Isolation of the Intercellular Glycoproteins of Desmosomes

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ABSTRACT To characterize the desmosome components that mediate intercellular adhesion and cytoskeletal-plasma membrane attachment, we prepared whole desmosomes and isolated desmosomal intercellular regions (desmosomal "cores") from the living cell layers of bovine muzzle epidermis. The tissue was disrupted in a nonionic detergent at low pH, sonicated, and the insoluble residue fractionated by differential centrifugation and metrizamide gradient centrifugation.

Transmission electron microscopic analyses reveal that a fraction obtained after differential centrifugation is greatly enriched in whole desmosomes that possess intracellular plaques. Metrizamide gradient centrifugation removes most of the plaque material, leaving the intercellular components and the adjoining plasma membranes. Sodium dodecyl sulfate polyacrylamide gel electrophoresis coupled with methods that reveal carbohydrate-containing moieties on gels demonstrate that certain proteins present in whole desmosomes are glycosylated. These glycoproteins are specifically and greatly enriched in the desmosome cores of which they are the principal protein constituents, and thus may function as the intercellular adhesive of the desmosome.

Adhesive interactions among cells and between cells and their substrata are thought to play an important role in governing morphogenetic behavior. In many types of epithelia, desmosomes (*maculae adhaerentes*) and hemidesmosomes serve as organelles specialized to anchor cells to one another and to the basal lamina, respectively (reviewed in references 18, 37).

Although desmosomes are often considered to be relatively permanent cell surface specializations, ultrastructural evidence indicates that they appear or disappear during certain morphogenetic events. This has been observed in embryonic systems such as the expanding chick blastoderm (2, 20) and in teleost blastoderms undergoing epiboly (16). It also has been observed in adult systems undergoing such continuing dynamic processes as the differentiation and migration of cell layers in the epidermis (1, 15), wound healing (13), and some types of malignant transformation (reviewed in reference 37). Furthermore, in vitro studies of Overton (21) and Strickler and Wiseman (35, 38) provide evidence that when different embryonic tissues capable of sorting out and forming desmosomes cooperatively are experimentally combined, the tissue which produces more desmosomes tends to segregate internally to the tissue which produces fewer of them. According to earlier analyses, the more cohesive of two segregating cell populations will tend to assume an internal position (reviewed in references 32 and 33). Thus, desmosomes or their components may mediate morphogenetic recognition and guidance of cells during tissue assembly.

Skerrow and Matoltsy (30, 31) isolated a desmosome preparation from cow nose epidermis and, upon sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) of this material, found some 24 protein bands. More recently, Drochmans et al. (3), using the same tissue, described an alternative procedure for obtaining fractions containing intact desmosomes and tonofilaments. Through modification of Skerrow and Matoltsy's technique, we have developed methods for rapid purification of milligram quantities of desmosome "cores" that consist primarily of the desmosomal intercellular material (which we term the "desmoglea") and associated regions of plasma membrane. These preparations show that the desmosomal intercellular components, which must include the adhesive macromolecules, consist of only a few glycoproteins.

MATERIALS AND METHODS

Isolation of Desmosomes and Cores

For each preparation, ~20 fresh bovine muzzles, obtained from a local slaughterhouse, were washed thoroughly in running tap water. The outer 0.2-mm layer of the epidermis, mainly *stratum corneum*, was removed with an electrokeratotome (Storz Instruments, St. Louis, Mo.) and discarded. The underlying 0.2-mm layer, which consists primarily of live *stratum spinosum*, was then

collected, and ~12–15 g wet weight of the tissue was added to a beaker with 20 ml of pH 2.6, 0.1 M citric acid–sodium citrate buffer containing 0.05% Nonidet P-40 (NP-40) detergent and 5 μg/ml each of pepstatin (Sigma Chemical Co., St. Louis, Mo.) and leupeptin (Peninsula Laboratories, Inc., San Carlos, Calif.), inhibitors of acid proteases (CASC-A). Pepstatin was stored as a 20 mg/ml stock solution in dimethyl sulfoxide. The *stratum spinosum* slices were finely minced with scissors, suspended in 500 ml of CASC-A, and stirred vigorously for 3 h at 4°C. The homogenate was passed through 51-μm mesh polyester netting (Tetko Inc., Elmsford, N. Y.) and the filtrate was then centrifuged at 13,000 *g* for 20 min in two 250-ml centrifuge bottles in a GSA rotor of a Sorvall RC-5 centrifuge (DuPont Instruments-Sorvall, DuPont Co. Newtown, Conn.). The pellet was resuspended in 80 ml of pH 2.6, 0.1 M citric acid–sodium citrate buffer containing 0.01% NP-40 and the protease inhibitors (CASC-B), transferred to a 100-ml beaker, and sonicated in the cuphorn of a Heat Systems W-220F sonicator (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.) at setting 7 for 10 treatments of 15 s each with 10-s rest intervals. The homogenate was centrifuged at 750 *g* for 20 min in two 50-ml polycarbonate tubes in an SS-34 rotor of the RC-5 centrifuge, and the pellet, containing residual *stratum corneum* cells and nuclei, was discarded. The supernate was then spun in the same rotor at 12,000 *g* for 20 min. A trilaminar pellet resulted with desmosomes at the top, pigment in the middle, and large cell debris and nuclei at the bottom. The upper layer was gently resuspended in 80 ml of CASC-B and recentrifuged at 12,000 *g* for 20 min. This process was repeated three more times. The pellet was finally resuspended in 2 ml of CASC-B, sonicated at setting 7 for three periods of 15 s each, applied to four 12-ml linear 10–45% (wt/vol) gradients of metrizamide (Grade C; Accurate Chemical & Scientific Corp., Hicksville, N. Y.) in cellulose nitrate tubes, and spun at 260,000 *g* for 3 h in the SW-41 rotor of a Beckman L5-75 ultracentrifuge (Beckman Instruments, Spinco-Div., Palo Alto, Calif.). A single dense white band formed in the lower third of each tube, and these bands were collected with a syringe attached to a short length of Teflon tubing. The material was suspended in 80 ml of CASC-B and centrifuged at 22,000 *g* for 30 min. The purified cores were washed twice more by resuspension in 80 ml of CASC-B and centrifugation at 22,000 *g* for 20 min.

Core fractions and fractions from other stages of the isolation procedure were used either immediately or stored frozen at −70° in small aliquots in CASC-B. Freezing and thawing do not affect either the ultrastructure observed by electron microscopy or the mobility of the constituent proteins on electrophoretic gels.

Protein Determinations

For protein determination, desmosomal fractions and bovine serum albumin standards in CASC-B were diluted 1:1 with 0.2% SDS in 0.3 N NaOH and processed according to Lowry et al. (17).

Electron Microscopy

For transmission electron microscopy, 0.1–0.2 mg of material was pelleted at 200,000 *g* for 45 min in hemi-hyperboloid BEEM capsules (Better Equipment for Electron Microscopy, Inc., Bronx, N. Y.) in a Beckman SW-41 rotor fitted with adaptors made from Epon according to the method of Goodenough (6). The pellets or intact *stratum spinosum* pieces were fixed for 1 h at room temperature in 2.5% glutaraldehyde buffered with 0.1 M cacodylate at pH 7.2. Postfixation was done in buffered 1% OsO_4 on ice for 30 min followed by *en bloc* staining with 2% aqueous uranyl acetate at room temperature for 1 h. Samples were dehydrated in a graded series of alcohols and embedded in Spurr's resin. Silver sections from various areas of the blocks were taken with glass or diamond knives, stained with uranyl acetate and lead citrate, and examined on a JEOL 100C transmission electron microscope.

Gel Electrophoresis

SDS PAGE was carried out under reducing conditions in gradient gels according to the general SDS–Tris glycine system of Laemmli (14). Proteins were stained with Coomassie Blue or, to detect carbohydrate, with periodic acid–dansyl hydrazine (PADH), according to the technique of Eckhardt et al. (4).

RESULTS

The living cell layers of bovine muzzle epidermis provide an ideal tissue for the purification and fractionation of desmosomes. Transmission electron microscopy shows that this tissue contains well developed desmosomes in great abundance (Fig. 1).

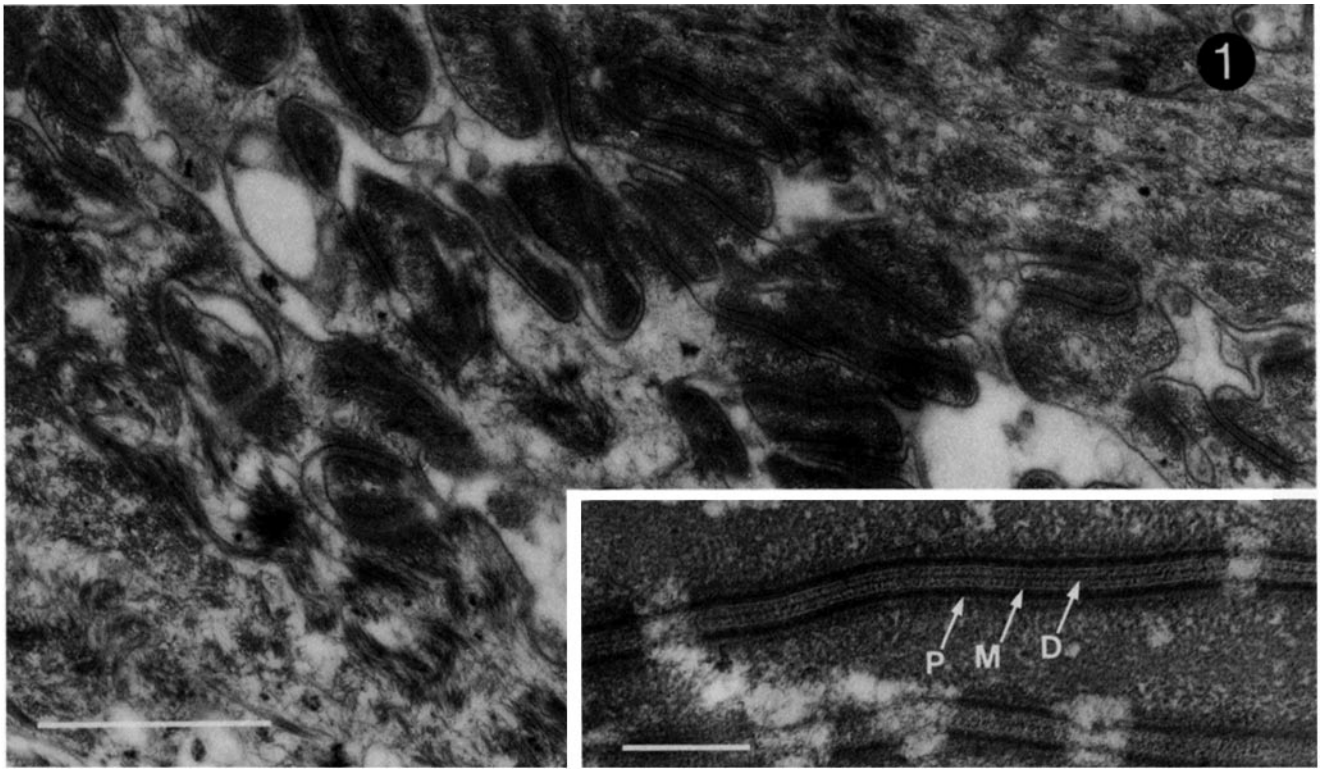

FIGURE 1 Section through intact *stratum spinosum* of bovine muzzle epidermis, showing extensive interdigitation of cell boundaries and the great number of desmosomes. Bar, 1.0 μm. × 30,000. *Inset:* desmosome of stratum spinosum at higher magnification, showing intracellular plaques (*P*), plasma membranes (*M*), and intercellular components or desmoglea (*D*). Bar, 0.2 μm. × 78,000.

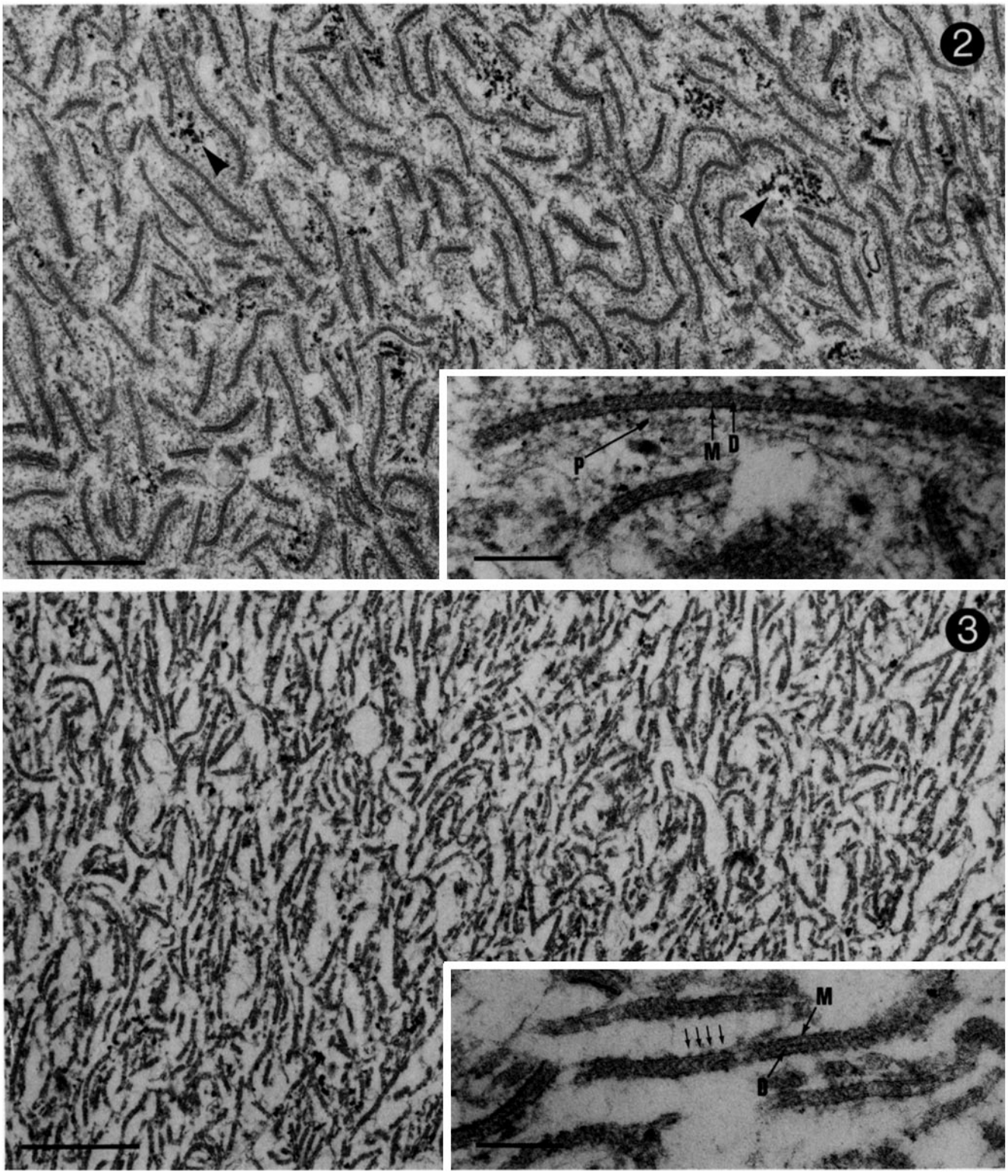

FIGURE 2 Whole desmosomes after completion of differential centrifugation. Loose filamentous layers which are presumably derived from the desmosomal plaques and/or the tonofilaments are apparent on the former cytoplasmic side of the plasma membrane. Some electron-dense contaminants are present (arrowheads). Bar, 1.0 μm. × 20,000. *Inset:* whole desmosomes at high magnification. Cytoplasmic desmosomal elements (*P*), presumably desmosomal plaques and/or tonofilaments, appear frayed out into filaments. Plasma membranes (*M*) and desmoglea components (*D*) are present. Bar, 0.2 μm. × 74,000.

FIGURE 3 Desmosome cores after metrizamide gradient centrifugation. The plaques are largely removed. Bar, 1.0 μm. × 20,000. *Inset:* desmosome cores at high magnification. Plaque filaments are almost entirely absent, leaving only small insertions which are sometimes spaced regularly on the cytoplasmic side of the plasma membrane (arrows). Plasma membranes (*M*) and desmoglea components (*D*) remain, although some disorganization of the intercellular laminae is apparent. Bar, 0.2 μm. × 74,000.

Vigorous stirring of spinous layer epidermis in pH 2.6 buffer in the presence of low concentrations of NP-40 dissolves the prekeratin tonofilaments and, coupled with sonication, severs the desmosomes from one another, from the adjoining plasma membrane, and from most intracellular constituents. After differential centrifugation, a relatively pure preparation of desmosomes, containing their intracellular plaques, is obtained with only minor contamination (Fig. 2). Whole desmosomes retain their plasma membranes and intracellular components as well as some intracellular filamentous material presumably derived from the desmosomal plaques and/or tonofilaments. After isopycnic centrifugation on metrizamide gradients, ~60–70% of the light-scattering material (OD_{500}) is recovered as a single dense white band. A small pigmented pellet normally contains 10–20% of the total light-scattering material in the tube, with the remainder distributed fairly evenly through the rest of the gradient. The major band contains both desmosomal nonglycoproteins and core glycoproteins. These are separated during the subsequent washing steps, in which the cores are pelleted while the nonglycosylated proteins are largely retained in the supernates. Ultrastructural observations indicate that centrifugation on metrizamide gradients and subsequent washing remove most of the intracellular plaques, leaving purified desmosome cores which consist of the desmosomal intercellular components sandwiched between plasma membranes, with some residual cytoplasmic filamentous material (Fig. 3). Some cores show a distinct periodicity of the residual plaque insertions on the cytoplasmic side of the plasma membrane. Isolation of the desmosomes is associated with some disorganization of the precise register of the laminae in the cores.

From 20 muzzles (12–15 g of *stratum spinosum*), ~15 mg of purified desmosomal cores are obtained. For both whole desmosomes and cores, sections taken from different regions of blocks of individual samples show no differences except in junction size and degree of packing.

SDS PAGE of whole desmosomes reveals at least 15 prominent protein bands. Those bands of 48,000–68,000 mol wt are largely prekeratin proteins derived from the tonofilaments (3, 5, 34). Preparations of purified cores are highly enriched in a very broad band ~150,000 mol wt, two broad bands of 115,000 and 100,000 mol wt, and a 22,000 mol wt component (Fig. 4). Occasionally the 150,000 mol wt band can be resolved into a triplet and the 100,000 mol wt component sometimes reveals a minor leading component in addition to the major band (Fig. 5). Staining of gels with periodic acid–dansyl hydrazine, which renders glycoproteins fluorescent (4), labels the major components of the cores and not the other proteins present in preparations of whole desmosomes (Fig. 4). Electrophoretic analysis also reveals that the nonglycosylated components prominent in whole desmosomes which retain their intracellular plaques are significantly reduced in cores which have been stripped of most intracellular material. These diminished bands include the prekeratins as well as bands at 230,000, 205,000, and, to a lesser extent, bands at 86,000 and 82,000 mol wt.

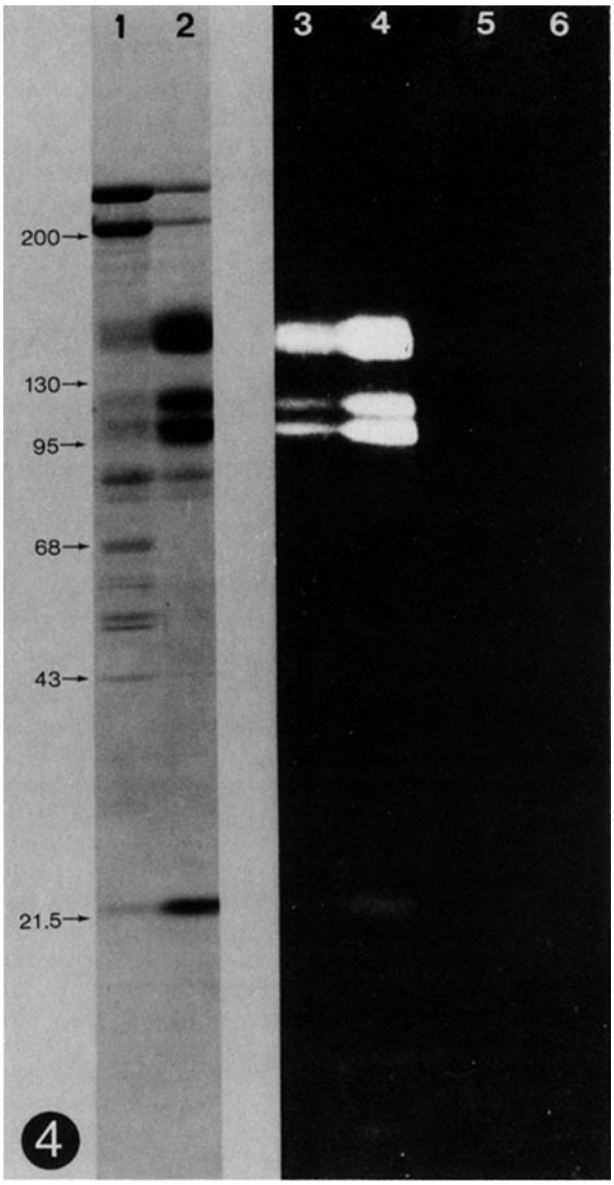

FIGURE 4 Desmosome fractions in polyacrylamide gradient gels loaded with 25 μg of protein/well. Lanes *1*, *3*, and *5*, whole desmosomes. Lanes *2*, *4*, and *6*, desmosomal cores. Lanes *1* and *2*, 5–20% gradients, Coomassie Blue staining for protein. Lanes *3–6*, 5–17% gradients, periodic acid–dansyl hydrazine staining for carbohydrate; only the major core proteins are labeled. In lanes *5* and *6*, periodate treatment was omitted.

DISCUSSION

Our modifications of the original technique for the isolation of desmosomes from bovine muzzle epidermis (30) include the use of a nonionic detergent and sonication to free desmosomes from intracellular organelles and from adjacent plasma membrane. Subsequently, through differential centrifugation, we obtain preparations of whole desmosomes morphologically and biochemically similar to the desmosome fractions of Skerrow and Matoltsy (31, 32), except that our whole desmosomes are comparatively enriched in the very high molecular weight nonglycosylated proteins (205,000 and 230,000 mol wt). We found these components to be especially sensitive to endogenous acid proteases, being degraded into a large number of components in the absence of protease inhibitors. The fact that our protocol uses inhibitors of proteolytic enzymes while the original technique did not may account for the relative enrichment of these moieties in our preparations.

We consider it likely that the core glycoproteins from the intercellular cross-bridges which have been observed in desmosome-containing tissues through the application of various electron microscopic techniques, including tannic acid–glutar-

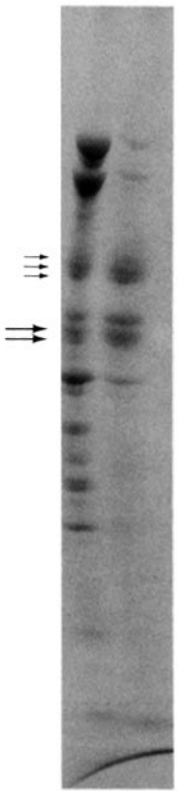

FIGURE 5 High resolution microslab gel showing three subcomponents of the 150,000 mol wt band (small arrows) and two subcomponents of the 100,000 mol wt band (large arrows). The gel was loaded with 8 μg of whole desmosomes (left lane) and 4 μg of desmosomal cores (right lane).

aldehyde fixation (12), lanthanum impregnation (23), and freeze fracture (12, 15).

Many workers have associated cell surface glycoproteins with adhesion-related activities in systems such as sponges (9), slime molds (19, 27), and avian and mammalian cells (7, 28, 36). Our finding that the function of an adhesive organelle, the desmosome, may be mediated by certain glycoproteins is also supported by ultrastructural localization of carbohydrate in the intercellular region of desmosomes, using various histochemical stains such as ruthenium red (11), phosphotungstic acid (8), and periodic acid–silver methenamine (8, 22).

Isopycnic centrifugation of whole desmosome preparations on metrizamide gradients, followed by washing in CASC-B, separates the desmosomal cores from most cytoplasmic components. The same protocol with a sucrose gradient of equivalent density produces no such purification of intercellular elements. Metrizamide is the common name for 2-(3-acetamido-5-N-methylacetamido-2,4,6 tri-iodobenzamide)-2-deoxy-D-glucose, essentially a tri-iodinated benzoic acid linked via an amide bond to a deoxyglucosamine. It has been used in a variety of purification procedures for organelles and macromolecules (reviewed in reference 24). Previous studies have shown that metrizamide at high concentrations interacts reversibly with proteins to form dense complexes (10, 25). Rickwood and Jones (26) have presented the most detailed study to date of the interaction of metrizamide with proteins, showing that high concentrations of metrizamide promote the release of proteins from ribosomes. They suggest that metrizamide combines with free proteins in equilibrium with proteins loosely bound to the ribosomes, driving the equilibrium toward dissociation. A similar phenomenon may take place during the metrizamide-induced release of cytoplasmic proteins from the inner face of the plasma membrane of desmosomes.

SDS PAGE reveals that desmosomal core preparations consist primarily of broad bands at 150,000, 115,000 and 100,000 mol wt, all of which are highly glycosylated. These bands can sometimes be resolved into a number of closely running subcomponents. They presumably correspond to the two periodic acid-Schiff-positive bands reported by Skerrow and Matoltsy (30, 31) in their electrophoretic analysis, in which stacking capabilities were lacking. In addition, we shown an additional desmosomal constituent not reported by these authors. This 22,000 mol wt component stains weakly for carbohydrate in the PADH reaction. Though we cannot yet eliminate the possibility that it is a cleavage product from a larger band, its regular presence in our preparations suggests that it is a true desmosomal element which the original workers failed to resolve with their electrophoretic gel system.

Certain nonglycosylated proteins with molecular weights of ~230,000, 205,000, 86,000, and 82,000, prominent in gels of whole desmosomes, are reduced in gels of desmosomal cores. This suggests that these molecules may be plaque elements. Skerrow (29) has reported that a 48-h extraction of whole desmosomes with deionized distilled water containing EDTA and mercaptoethanol leads to the disappearance of high molecular weight bands (230,000 and 210,000 mol wt) from densitometer traces of SDS acrylamide gels. However, her electron micrographs show that elimination of these bands is accompanied by only a partial removal of plaque elements, leaving considerable quantities of intracellular desmosomal material remaining.

Alternatively, these nonglycosylated proteins may be derived from contaminants (Fig. 2, arrowheads) present in whole desmosomes. Though this possibility cannot be entirely eliminated, it seems unlikely in view of the high concentration of these proteins revealed through SDS polyacrylamide electrophoresis of whole desmosomes relative to the low concentration of contaminants visible by electron microscopic observation.

In experiments in progress we are employing specific affinity probes to elucidate the organization, tissue distribution, and function of desmosomal components in junctional assembly and cell recognition.

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