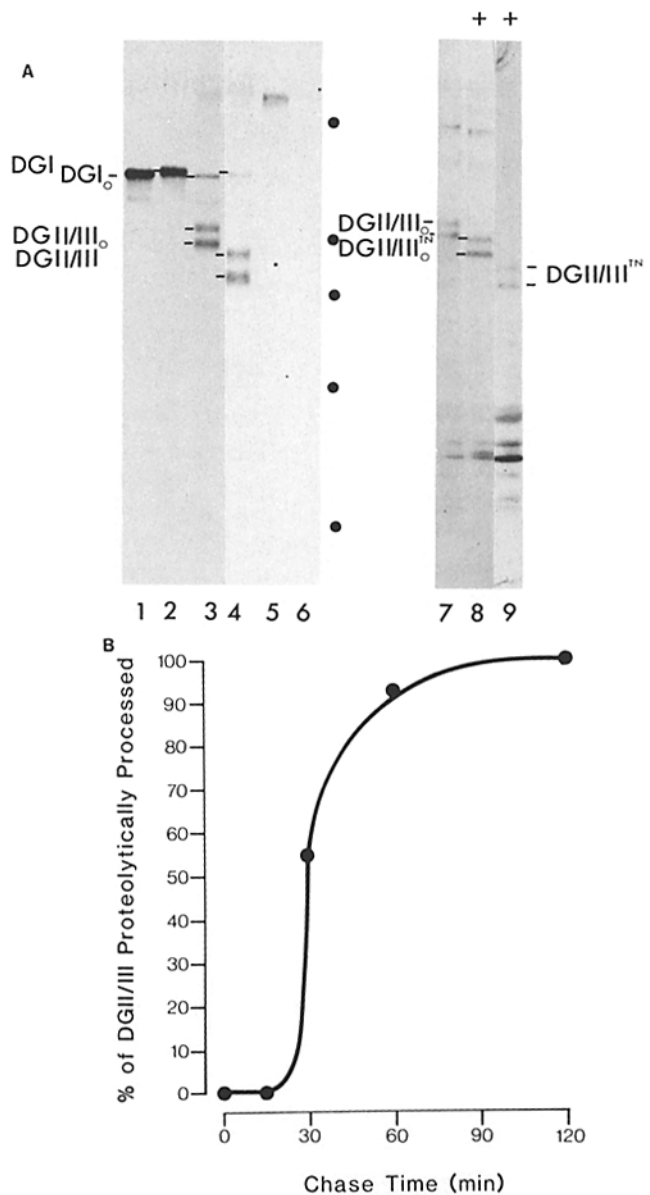


Figure 6. Proteolytic processing of DGII/III in MDCK cells. MDCK cells were pulse labeled with [35 S]methionine for 10 min, and chased for 0 (lanes 1, 3, 5, 7, and 8) or 2 h (lanes 2, 4, 6, and 9) (described in Materials and Methods). Cell lysates were immunoprecipitated with anti-DGI (lanes 1 and 2), anti-DGII/III (lanes 3, 4, 7, 8, and 9), or normal guinea pig serum (5 and 6). Lanes 8 and 9 show the effect of tunicamycin on proteolytic processing (+). Subscript O and the superscript TN are as used in Fig. 5. The rate of proteolytic processing (B) was determined using chase times as shown. Cell lysates were immunoprecipitated with anti-DGII/III antiserum and the percentage of DGII/III in the proteolytically processed form determined by scanning the appropriate region of the fluorograph.

120 min most was proteolytically processed. The half-time for proteolytic processing was \sim 30 min. A similar rate was observed for other cell types (not shown).

Endo H and Alkali Treatment of DGI and DGII/III

Another approach to determine the presence of high mannose cores and also time their processing to complex type

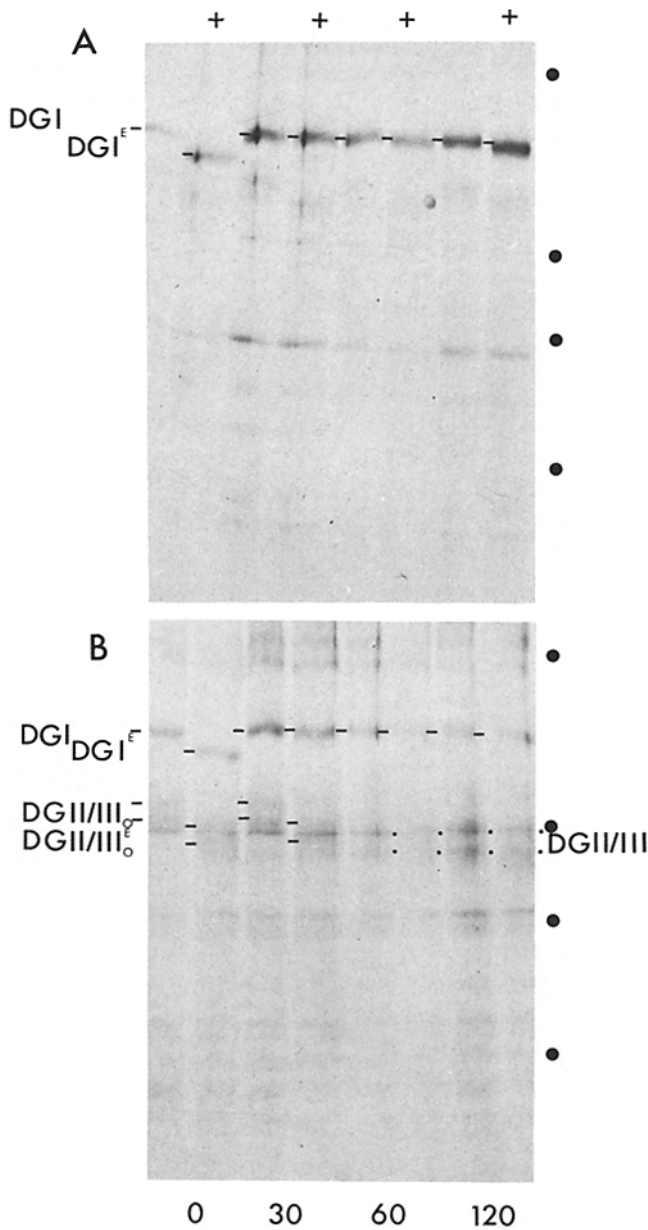


Figure 7. Endo H sensitivity of the DG polypeptides in MDCK cells. Immunoprecipitates were prepared using anti-DGI or anti-DGII/III antisera from cells pulse labeled for 10 min then chased for times indicated in minutes. Each sample was divided; half treated with endo H (+) and half with protease inhibitors alone as described in Materials and Methods. The superscript E refers to bands whose mobility has been altered by endo H treatment. Molecular masses in descending order are: 200; 116; 92; 68 kD.

chains was to investigate the sensitivity of the immunoprecipitated DGs to endo H. This enzyme selectively removes only high mannose cores containing 5–9 mannose residues leaving a single *N*-acetyl glucosamine residue linked to the peptide asparagine (Tarentino and Maley, 1974). The effect (Fig. 7), as determined by polypeptide mobility on SDS-PAGE, was very similar to that produced by tunicamycin (only one *N*-acetyl glucosamine/carbohydrate chain different) confirming the estimation of ~2–4 *N*-linked chains on each desmosome glycoprotein. In both MDCK cells and ker-

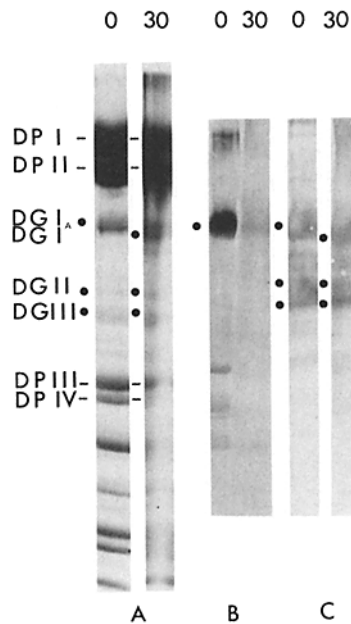


Figure 8. The detection of O-linked oligosaccharide chains on DGI. Lanes A–C show the effect of incubating 50 μ g of whole desmosomes for 0 or 30 min in 0.1 M NaOH at 45°C. Samples were neutralized with HCl and analyzed by SDS-PAGE. (A) Coomassie Blue-stained gel (B and C) shows protein transferred to nitrocellulose and probed with (B) peanut lectin or (C) concanavalin A (see Materials and Methods section). Superscript A indicates DGI minus the O-linked sugars.

atinocytes (not shown) all the desmosome glycoproteins became resistant to endo H treatment, albeit at different rates, implying that in the mature proteins mainly complex type oligosaccharide chains are present (this procedure would not have detected small amounts of high mannose oligosaccharides). In MDCK cells, DGI was found to be endo H resistant within the 30-min chase, DGII and III between 30–60-min chase by which time they were also proteolytically processed.

O-glycosylation is believed to occur in a late Golgi compartment (Johnson and Spear, 1983); however, there are no inhibitors available, as for *N*-linked glycosylation, for this process. The presence of O-linked chains on DGI of bovine muzzle epidermis has been suggested by its staining with the lectin from *Arachis hypogaea* (peanut) (our unpublished data; Cohen et al., 1983) and carbohydrate analysis (Kapprell et al., 1985). We confirmed this by exploiting the sensitivity of O-linked oligosaccharides to alkali. Treatment of bovine epidermal desmosomes in a β -elimination reaction (Fig. 8) shifted the molecular mass of DGI by 5 kD, resulting in a loss of peanut lectin staining (Fig. 8 B), but not concanavalin A staining (Fig. 8 C) indicating that the *N*-linked oligosaccharides were still intact. Treatment of DGI immunoprecipitates from MDCK cells and human keratinocytes in a similar manner was without any effect on the mobility of the [³⁵S]methionine-labeled polypeptides (not shown). Furthermore, labeling MDCK cells or keratinocytes for 6 h with [³H]glucosamine to label both *N*-linked (via *N*-acetyl glucosamine) and O-linked (via conversion to *N*-acetyl galactosamine) chains and using tunicamycin to inhibit the *N*-linked glycosylation, no label could be detected in a polypeptide corresponding in mobility to DGI minus the *N*-linked chains (data not shown).

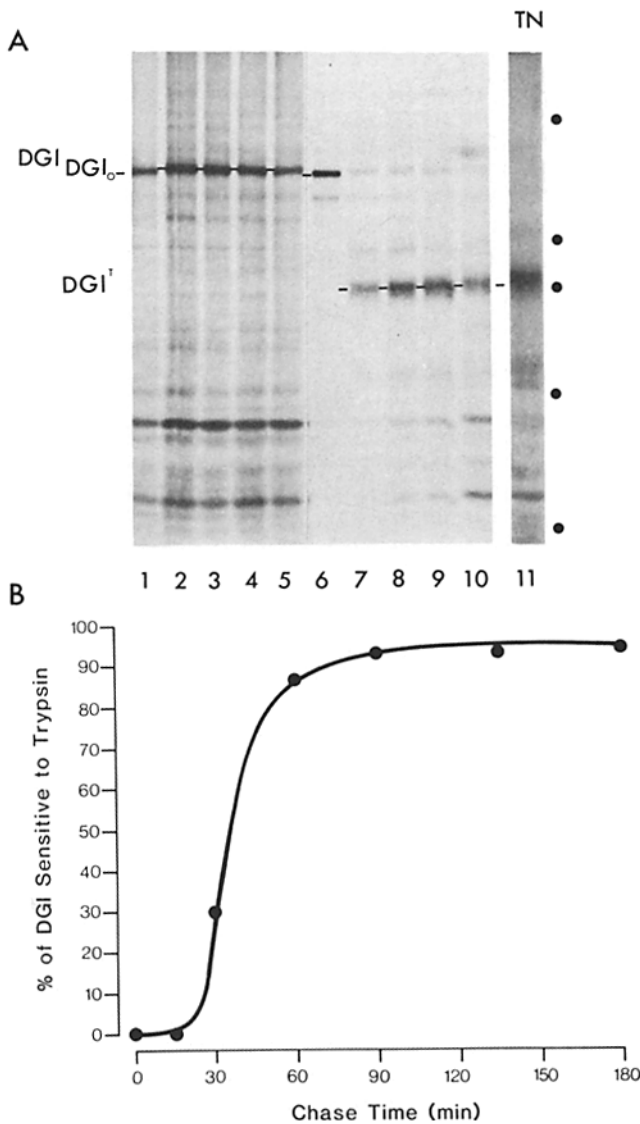


Figure 9. Susceptibility of DGI to trypsin in MDCK cells. (A) Duplicate 35-mm dishes of MDCK cells were labeled for 10 min then chased for 0 (lanes 1 and 6), 1 (2 and 7), 1.5 (3 and 8), 2 (4 and 9), and 3 h (5 and 10). Half were trypsinized (lanes 6–11) as described in Materials and Methods. In the lane marked TN the cells had been treated with tunicamycin as well as trypsinized. Lysates were immunoprecipitated with anti-DGI antiserum. Trypsinized polypeptides are indicated by the superscript T. The dots correspond to molecular mass markers from top to bottom as follows: 200; 116; 92; 68; 45 kD. To determine the rate of transport of DGI to the cell surface (B) MDCK cells were labeled as above and chased for the indicated times before trypsinization. The percentage of DGI remaining was determined by scanning the fluorograph.

Appearance of DGI on the Cell Surface in MDCK Cells

The appearance of DGI on the cell surface was studied using the pulse-chase procedure on duplicate plates of cells and one plate of each pair was trypsinized (Fig. 9 A). The portion of each polypeptide protected by the plasma membrane was then determined by immunoprecipitation and SDS-PAGE. Digestion of cell surface DGI resulted in loss of the 140-kD polypeptide and appearance of several smaller polypeptides, the faster migrating of which was interpreted to represent the

maximum extent of the trypsinization with slower migrating bands most likely being the result of partial digestion at several external susceptible sites (Fig. 9 A). The trypsinization procedure did not render the cells leaky as demonstrated by the failure of trypsin to attack newly synthesized DGI (0-h chase; Fig. 9 A, lane 6). The minimum molecular mass band consistently observed was ~92 kD giving a maximum estimate for the size of the protected domain with up to 50 kD exposed on the outside. From the tunicamycin data only 5–10 kD of this would be attributed to carbohydrate structure. Similar experiments monitoring trypsinization of cell surface DGII and III were difficult to interpret due to the antiserum recognizing the tryptic fragments of DGI also, though the DGII/III polypeptides clearly disappeared on treatment with trypsin though at a slower rate than for DGI (not shown). These results suggest that the desmosome glycoprotein polypeptides have at least some portion exposed on the cell surface. The timing of the transport of DGI to the cell surface was measured by monitoring the disappearance of the DGI polypeptide with increasing chase times (Fig. 9 B). The half-time for DGI transport to the cell surface was ~40 min.

Tunicamycin treatment of MDCK cells was not found to inhibit the susceptibility of DGI to trypsinization indicating that glycosylation is not required for transport to the cell surface (Fig. 9 A, lane 11).

Discussion

Using antisera raised against the major desmosome proteins and glycoproteins of bovine muzzle epidermis we were able to identify cross-reacting components in a number of cultured epithelial cell types. Although the components in isolated bovine muzzle desmosomes appear to be firmly locked into the junction, in cultured epithelial cells and tissues there is evidence that desmosomes may be more dynamic structures being assembled and dispersed (Dulbecco et al., 1984).

Using a combination of metabolic labeling of cells and immunoprecipitation techniques, we found DPI to be common to all epithelial desmosome-forming systems as already reported by others (Giudice et al., 1984; Cowin et al., 1985a; Suhrbier and Garrod, 1986). DPII however, appeared to either be absent from nonstratifying cell types or present in greatly reduced amounts. We found DGI in MDCK, MDBK, A431 cells, and human keratinocytes to be indistinguishable in molecular mass on SDS-PAGE, number of N-linked carbohydrate chains, and immunological cross-reactivity. DGII/III, by contrast, showed some variation in molecular mass on SDS-PAGE between the human cell types and the others, but no variation in the number of N-linked chains could be detected. Preliminary data on other nonstratifying cell lines (not shown) suggested that the same was true for a human hepatoma line (HepG2), a *Xenopus laevis* line (XL2), and an SV-40-transformed keratinocyte line (SVK 14). Primary keratinocytes differed in that they expressed the additional component DPII. In this they more closely resembled the desmosomes from bovine muzzle epidermis which also have DPII.

Our results using DPI/II antiserum are in agreement with those of Cowin et al. (1985a) who used a monoclonal antibody to DPI/II. Another group has failed to detect DPII in stratified tissues other than epidermis (Giudice et al., 1984)

and one has reported the presence of D_{PII} in MDCK and MDBK cells (Suhriebier and Garrod, 1986). These discrepancies may have arisen from the necessity of preparing desmosome enriched fractions during which D_{PII} might be selectively lost or D_{PI} become degraded to peptides of similar mobility to D_{PII}. The multiple faint bands we observed migrating slightly faster than D_{PI} could well be due to proteolysis because even though the cells were harvested directly into SDS-containing buffer to minimize proteolysis, the subsequent immunoprecipitation reaction was performed with the SDS concentration reduced to 0.1%. Similar results were obtained with immunoblotting on MDCK cells (Fig. 2). A monoclonal antibody capable of recognizing D_{PI} only would allow these discrepancies to be resolved. The advantage of this technique over immunoblotting is that greater amounts of cell protein can be present in immunoprecipitation reactions than can be separated by SDS-PAGE for blotting even when desmosome-enriched fractions are used, resulting in high sensitivity and resolution.

Certain desmosome components may be restricted to stratifying cell types, e.g., D_{PII} and a 140-kD glycoprotein (Franke et al., 1982; Jones et al., 1986). Indeed Cowin et al. (1985a) have proposed that D_{PII} is under cell type regulation related to the stratification process. In support of this we have found that an SV-40-transformed keratinocyte line (SVK 14) that fails to stratify in culture (Taylor-Papadimitriou et al., 1982) expresses mainly D_{PI} (data not shown).

Suhriebier and Garrod (1986), using immunoblotting, observed major D_{GI}-related bands of molecular mass >200 kD while we did not. We have confirmed our result by preparation of a cytoskeletal fraction from MDCK cells and immunoblotting (Fig. 2). Other groups, however, have identified a D_{GI} polypeptide of similar molecular mass (140 kD) in a wide variety of different species and tissues. Only in bovine muzzle epidermis does D_{GI} appear to be a triplet (Cohen et al., 1983; Mueller and Franke, 1983). Using monoclonal antibodies, both highly conserved (Schmelz et al., 1986) and tissue specific epitopes (Giudice et al., 1984) have been identified. Whether the differences are at the amino acid sequence level or in glycosylation is not yet clear.

D_{GII} and III, by contrast, appear to exhibit far more tissue and species variation. Giudice et al. (1984) found that out of four monoclonal antibodies raised to the bovine muzzle D_{GII/III} only one was able to recognize cross-reactive polypeptides in cornea and esophagus by immunoblotting. Furthermore, Cowin et al. (1985b) found that none of the monoclonal antibodies they raised to the bovine muzzle D_{GII/III} would recognize D_{GII/III} in simple epithelia. However, D_{GII/III} have been detected in simple epithelia using polyclonal antisera by immunofluorescence (Cowin et al., 1983; 1984a, b) and immunoblotting (Suhriebier and Garrod, 1986). Since these have been proposed to be the adhesive molecules (Cowin et al., 1984b) their variability may relate to tissue specific modulation of adhesion.

The major desmosome glycoprotein families appear to be synthesized in the same manner as reported for other membrane glycoproteins (for review see Kornfeld and Kornfeld, 1985). Two to four high mannose cores are added rapidly to newly synthesized D_G polypeptides probably co-translationally in the rough endoplasmic reticulum (Hubbard and Ivatt, 1981). All high mannose chains appear to be processed into complex type chains in the Golgi apparatus as determined by

loss of sensitivity to endo H (Tabas and Kornfeld, 1978; Bretz et al., 1980; Bennett and O'Shaughnessy, 1981; Roth and Berger, 1982). This step in the pathway is complete by 30 min for D_{GI} and 60 min for D_{GII/III} in MDCK cells, leading to the interesting conclusion that the two families of D_G move through the cell at different rates. So far, we have no evidence for the addition of O-linked oligosaccharide chains to D_{GI} in the various cell types examined here and this may indicate a difference to the bovine epidermal D_{GI} that does appear to have O-linked sugars (see Results). We have initiated detailed analysis of the carbohydrate moieties that will be required to confirm this and elucidate the structure of N-linked chains present. Other late processing events that can modify membrane glycoproteins include sulphation (Young, 1973) and fatty acylation (Magee and Schlesinger, 1982). We have been unable to detect the incorporation of ³⁵S-sulphate, [³H]-myristic acid, or [³H]-palmitic acid into desmosome components (results not shown). The major desmosome components have however, been shown to be phosphorylated (Mueller and Franke, 1983; Magee, A. I., unpublished data), a posttranslational modification found to modulate enzyme activity (Cohen, 1982) and protein-protein interaction as in intermediate filament assembly (Lazarides, 1980).

The desmosome glycoproteins move from the Golgi to the cell surface within 1 h for D_{GI} and possibly slightly longer for D_{GII/III}; these are among the fastest rates observed for membrane glycoproteins, similar to erythrocyte anion transport protein (Braell and Lodish, 1981), 5'-nucleotidase (Wada et al., 1986), HLA-A and -B antigens (Owen et al., 1980), and low density lipoprotein receptor (Tolleshaug et al., 1982). By contrast some glycoproteins take several times as long (insulin receptor, [Salzman et al., 1984]; HLA-DR antigen [Owen et al., 1981]). Treatment of cells with tunicamycin does not prevent expression of the D_Gs at the cell surface as is also observed for HLA-A and -B antigens (Owen et al., 1980). However, this effect is not universal; vesicular stomatitis virus G protein although correctly inserted as a transmembrane protein (Rothman et al., 1978) is not expressed at the cell surface unless glycosylated (Leavitt et al., 1977). Whether D_Gs lacking the correctly glycosylated residues can assemble into functional desmosomes is not entirely clear. Overton (1982) has found that the frequency of desmosome formation in aggregating chick corneal cells is reduced by tunicamycin. However, since this effect is reversed by leupeptin the major role of glycosylation may be to stabilize the polypeptides against proteolytic attack especially since removal of carbohydrate substituents may facilitate proteolysis as suggested for other proteins (Olden et al., 1978; Schwarz et al., 1976).

The presence of substantial amounts of desmosome glycoprotein polypeptide as well as carbohydrate moieties on the cell surface agrees with electron microscopic immunolocalization studies (Steinberg et al., 1987). An adhesive function has been proposed for D_{GII} and/or III; antiserum recognizing D_{GII/III} stains the surface of MDBK cells without permeabilization. Moreover, culturing such cells in the presence of Fab fragments from these antibodies inhibits desmosome formation (Cowin et al., 1984b). Failure of D_{GI} antiserum to stain the surface of living cells, which was at first interpreted as a lack of D_{GI} at the cell surface, probably reflects a problem of accessibility since our studies show that as much as 50 kD or more may be externally exposed. This

is also consistent with recent results from immunoelectron microscopy on tissue sections (Steinberg et al., 1987).

The role of the late proteolytic processing of DGII/III remains unclear. The observation that the two components appear to be processed similarly and in parallel confirms the close relationship previously apparent from their cross-reactivity with monoclonal antibodies and peptide maps (Cohen et al., 1983); our pulse-chase experiments show quite clearly that they are separate translation products. Such proteolytic processing has been reported for a number of membrane glycoproteins such as insulin receptor (Hedo et al., 1983), rat intestinal sucrase-isomaltase (Hauri et al., 1979), and the glycoproteins of several viruses including influenza (Bosch et al., 1981), Newcastle disease virus (Kohama et al., 1981), and Semliki forest virus (Garoff et al., 1980). In the case of the virus glycoproteins the proteolytic processing is critically timed and essential to the assembly process. With influenza, strain-specific differences in the susceptibility of haemagglutinin to cleavage account for differences in host range and pathogenicity (Bosch et al., 1981). In these examples the pattern of cleavage observed appears to be similar to that seen for the prohormones, namely at sites occupied by two successive basic amino acids using trypsin and carboxypeptidase B-like enzymes (Docherty and Steiner, 1982; Garten et al., 1981). It is possible that the proteolytic cleavage of the desmosome proglycoprotein has a role in desmosome assembly, allowing correct interaction of the components during their transport to the cell surface.

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