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Isolation of the non-glycosylated proteins of desmosomes and immunolocalization of a third plaque protein: Desmoplakin III

(macula adhaerens/immunoelectron microscopy)

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ABSTRACT **The cytoplasmic plaque of the spot desmosome or macula adhaerens mediates the attachment of bundles of intermediate filaments to the plasma membrane. We have isolated from a bovine epidermal desmosome preparation a fraction that is highly enriched in the non-glycosylated desmosomal proteins. Plastic-embedded and thin-sectioned high-speed pellets of this fraction reveal closely packed filaments that resemble plaque regions of the low pH whole desmosome preparation from which they are derived. NaDodSO$_4$/polyacrylamide gel electrophoresis reveals four major, non-glycosylated proteins of 240, 210, 81, and 77 kDa. In agreement with a previous study, we find the 240- and 210-kDa proteins (desmoplakins I and II) to be closely related, whereas the 81- and 77-kDa proteins are unique. This is shown both immunologically and by one-dimensional proteolytic peptide mapping. Monospecific, polyclonal rabbit antibodies were prepared against the 81-kDa protein and used, in conjunction with protein A-complexed colloidal gold particles (PAG), to immunolocalize this antigen on ultrathin sections of bovine muzzle epidermis. On antibody-labeled sections, PAG particles were associated principally with the desmosomal cytoplasmic plaque. Sections exposed to preimmune serum showed little or no labeling. We conclude that the 81-kDa protein, like the 240/210-kDa protein family, is one of the major components of the desmosomal plaque. We designate it as "desmoplakin III." The location of the 77-kDa protein remains to be definitively established.**

Because of its well-defined ultrastructure, the spot desmosome provides a useful system in which to study the attachment of intermediate filaments to the plasma membrane. The intermediate filament cytoskeleton of epithelial cells is linked to the desmosomal plasma membrane by the desmosomal cytoplasmic plaque (1). The plaque appears in thin sections as an electron-dense layer adjacent to the cytoplasmic surface of the plasma membrane, often separated from it by a thin, electron-lucent zone. Although the substance of the plaque is often too dense to reveal structural details, a number of authors have reported the plaque to contain or consist of fine filaments (2–5). In desmosomes isolated by the low pH procedure, in which the plaque components have been subject to a degree of denaturation, a filamentous plaque substructure is clearly evident (6–8).

The major protein components of the bovine epidermal desmosome have recently been identified and partially characterized. Gorbsky and Steinberg (6), using a modification of the desmosome isolation procedure of Skerrow and Matoltsy (8), have identified 10 major desmosomal protein bands resolved by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (NaDodSO$_4$/PAGE). Six of the major bands are glycosylated, and these have been classified into three glycoprotein "families" based upon immunological relationships and one-dimensional peptide mapping analysis (9). These families have been termed desmoglein I (a triplet, average molecular mass = 150 kDa), desmoglein II (a doublet, 118 and 97 kDa), and desmoglein III (a single band, 22 kDa) (10, 11). The 4 major non-glycosylated proteins have also been analyzed immunologically and biochemically. Two of the proteins that have molecular masses of >200 kDa have been shown to be structurally closely related by both immunological criteria and peptide mapping analysis (7). The other 2 non-glycosylated proteins differ greatly both from the former proteins and from each other as shown by immunoblot labeling patterns, tryptic peptide mapping, isoelectric point comparisons, and biochemical characterization of *in vitro* translation products of bovine epidermal mRNA (7, 12). Estimates of the apparent molecular weights of corresponding desmosomal proteins differ somewhat (see table I in ref. 11). Our current values for the 4 major non-glycosylated proteins of the bovine muzzle epidermal desmosome are 240, 210, 81, and 77 kDa.

We have previously reported the preparation of a desmosomal fraction appearing in the electron microscope to consist almost exclusively of the desmosomal intercellular region or "core." This preparation is greatly enriched in all of the desmosomal glycoproteins (6). We describe here the complementary preparation, highly enriched in the four major non-glycosylated proteins. Mueller and Franke have earlier reported the immunofluorescent localization of antibodies against the >200-kDa protein pair specifically to desmosomes and hemidesmosomes and the electron microscopic localization of these antibodies to the corresponding plaques by a pre-embedding immunoperoxidase technique (7, 13). They have consequently designated these proteins as "desmoplakins (DPs) I and II."

In the present study, we introduce a post-embedding immunoelectron microscopic labeling technique that we have used to localize the 81-kDa protein on ultrathin sections of bovine muzzle epidermis. We show that this protein is restricted to the desmosomal plaque and propose for it the designation "DP III." The location of the 77-kDa protein

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Abbreviations: DP, desmoplakin; PAG, protein A-conjugated gold; PMS, post-metrizamide supernatant.

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remains to be established. Should it too prove to be a plaque protein, we would propose to designate it as "DP IV."

MATERIALS AND METHODS

Isolation of Desmosomal Fractions. Whole desmosomes and desmosomal cores were isolated as described (6) with some modifications. About 20 fresh calf muzzles were washed thoroughly in tap water. The upper 0.2-mm layer of the skin was removed with an electrokeratotome (Storz Instruments, St. Louis, MO) and discarded. The underlying 0.2-mm layer was removed, and about 12–15 g of this tissue was minced with scissors. The pieces were then homogenized by rapid stirring for 3 hr at 4°C in 500 ml of 0.1 M citrate (pH 2.6) buffer containing 0.05% Nonidet P-40 (NP-40) and 5 μg of the protease inhibitors pepstatin and leupeptin per ml (citrate buffer A). After filtration through 51-μm mesh polyester netting to remove large debris, the homogenate was centrifuged at 13,000 × *g* for 20 min in two 250-ml centrifuge bottles in a GSA rotor of a Sorvall RC-5 centrifuge. (All centrifugations are listed as maximal acceleration.) The pellet was resuspended in 80 ml of 0.1 M citrate (pH 2.6) buffer with 0.01% NP-40 and the protease inhibitors (citrate buffer B) and sonicated in a cuphorn of a Heat Systems W-220 F sonicator (Heat System/Ultrasonics, Plainview, NY) at setting 7 for 10 treatments of 15 sec each separated by 10-sec rest intervals. The resulting finely dispersed homogenate was centrifuged at 750 × *g* for 20 min in an SS-34 rotor of the RC-5 centrifuge to pellet large fragments. The supernatant was transferred to four 50-ml polycarbonate tubes, underlayered with 15 ml of 50% sucrose in citrate buffer B per tube, and centrifuged at 24,000 × *g* in an HB-4 swinging bucket rotor for 30 min. Desmosomes collect at the interface, while pigment granules and some contaminating debris are pelleted. The desmosome band was collected, resuspended in ≈80 ml of citrate buffer B, and washed twice by centrifugation at 12,000 × *g* in citrate buffer B. The pellet contains purified whole desmosomes.

About 80% of the whole desmosomes from an isolation were resuspended in 3 ml of citrate buffer B, sonicated briefly (setting 7 for three 10-sec treatments), and applied to six 12-ml 10–50% (wt/vol) linear gradients of metrizamide (grade C; Accurate Chemicals, Hicksville, NY) in citrate buffer B in cellulose nitrate tubes. Gradients were poured using a six-channel gradient maker (Molecular Instruments, Evanston, IL). The tubes were centrifuged at 260,000 × *g* for 3 hr in an SW 41 rotor of a Beckman L5-75 ultracentrifuge. A single dense white band formed in the lower third of each tube. About 3 ml of the gradient from the lower third of each tube, including the white band, was collected by the use of a syringe attached to a short length of Teflon tubing. This material was diluted to 80 ml with citrate buffer B and centrifuged at 22,000 × *g* for 20 min. The desmosomal cores pellet while the remaining elements stay in suspension. The material in the post-metrizamide supernatant (PMS) was pressure-concentrated and dialyzed on a PM-30 filter in a stirred cell apparatus (Amicon) to remove residual metrizamide. The yield averaged 6 mg from 12–15 g of spinous epithelial tissue. Whole desmosomes, cores, and PMS fractions were suspended in small volumes of citrate buffer B and either used immediately or stored frozen at −70°C in small aliquots.

Protein Determinations. Desmosomal fractions and bovine serum albumin standards in citrate buffer B were diluted 1:1 with 0.2% $NaDodSO_4$ in 0.3 M NaOH and processed according to Lowry *et al.* (14).

Gel Electrophoresis. $NaDodSO_4$/PAGE was carried out under reducing conditions using the $NaDodSO_4$/Tris/glycine buffer system of Laemmli (15). Proteins were stained with Coomassie blue.

Proteolytic Peptide Mapping. Partial proteolytic peptide mapping of desmosomal plaque proteins was carried out according to the procedure of Cleveland *et al.* (16) using the modifications described for mapping desmosomal glycoproteins (9). Briefly, gel slices containing iodinated desmosomal plaque proteins were digested with either *Staphylococcus aureus* protease (Miles) or elastase (2× crystallized; Nutritional Biochemicals). The amounts of protease added are indicated in the legend to Fig. 3. Addition of 0.5 μl of 2-mercaptoethanol to each well along with the buffer solution used to overlay the gel slice improved resolution of the digested peptides. Digestion was carried out as the proteins were run through the stacking gel and the resulting peptide fragments were resolved in 10–20% acrylamide gradient gels.

Preparation of Antibodies and Labeling of Nitrocellulose Blots. Monospecific polyclonal (11) and monoclonal (9) antibodies were prepared as described. The specificity of all antibody preparations was demonstrated by the labeling of nitrocellulose blots (Fig. 4 and ref. 11).

Electron Microscopy. Whole desmosomes and PMS fractions were pelleted at 35,000 rpm for 45 min in hemihyperboloid BEEM capsules in a Beckman SW 41 rotor fitted with Epon adapters as described by Goodenough (17). The pellets were processed for electron microscopy as described (6).

Immunocytochemistry. A thin slice of fresh bovine muzzle, consisting mainly of stratum spinosum, was fixed with a solution of 0.2% glutaraldehyde/4% formaldehyde/0.1 M phosphate buffer, pH 7.3 (18), for 1 hr at 4°C. The fixed tissue was dehydrated as usual through an ethanol series and infiltrated with JB-4 solution A containing 0.9% catalyst at 8°C overnight. The infiltrated samples were transferred to a mixture of 20 parts freshly prepared JB-4 A, 1 part divinylbenzene (Polyscience, Warrington, PA), 1 part methyl methacrylate, and 1 part JB-4 B, placed in BEEM capsules, and sealed with paraffin. The resin was polymerized at 8°C for at least 6 hr followed by UV irradiation in a curing chamber (Ladd Research Industries, Burlington, VT) at room temperature overnight.

To prevent the hydrophilic resin from wetting during sectioning, the sides of the block were coated with a thin film of silicone grease. Gold sections were cut at 0.6–0.9 mm/sec with a Porter-Blum MT-2 ultramicrotome using glass knives. The sections were mounted on gold grids. Copper grids were avoided because a precipitate forms on the copper surface during the antibody labeling procedure. Nickel grids can be substituted for the gold grids used here.

The grids containing the ultrathin tissue sections were placed in phosphate-buffered saline (P_i/NaCl) and were then incubated in 0.5% bovine serum albumin (Sigma) in P_i/NaCl for 30 min to block nonspecific binding of protein. The sections were then incubated in the first antibody or preimmune serum diluted in P_i/NaCl containing 0.5% bovine serum albumin. Maximal labeling was obtained with a 6-hr incubation period. After two 15-min washes in P_i/NaCl, the grids were incubated in 0.5% bovine serum albumin in P_i/NaCl and were then labeled for 1 hr with a suspension of protein A-conjugated gold (PAG) particles (diameter = 5–15 nm; kindly supplied by S. Yokota, Dept. of Anatomy, Univ. of Yamanashi Medical School) diluted in Dulbecco's P_i/NaCl prepared without calcium and magnesium salts. The labeled grids were washed twice with P_i/NaCl for 10 min and then with distilled water for 10 min. Finally, the labeled sections were stained with 1% uranyl acetate for 15 min and with Reynolds' lead citrate for 5 min. The samples were examined with a Hitachi H-600 or JEOL 100C transmission electron microscope at an accelerating voltage of 100 kV.

RESULTS

Isolation and Electrophoretic Characterization of Desmosomal Non-glycosylated Proteins. Fig. 1 shows a comparison of the $NaDodSO_4$/polyacrylamide gel profiles of the desmosomal fractions. The whole desmosome fraction contains about 15 proteins. Three immunologically distinct families of glycoproteins are components of the intercellular region of the desmosome and are concentrated in the desmosomal core fraction (6, 9). Four non-glycosylated proteins of apparent molecular masses of 240, 210, 81, and 77 kDa present in whole desmosomes are specifically depleted from desmosomal cores and concentrated in the PMS fraction. Transmission electron microscopy showed that desmosomal cores have lost most of the filamentous plaque material observed in whole desmosomes (figure 3 in ref. 6), suggesting that the depleted non-glycosylated proteins, present in the PMS fraction, may be components of the plaque. The PMS fraction also contains proteins with molecular masses characteristic of actin and the prekeratin components of intermediate filaments (45–68 kDa; refs. 3 and 19).

Ultrastructural Examination of the PMS Fraction. The larger complexes present in the concentrated PMS fraction could be pelleted by high-speed centrifugation and were fixed, embedded, and sectioned. In thin sections the pelleted material shows a convoluted fibrillar organization (Fig. 2*A*) closely resembling that observed in the plaque region of isolated whole desmosomes (Fig. 2*B*). Different regions of the high-speed PMS pellet were ultrastructurally similar when processed for transmission electron microscopy, with the exception that the bottom of the pellet also contained small amounts of core fragments, pieces of membrane, and unidentified amorphous material. Apart from slight relative enrichment of the highest molecular weight proteins, electrophoretic profiles of the high-speed pelleted PMS fraction were identical to those of the original PMS fraction (not shown).

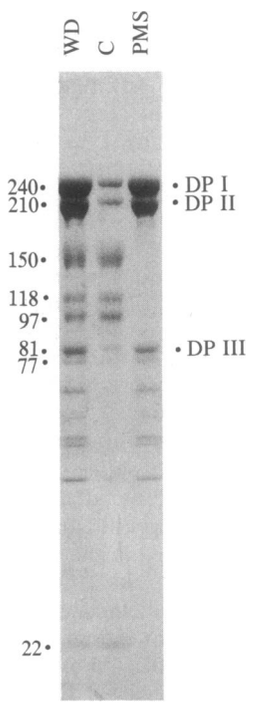

FIG. 1. $NaDodSO_4$ gel electrophoresis of desmosomal fractions in 5–20% polyacrylamide gels. WD, whole desmosomes; C, desmosomal cores; PMS, PMS fractions. Bands at 240, 210, 81, and 77 kDa are highly enriched in the PMS fraction. Also present in both the whole desmosome preparation and its PMS fraction are prekeratins (45–68 kDa) and a protein that comigrates with actin (43 kDa). Lanes containing whole desmosomes and cores were loaded to equalize the concentration of desmosomal glycoprotein. This allows visualization of the relative depletion of non-glycosylated proteins in the desmosomal core fraction.

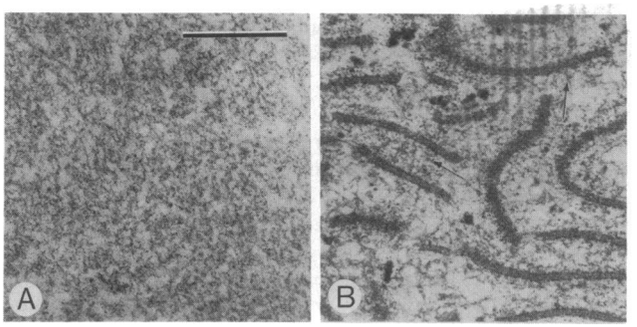

FIG. 2. Transmission electron micrographs of thin sections of desmosomal fractions. (*A*) PMS fraction pelleted by high-speed centrifugation. (*B*) Whole desmosomes. The material of the PMS fraction resembles the fibrillar plaque elements (arrows) that insert into the cytoplasmic surfaces of the plasma membrane of whole desmosomes. (Bar = 0.5 μm.)

Comparison of the Non-glycosylated Desmosomal Proteins. Because our molecular mass estimates for the four major non-glycosylated proteins we detect differ slightly from those of Mueller and Franke (7), and because of the latter authors' experience that quite different proteins may migrate with mobilities close to those of the major desmosomal proteins it is our object to study, we performed these tests as checks to ascertain that our 240/210-kDa protein pair, like their 250/215-kDa pair, are indeed closely similar and that our 81-kDa and 77-kDa proteins, like their 83-kDa and 75-kDa proteins, differed both from the larger proteins and from each other.

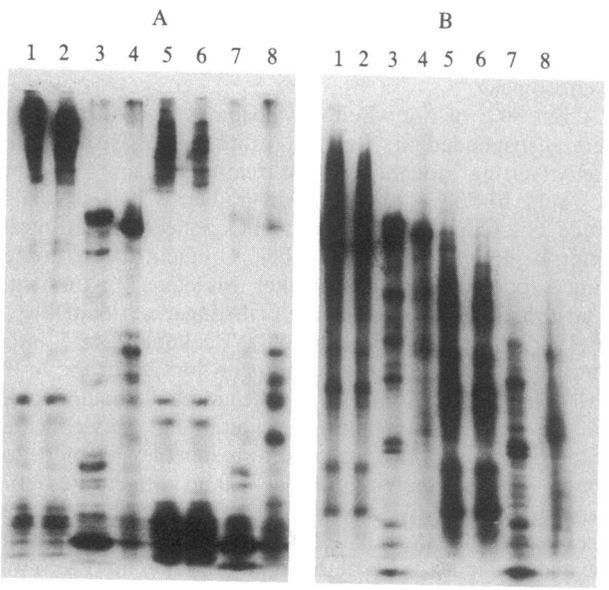

FIG. 3. Partial proteolytic peptide mapping of desmosomal non-glycosylated proteins. Lanes 1–4 and 5–8 contain, respectively, the 240-, 210-, 81-, and 77-kDa non-glycosylated proteins digested with the following amounts of protease per well. (*A*) *S. aureus* protease at 0.01 μg (lanes 1–4) and 0.05 μg (lanes 5–8). (*B*) Elastase at 0.001 μg (lanes 1–4) and 0.003 μg (lanes 5–8). Only minor differences can be detected between the 240- and 210-kDa proteins in the very high molecular mass peptide fragments of the *S. aureus* digest. No difference can be detected between these proteins in the elastase digests. The maps of the 81- and 77-kDa proteins are significantly different from each other with the exception of some very low molecular mass peptides in the *S. aureus* digest. They are also quite different from the maps of the 240- and 210-kDa pair.

Proteolytic peptide mapping of the 240- and 210-kDa desmosomal plaque proteins using both *S. aureus* protease and elastase indicates that these proteins are indeed extremely similar (Fig. 3). Only minor differences in very high molecular mass peptide fragments were detected (compare lanes 5 and 6, Fig. 3*A*). No differences were detected between the 240- and 210-kDa proteins in the elastase digests (Fig. 3*B*). The overall patterns of *S. aureus* protease digestion of the 81- and 77-kDa plaque proteins are quite different. These 2 proteins do not appear to be closely related to each other or to the 240/210-kDa pair. The apparent similarity, in the very low molecular mass *S. aureus* protease peptide fragments of the 81- and 77-kDa proteins does not necessarily indicate a relationship between these proteins.

The results of the peptide mapping were confirmed by immunological comparison of the non-glycosylated proteins. We prepared monospecific polyclonal and monoclonal antibodies that react with the desmosomal plaque proteins. Both polyclonal antibody R_pDPI/II-1 and monoclonal antibody M_mDPI/II-1 react with both the 240- and 210-kDa proteins (Fig. 4, lanes 2 and 3). Another monoclonal antibody, designated M_mDPI-1, reacts only with the 240-kDa protein but not with the 210-kDa protein (Fig. 4, lane 4). Immunoaffinity-purified polyclonal antibody (R_pDPIII-1 reacts exclusively with the 81-kDa protein (Fig. 4, lane 5). These experiments indicate that the 240- and 210-kDa proteins are related but that at least one antigenic determinant present on the former is not present on the latter. They also confirm that the 240/210-kDa pair, the 81-kDa protein, and the 77-kDa protein are three quite distinct protein entities or families.

Immunoelectron Microscopic Localization of the 81-kDa Protein. The monospecific, polyclonal antibody directed against the 81-kDa protein, applied to thin sections of bovine muzzle epidermis and followed with PAG, produced the labeling patterns shown in Fig. 5 *A* and *C*. Not only was labeling confined almost exclusively to the desmosomes but it also was further restricted quite closely to the cytoplasmic plaque regions. Because of the distance between the center of a bound colloidal gold particle and the antigenic site to which it is linked via protein A and immunoglobulin, gold particles are expected to be localized both directly over and adjacent to their antigenic targets. A quantitative treatment of this and other labeling patterns will be presented elsewhere. Preimmune serum showed virtually no labeling in any area of the tissue (Fig. 5*B*).

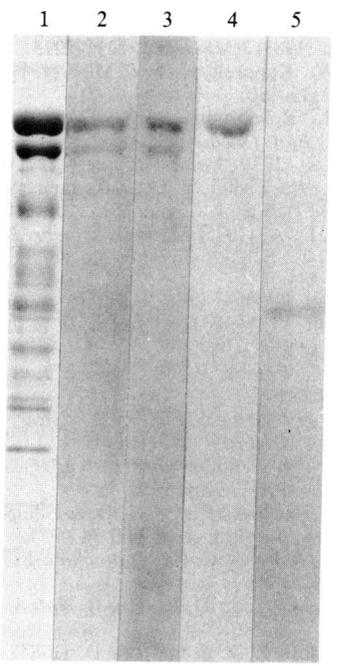

FIG. 4. Immunoblots of desmosomal proteins electrophoresed on 5–20% polyacrylamide gels. Lane 1, Coomassie blue staining pattern of a whole desmosome preparation. Lanes 2–5, immunoblots of the same preparation labeled with the following antibodies: R_pDPI/II-1 (lane 2), M_mDPI/II-1 (lane 3), M_mDPI-1 (lane 4), and R_pDPIII-1 (lane 5). Both DP I and DP II are recognized by both polyclonal antibody R_pDPI/II-1 and monoclonal antibody M_mDPI/II-1 (lanes 2 and 3). Monoclonal antibody M_mDPI-1 reacts with DP I but not with DP II (lane 4). R_pDPIII-1 is a polyclonal antibody that reacts specifically with the 81-kDa protein, DP III.

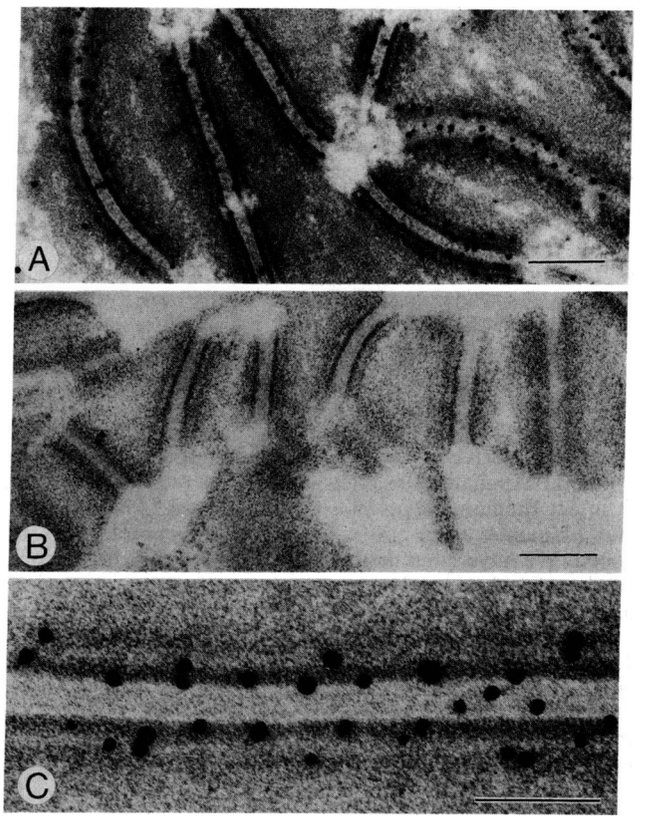

FIG. 5. Immunoelectron microscopic localization of the 81-kDa protein. (*A*) Electron micrograph of bovine muzzle epidermis labeled with a polyclonal antibody directed against the 81-kDa protein (see Fig. 4, lane 5). The PAG particles are almost completely restricted to the desmosomal plaque region. (*C*) The antibody labeling pattern at a higher magnification. Preimmune serum substituted for the primary antibody gives almost no labeling (*B*). (Bars in *A* and *B* = 200 nm; the bar in *C* = 100 nm.)

DISCUSSION

The interaction of intermediate filament systems with components of the cell membrane is a matter of great current interest. The desmosomal cytoplasmic plaque is the membrane-associated attachment zone for intermediate filaments—of the keratin type in epithelial cells (1), of desmin in cardiac muscle cells (20), and of vimentin in certain cells of arachnoidal origin (21). The desmosomal plaque is resistant to solubilization by concentrations of urea as high as 9 M or by inclusion of as much as 300 mM mercaptoethanol in the extraction solutions (7, 13). Nevertheless, all 4 non-glycosylated proteins, 3 of which are now known to be plaque components, are released from the desmosomal plasma membrane during centrifugation after exposure to metrizamide [2-(3-acetamido-5-*N*-methylacetamido-2,4,6-triiodobenzamide)-2-deoxy-D-glucose]. Extraction of whole

desmosomes with metrizamide without centrifugation is much less effective in removing these proteins (data not shown). Metrizamide has been reported to form reversible dense complexes with proteins. For example, it forms a dense complex with catalase with a half-dissociation time of <5 min (22). Similar complexes are formed with α-casein and with bovine serum albumin, the metrizamide comprising up to 40% of the complex (23). Rickwood and Jones (24) have shown that high concentrations of metrizamide cause loosely associated proteins to be released from ribosomes. Metrizamide seems to interact in a similar manner with desmosomes, causing the loosening of the non-glycosylated proteins from the desmosomal plasma membrane. Material of the PMS fraction that was pelleted by high-speed centrifugation and embedded for transmission electron microscopy ultrastructurally resembles plaque regions of whole desmosomes, isolated by the low pH method, from which this fraction is derived. We have not determined whether the isolated PMS material is released from the plasma membrane as intact filaments or as dissociated subunits that assemble into filaments when the metrizamide concentration is reduced. We have been unable to obtain electron microscopic images of this material in the presence of high concentrations of metrizamide.

Based upon biochemical and immunological analyses, the 4 major non-glycosylated desmosomal proteins can be classified into 3 major protein families, which have been named DP I/II, DP III, and, provisionally, DP IV (7, 11). DP III and DP IV differ from each other and from DP I/II, as shown by peptide mapping, absence of immunological cross-reactivity (refs. 7 and 12 and this report), and analysis of *in vitro* translation products of bovine epidermal mRNA (12). Although an antiserum raised by Cowin and Garrod (25) against DP III reacts with both DP III and DP IV, this has presumably resulted from incomplete separation of the two closely spaced antigens and the omission of cross-adsorption of the antiserum against the lower band, a possibility these authors have recognized. Cowin *et al.* have subsequently reported the preparation of a polyclonal antiserum that reacts exclusively with DP III (26).

DPs I and II are very similar, according to both their peptide maps and their cross-reactivity with a rabbit antiserum and a monoclonal antibody. They are not identical, since one appears larger by 30 kDa and possesses at least one antigenic determinant not shared by the other; moreover, DP II also yields some unique peptide fragments detectable on two-dimensional peptide maps (7).

The high molecular mass doublet, DP I/II, has been localized previously to the desmosomal plaque (refs. 7 and 13; unpublished observation). It has been speculated previously that DP III is also a plaque protein (12, 25, 26). However, the only published evidence regarding its localization, prior to the present direct demonstration, came from our earlier study in which it was shown that the metrizamide-centrifugation procedure depleted desmosomal cores of all of the non-glycosylated proteins in the course of removing the plaques. The metrizamide-centrifugation procedure utilized here provides a means for isolating significant quantities of the non-glycosylated proteins of the desmosome. The availability of a procedure for isolating a small group of proteins, one or more of which may serve to mediate the connection between intermediate filaments and the cell membrane, should facilitate the investigation of these interactions.

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