Restricted Tissue Distribution of a 37-kD Possible Adherens Junction Protein

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Abstract. A major polypeptide of M_r 37,000 was purified from a desmosome-enriched citric acid–insoluble pellet of pig tongue epithelium. The polypeptide was solubilized from the 4-M urea-insoluble pellet with 9 M urea, and extracts were separated by carboxymethyl cellulose and gel filtration chromatography. The 37-kD protein was obtained in milligram quantities as a single band on two-dimensional gels in 30% yield after 21-fold purification from the citric acid–insoluble fraction. The protein is not glycosylated and has a pI of ∼8.7. Although isolated from a fraction rich in desmosomes, the 37-kD protein is not a desmosomal protein. Indirect immunofluorescence analysis of frozen sections of tongue and other tissues demonstrated that antibodies raised to the 37-kD protein bound only to suprabasal cell layers at punctate regions of the periphery of the cell and was absent from most regions of epidermis, whereas antibodies to desmoplakins I and II, desmosomal proteins, bound similarly but in all epidermal layers. Immunoelectron microscopy localized the 37-kD protein to the cell periphery in regions between, but never in, desmosomes. By immunofluorescence, the 37-kD protein colocalized with actin as well as with vinculin and uvomorulin in oral tissues. Like the 37-kD protein, vinculin and uvomorulin were absent from the basal layer. Based on its appearance, localization, and solubility properties, the 37-kD protein is probably a component of adherens junctions; its restriction to suprabasal cells and exclusion from the epidermis are unique.

INTERCELLULAR junctions were originally defined by their electron microscopic appearance (Staehelin, 1974; Farquhar and Palade, 1963; McNutt and Weinstein, 1973), but recent studies involving purification of junctions and their proteins or the use of monoclonal antibodies specific for junctional proteins have led to more detailed understanding of the component proteins of the "adhering" types of intercellular junctions, desmosomes and adherens junctions. In cultured cells adherens junction proteins are found both in the focal contact, an in vitro structure (in both fibroblasts and epithelial cells) and in intercellular junctions (in epithelial cells only). In tissues they are located in characteristic intercellular junctions. All contain actin, α-actinin, and vinculin (Burridge et al., 1988). The belt desmosome, or *zonula adherens*, an apical structure of the gut epithelium, contains a circumferential belt of actin cables associated with the actin in the microvilli of the epithelial cells and appears to insert into vinculin-containing junctions. Smaller structures known by various names (McNutt and Weinstein, 1973; Staehelin, 1974; Geiger et al., 1983; Drenckhahn and Franz, 1986) are buttonlike structures similar to desmosomes (*macula adherens*) in size and shape and have been described in stratified epithelia. The detailed structure of these adherens-type junctions has been studied most in focal contacts and remains controversial, but certain structural features have been elucidated (Burridge et al., 1988; Geiger et al., 1990); for example, it is clear that the intracellular proteins such as vinculin, actin, and α-actinin are associated with transmembrane proteins such as uvomorulin (E-cadherin) (Boller et al., 1985), which exhibit homophilic binding and are, in some cases at least, responsible for cell–cell adhesion (Takeichi, 1988). The component proteins of adherens junctions are present early in development, especially in ectoderm (Takeichi, 1988, 1990; Choi and Gumbiner, 1989). The structure, distribution, and protein components of adherens junctions in tissues have only recently begun to be elucidated.

In addition to these morphological subtypes of adherens junctions in tissues, heterogeneity between the component proteins of the adherens junction and the focal contact has been described. The focal contact, a structure apposed to the basal aspect of cultured cells that is electron dense and has been visualized by interference reflection microscopy, shares many of the component proteins of the adherens junction and, like the adherens junction, is intimately associated with actin filaments (Burridge et al., 1988). Although both cell–cell junctions (adherens junctions) and cell–substratum junctional structures (focal contacts) contain vinculin (Geiger et al., 1980, 1985), some intracellular proteins of focal contacts, such as talin (Geiger et al., 1985) and paxillin (Turner et al., 1990), are not found in cell–cell junctions. Second, a 135-kD protein of lens (Geiger et al., 1985) and radixin, an adherens junction protein from rat liver (Tsukita et al., 1989c), have been found in cell–cell junctions but not in fo-

cal adhesions. Heterogeneity in the proteins of adherens junctions in epithelial tissues in vivo has not been described, however. Except for proteins related to the cadherins, known adherens junctions proteins are intracellular (e.g., McCrea and Gumbiner, 1991; Tsukita et al., 1989a; Tsukita et al., 1989b; Nagafuchi et al., 1991).

We describe the purification of a major 37-kD insoluble basic protein that appears to be a component of intercellular junctions from pig tongue epithelium, a stratified squamous epithelium. Although the protein was isolated from a citric acid–nonionic detergent insoluble fraction rich in desmosomes, it appears not to be a desmosome protein by immunofluorescence and immunoelectron microscopic criteria. This protein was detected only in suprabasal layers of the epithelium and was absent from most areas of the epidermis, suggesting that it is a component of a type of junction present only in a restricted group of epithelial tissues or, alternatively, that it is expressed only in a subpopulation of a generally distributed type of junction. Like the 37-kD protein, both uvomorulin and vinculin were present only in suprabasal layers, and the 37-kD protein is closely associated with actin. The data suggest that the 37-kD protein is an adherens junction protein and consequently that adherens junctions, or a subgroup of adherens junctions, may be limited to suprabasal layers in this tissue. The findings suggest a previously undescribed type of heterogeneity in epithelial junctions.

Materials and Methods

Materials

Pig tongues were obtained fresh from a slaughterhouse. Carboxymethyl cellulose (CM52) was from Whatman Inc. (Hillsboro, OR). Fractogel and Protein A were from Pharmacia LKB Biotechnology (Piscataway, NJ). Rat monoclonal anti-uvomorulin, mouse monoclonal anti–human vinculin, and goat anti–rat IgG fluorescein conjugate were from Sigma Chemical Co. (St. Louis, MO). RNAse A, monoclonal anti-desmoplakin I and II, and the glycan detection kit were from Boehringer Mannheim Corp. (Indianapolis, IN). Goat anti–mouse IgG fluorescein conjugate and goat anti–rabbit IgG (H+L) rhodamine conjugate were from Cappel Organon Teknika Corporation (West Chester, PA). Rhodamine-phalloidin was from Molecular Probes Inc. (Eugene, OR). O.C.T. Compound was from Miles Inc. (Elkhart, IN). Rabbit anti-keratin was from Biomedical Technologies (Stoughton, MA). Goat anti–mouse IgG (H+L) alkaline phosphatase conjugate was from Bio-Rad Laboratories (Richmond, CA).

Antibodies to the 37-kD protein were produced with protein that was partially purified (9-M urea extractable fraction; Fig. 1) and electrophoresed on gradient SDS polyacrylamide slab gels. The band corresponding to the 37-kD protein was excised and used to immunize rabbits as described (O'Keefe et al., 1984).

PAGE and Immunoblotting

PAGE was performed on 1.5-mm-thick 3.5–17% gradient gels with the buffer system of Fairbanks et al. (1971). Gels were stained in methanol/acetic acid/water (50:10:40) with 0.1% Coomassie blue R-250 and destained in methanol/acetic acid/water (10:7:83). Transfer of electrophoresed fractions for immunoblotting was according to Towbin et al. (1979). The use of the antiserum for immunoblotting with ^{125}I-labeled protein A was as described previously (O'Keefe et al., 1984).

Two-Dimensional Gel Electrophoresis

Nonequilibrium pH gradient gel electrophoresis (NEPHGE)[1] was em-

1. Abbreviations used in this paper: DPBS, Dulbecco's phosphate buffered saline; NEPHGE, nonequilibrium pH gradient gel electrophoresis.
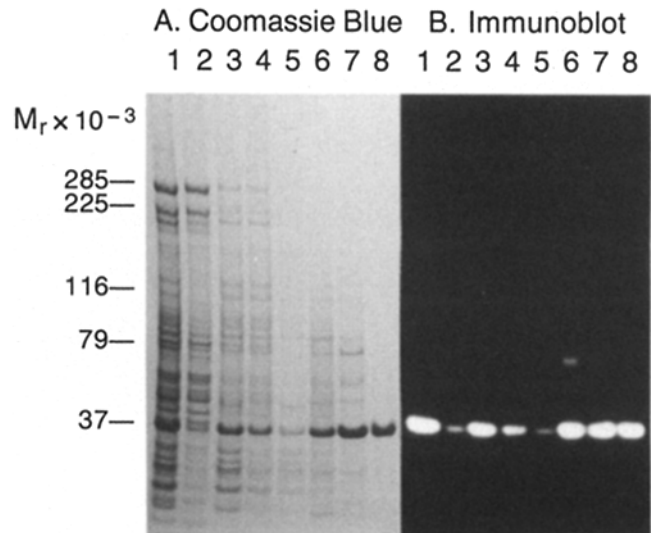

Figure 1. Purification of the 37-kD protein. (A) Coomassie blue stained gels. (B) Immunoblots of SDS polyacrylamide gel corresponding to A. (Lanes 1) junctional preparations; (lanes 2) 4-M urea extract of junctional preparations; (lanes 3) pellet after 4-M urea extraction; (lanes 4) 9-M urea extract of pellet shown in lane 3; (lane 5) pellet after 9-M urea extraction of material shown in lane 3; (lane 6) eluate of Fractogel column; (lanes 7) eluate of carboxymethyl cellulose column; (lanes 8) eluate of second Fractogel column run.

ployed in the first dimension using a 3 × 100 mm tube gel (O'Farrel et al., 1977) and was followed by SDS-PAGE (Laemmli, 1970) with a 12.5% acrylamide separating gel and a 3% acrylamide stacking gel. The cylindrical gel was prepared as described with ampholines of pH 3–10, and electrophoresis was toward the cathode with the acidic reservoir at the anodal end and the basic reservoir at the cathodal end of the gel. 20 µg of cytochrome c was applied as a marker, and the proteins were electrophoresed at 450 V without cooling until cytochrome c was 2.5 cm from the bottom. The gels were recovered from tubes, equilibrated for 30 min in buffer (0.0625 M tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.01% bromphenol blue, 10 mM dithiothreitol or 5% β-mercaptoethanol), placed on the second dimensional slab gel, and electrophoresed as described with 60 mA constant current.

Binding of Proteins to Peptides on Western Blots

To renature the proteins, we dialyzed purified keratin [purified according to a modification (O'Keefe et al., 1989) of the method of Steinert (1976)] or purified 37-kD protein dissolved in 9 M urea in buffer A (10 mM Na phosphate, pH 8.0, containing 10 mM glycine, 1 mM EDTA, 0.05% Tween 20, 1 mM NaN₃) with 0.1% β-mercaptoethanol and 0.1% BSA at 20 µg/ml against 4 M urea in the same buffer for 20 min at room temperature and then for 1.5 h at 4°C. The samples were then dialyzed against 2 M urea in the same buffer with 0.1% β-mercaptoethanol for 2 h at 4°C, and the process was repeated using 1 M urea in "blot buffer" (0.15 M NaCl, 10 mM Na phosphate, pH 7.5, 1 mM EDTA, 0.2% Triton X-100, 1 mM NaN₃, with 0.1% β-mercaptoethanol to further reduce the concentration of urea to ∼1 M. Finally, the protein was dialyzed against blot buffer with 0.1% β-mercaptoethanol at 4°C with several changes of buffer to remove urea. Desmoplakin I purified to homogeneity as described previously (O'Keefe et al., 1989) was dialyzed directly against blot buffer.

Nitrocellulose membranes containing transferred proteins were preincubated in blot buffer containing 4% BSA at room temperature for 20 min with gentle shaking and then placed in blot buffer with 4% BSA containing 0.5–2 µg renatured protein per ml and incubated overnight at 4°C on a rocker. The membrane was washed in blot buffer three times at room temperature for 10 min and in 2 M urea–glycine buffer (10 mM sodium phosphate, pH 7.4, containing 2 M urea, 0.1 M glycine, 1% Triton X-100, 1 mM NaN₃) for 30 min to remove excess protein. It was then rinsed in briefly in deionized water and in blot buffer for 5 min. Subsequent incubation in

blot buffer with 4% BSA containing 0.1% (v/v) antiserum overnight at 4°C, incubation with ^{125}I-protein A, and autoradiography were as described (O'Keefe et al., 1984).

Detection of Vinculin Immobilized on Nitrocellulose

Proteins transferred to nitrocellulose were incubated with mouse monoclonal anti–human vinculin (1:1,000), and the antigen antibody complex was detected with goat anti–mouse IgG (H+L) alkaline phosphatase conjugate (Blake et al., 1984).

Quantitation of Protein

Protein in the various insoluble fractions was estimated by the procedure of Lowry et al. (1951) using 4.5% SDS for solubilization and BSA as standard. The yield of the 37-kD band was estimated on SDS gels by extracting bound Coomassie blue from slices of the stained gel with dimethylsulfoxide containing 1% HCl and determining absorption at 577 nm; 1 μg of purified 37-kD protein/ml as determined by the Lowry procedure gave an absorbance of bound dye of 0.073. Because two-dimensional gels showed that only one polypeptide migrated in the region of the 37-kD band in the desmosome preparation, measurement of the dye bound by this band was used to estimate the amount of the 37-kD protein in various fractions.

Immunostaining of Tissues

Fresh tissue samples were embedded in O.C.T. compound, frozen in dry ice, and stored at −70°C. Frozen sections (6 μm) were placed on gelatin-coated microscope slides and fixed in methanol/acetone (1:1) at −20°C for 10 min, dried, and incubated in Dulbecco's phosphate buffered saline (DPBS; 0.137 M NaCl, 2.7 mM KCl, 0.9 mM CaCl$_2$, 0.5 mM MgCl$_2$, 6.5 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$; Dulbecco and Vogt, 1954) for 10 min and subsequently in DPBS containing 2% BSA for 15 min at room temperature. Primary antibodies were diluted 25–500 times in DPBS containing 0.01% Tween 20 and 2% BSA and incubated with tissue sections in a humidified container at 4°C overnight, washed in cold DPBS, incubated with rhodamine- or fluorescein-conjugated goat secondary antibodies for 2 h in a dark, humidified chamber, washed, and mounted in polyvinyl alcohol. Confocal microscopy was performed on a BioRad MRC 600 laser confocal microscope.

Immunoelectron Microscopy

Frozen sections (6 μm) of pig epithelium were placed on microscope slides

and fixed in acetone at −20°C for 5 min and then air-dried and incubated overnight in antibody (1:50 in DPBS). After extensive washing in DPBS, sections were incubated for 30 min in normal goat serum (1:25 in DPBS) and then for 4 h in 5-mm gold-conjugated goat anti-rabbit IgG (Amersham International, Amersham, UK). The sections were then washed in DPBS, fixed in 1% glutaraldehyde in DPBS for 30 min, and postfixed in 1% OsO$_4$ in DPBS for 30 min. The sections were dehydrated in a graded series of alcohols and embedded in inverted BEEM capsules in Epon-Araldite (Tousimis Research Corporation, Rockville, MD). After curing at 60°C, the BEEM capsules containing the sections were removed from the microscope slides by immersion in liquid nitrogen. Thin sections (50 nm) of the embedded material were placed on copper 400 mesh grids and then stained with uranyl acetate and lead citrate. The sections were viewed in a JEOL 100CX electron microscope at 60 kV.

Results

Purification of the 37-kD Protein

Extraction of the Protein. Pig tongues were keratomed to remove the dorsal epithelium, the tissue was extracted in sodium citrate buffer, pH 2.6, and the desmosome-rich pellet was collected by centrifugation according to a modification of the methods of Skerrow and Matoltsy (1974) and Gorbsky and Steinberg (1981), as previously described (O'Keefe et al., 1989) (Fig. 1, lanes *1*). The pellet obtained from three pig tongues was suspended by sonication in 25 ml of ice cold buffer (buffer A; 10 mM Na phosphate, pH 8.0, 10 mM glycine, 1 mM EDTA, 0.05% Tween-20, 1 mM NaN$_3$) containing 4 M urea, 1 mM DTT, 1 mM PMSF and 40 μg/ml leupeptin, extracted overnight at 4°C, and centrifuged at 50,000 rpm in a Beckman Ti 65 rotor for 1 h. The pellet was resuspended in the same solution, sonicated, and centrifuged again. After extraction in 4 M urea, the pellet, which contained most of the 37-kD protein (Fig. 1, lanes *3*), was resuspended and sonicated in 7.5-ml buffer A containing 9 M urea and 0.1% β-mercaptoethanol, 1 mM PMSF, and 50 μg leupeptin per ml, extracted at room temperature for
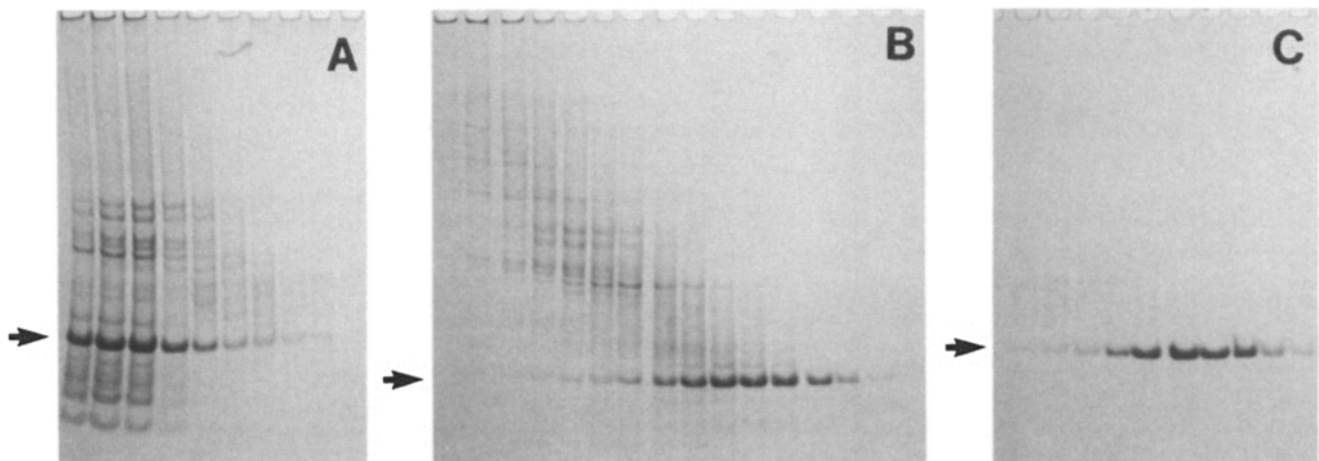

Figure 2. Altered Stokes radius of the 37-kD protein in the absence or presence of SDS. The 4-M urea-insoluble fraction of the junction preparation (Fig. 1, lanes *3*) was resuspended in buffer A (see text) containing 9 M urea and 0.1% β-mercaptoethanol, 1 mM PMSF, and 50 μg leupeptin per ml, extracted at room temperature for 3 h, and centrifuged at 50,000 rpm in a Beckman TLA 100.3 rotor for 45 min at 23°C. Similar samples were separated on a Fractogel column (see text) (*A*) without SDS or (*B*) after addition of SDS to a final concentration of 0.2% and heating to 70°C for 10 min. The pooled peak of 37-kD protein from (*B*) was applied to carboxymethyl cellulose and eluted as described in the text and rechromatographed on the same gel filtration column; the eluate is shown in (*C*). Samples were analyzed by SDS-PAGE. The arrow indicates the position of the 37-kD protein.

3 h, and centrifuged at 50,000 rpm in a Beckman TLA 100.3 rotor for 45 min at 23°C. To the supernatant, which contained most of the 37-kD protein (Fig. 1, lanes 4), four volumes of cold acetone was added on ice. After 1 h, the sample was centrifuged at 19,000 rpm for 15 min at 4°C in a Sorvall SS-34 rotor. The precipitate was dissolved in 6 ml of the same buffer and was added to 1.5 ml 5× Fairbanks' sample buffer (5% SDS, 25% sucrose, 50 mM Tris, pH 8.0, 5 mM EDTA, 10 mM DTT; Fairbanks et al., 1971), heated to 70°C for 10 min, and made 1 mM in PMSF, and then 100 μg leupeptin/ml was added.

Column Chromatography of the 37-kD Protein. It was found that the 37-kD protein was excluded from Fractogel TSK HW-55 (F) in the absence of SDS (Fig. 2 A) but migrated with a Stokes radius of appropriate for a 37-kD protein in the presence of SDS (Fig. 2 B and C). It was necessary, therefore, to include SDS in the chromatographic buffers. One half of the solubilized precipitate (3.75 ml) was applied at room temperature at 2 ml/h to a Fractogel column (16 × 600 mm) previously equilibrated with 10 mM Na phosphate, pH 7.0, containing 0.2% SDS, 9 M urea, 10 mM glycine, 1 mM EDTA, 1 mM NaN₃, 0.1% β-mercapto-ethanol (buffer B), and developed with the same buffer at 3 ml/h. The eluate was monitored by absorption at 280 nm and by SDS-PAGE, and fractions containing the 37-kD protein

were combined. In the presence of SDS, the 37-kD protein was separated according to size (Fig. 2 B; Fig. 1, lanes 6). The protein was concentrated by precipitation in four volumes of cold acetone (−20°C) and centrifuged, and the precipitate was washed twice by suspension in 39 ml of cold acetone containing 1 ml of deionized water and then centrifuged at 4°C at 19,000 rpm in a Sorvall SS-34 rotor for 15 min to remove SDS. The second half of the sample was processed similarly. The combined precipitate was dissolved in 20 ml of tricine buffer (20 mM tricine, pH 6.85, 1 mM EDTA, 0.05% Tween 20, 1 mM NaN₃, 0.1% β-mercapto-ethanol), containing 9 M urea and 1 mM PMSF, and 25 μg leupeptin per ml was added. The sample was applied to a carboxymethyl cellulose (CM52) column (5-ml bed volume) previously equilibrated with same buffer at 0.25 ml/min, washed, eluted with the same buffer containing 0.1 M guanidine-HCl at pH 8.5, and analyzed by SDS-PAGE (Fig. 1, lanes 7). Fractions containing the 37-kD protein were combined, concentrated with four volumes of acetone (−20°C), dissolved in 1.2 ml of buffer B plus 0.3 ml of 5× Fairbanks sample buffer, and heated to 70°C for 10 min. The clear solution was applied to the Fractogel column again and eluted as described, after which the 37-kD protein was electrophoretically homogeneous (Fig. 1, lanes 8; Fig. 2 C; Fig. 3 A). From the epithelium of three tongues, as is shown in
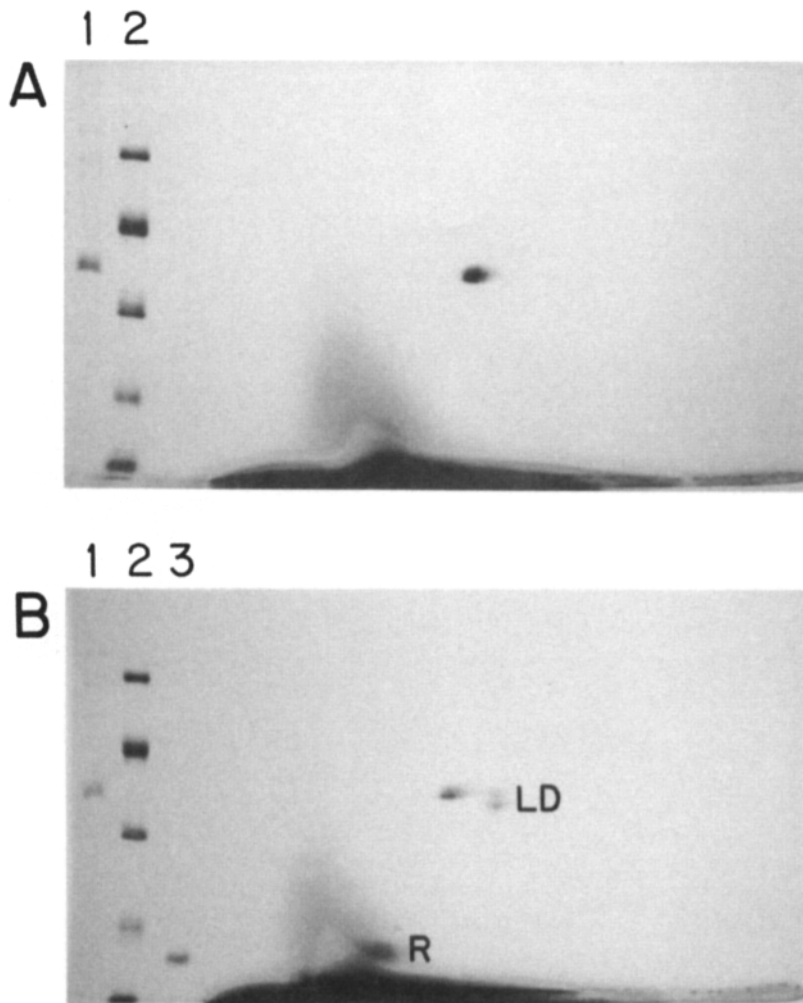

Figure 3. Two-dimensional gel of purified 37-kD protein. NEPHGE was used in the first dimension and SDS-PAGE in the second. (A) 4 μg of the 37-kD protein was applied and run as described in Experimental Procedures. Lanes 1 and 2 show one-dimensional SDS-PAGE. (Lane 1) 9 M urea extract of junctional preparation (Fig. 1, lanes 4); (lane 2) molecular weight standards. Standards were BSA (67 kD), ovalbumin (42.7 kD), carbonic anhydrase (31 kD), soybean trypsin inhibitor (21.5 kD), and cytochrome C (12.1 kD), 5 μg each. (B) The 37-kD protein was mixed with lactate dehydrogenase (LD, 24 μg) ribonuclease A (R, 12 μg) and cytochrome C (20 μg) and run as in A. (Lane 1) 9 M urea extract of junctional preparation (Fig. 1, lanes 4); (lane 2) molecular weight standards; (lane 3) ribonuclease A (3 μg).

Table I. Purification of the 37-kD Protein

Purification Step	Protein	37 kD	Yield	Purification-fold
	mg	*mg*	*%*	
Citric acid insoluble	67.5	3.2	100	1.0
4-M urea residue	32.3	2.8	87	1.8
9-M urea extract	22.0	2.4	75	2.3
First gel filtration	4.9	1.9	59	8.2
Ion exchange eluate	1.6	1.2	38	15.8
Second gel filtration	1.0	1.0	31	21.1

Table I, we obtained 67 mg of citric acid buffer–insoluble protein. In the final step we obtained a total of 1 mg of the 37-kD protein in 31% yield after a 21-fold purification. Amino acid sequencing of the purified protein was unsuccessful, indicating that the amino-terminus appears to be blocked. Sequencing of tryptic digests yielded the sequences VVVNGXPFXE and NQQPCNLPC. When these sequences were compared with sequences in the GenBank, EMBL, and SwissProt databases with the University of Wisconsin Genetics Computer Group, Inc., programs TFASTA and FIND-PATTERNS, no identical patterns were found.

Characterization of 37-kD Protein

NEPHGE pH Gradient and Binding to Immobilized Proteins. To examine the 37-kD protein for the presence of carbohydrate, protein from the final purification step and the 4 M urea-insoluble material were electrophoresed in separate lanes of an SDS gel, transferred to nitrocellulose, subjected to periodate oxidation, and tested for the presence of reactive aldehydes according to the manufacturer of the glycan detection kit. Although some proteins in the 4 M urea-insoluble material (Fig. 1, lanes *3*) were shown to contain carbohydrate by this method, the purified 37-kD protein run on the same blot did not react (data not shown) and therefore lacked

Table II. Amino Acid Composition of the 37-kD Protein

Amino acid	mol
	%
Asx	10.0
Glx	11.7
Ser	6.4
Gly	13.0
His	1.3
Arg	4.9
Thr	3.2
Ala	6.0
Pro	10.5
Tyr	2.2
Val	9.0
Met	1.7
Ile	3.9
Leu	6.4
Phe	5.6
Lys	4.6
Cys	ND
Trp	ND

ND, not done.

carbohydrate. The isoelectric point of the 37-kD protein by NEPHGE was ∼8.7 as determined by comparison with RNase and lactic acid dehydrogenase (Fig. 3). Amino acid analysis is presented in Table II.

At neutral pH, the 37-kD protein was tightly bound to other proteins but was extractable in solutions of low ionic strength containing 9 M urea. It was apparently possible to renature the extracted purified protein by dialyzing it at dilute concentrations serially against buffers with reduced concentrations of urea and then against buffers of physiological ionic strength. The 37-kD protein treated in this fashion at subnanomolar concentrations remained in solution, since it was able to bind some proteins of the citric acid–insoluble fraction immobilized on nitrocellulose (Fig. 4, lane *4*). Keratin polypeptides from pig tongue purified and similarly dialyzed at low concentrations (20 μg/ml) against buffers containing reduced concentrations of urea remained in solution under the conditions of the experiment, since they bound to immobilized junction proteins in distinctive fashion (see below).

The binding of the 37-kD protein to immobilized proteins from the junction preparation appeared to be specific (Fig. 4), because desmoplakin I (lane *2*), the 37-kD protein (lane *4*), or keratin (lane *6*) bound to different proteins on blots containing the same immobilized junction proteins. Only the 37-kD protein bound proteins of approximate molecular weight of 135 and 115 kD, and slightly to a protein of 105 kD (lane *4*). A similar result was obtained when immobilized proteins were from the 9 M urea-soluble fraction (data not shown). Soluble desmoplakin I (lane *2*) and keratins in solution (lane *6*) did not bind to these proteins. We attempted to determine whether cadherins, catenins, or adherens proteins such as vinculin are associated with the 37-kD protein. Because of insolubility of the junction preparation, we were unable to immunoprecipitate the 37-kD protein from the junction preparation to determine what proteins might be associated with it. Because immunoprecipitation might be possible with cultured cells, we cultured pig oral epithelial keratinocytes and performed immunoblots with antibody to the 37-kD protein; the 37-kD protein was not expressed in culture either in monolayers cultured in medium containing 0.1 mM Ca²⁺ or in stratified cultures in medium containing 1.0 mM Ca²⁺ (data not shown). A 130-kD protein was detected in the junction preparation and in the 9-M urea extract of the junction preparation, however, by immunoblotting with antibody to human vinculin against immobilized junction proteins (data not shown).

Distribution of 37-kD Protein. When sections of various pig tissues were examined by indirect immunofluorescence, the 37-kD protein was found to be limited to specific regions and certain tissues. Because the 37-kD protein was obtained from a preparation highly enriched in desmosomes, we compared the 37-kD protein and desmoplakins I and II by double immunofluorescence (Fig. 5). Antibodies to the 37-kD protein stained the periphery of epithelial cells in a punctate or linear fashion, very similar to the staining of desmoplakins, but, unlike desmoplakins, which were present in all epidermal layers, the protein was found only in suprabasal layers of the epithelium. In esophageal mucosa (Fig. 5 *a*) and gingiva (Fig. 5 *c*), the 37-kD protein was absent in the basal layer and present in all suprabasal layers, whereas desmoplakin I/II was present in all layers (Fig. 5 *b* and *d*). In
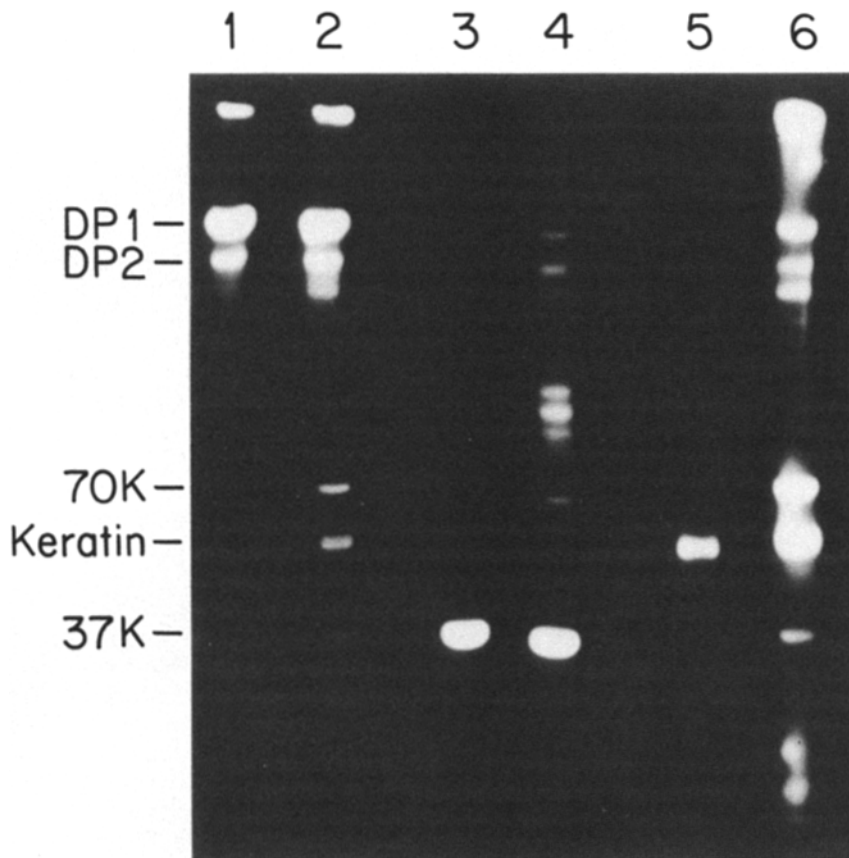
Figure 4. Distinctive binding of desmoplakin I, the 37-kD protein, or keratins to desmosomal proteins immobilized on nitrocellulose. A pig tongue desmosome preparation (4.7 μg protein) (Fig. 1, lanes *1*) was electrophoresed on multiple lanes of a 3.5–17% gradient gel, transferred to nitrocellulose as described, and cut into strips. Strips were incubated in blot buffer containing 4% BSA with or without added proteins at 4°C overnight with rocking, washed, and then incubated with appropriate antibodies to the proteins added in solution. Nitrocellulose strips were then washed in blot buffer and then incubated with ^{125}I-labeled protein A (see Experimental Procedures). (Lanes *1, 3,* and *5*) Strips incubated in blot buffer containing 4% BSA; (lanes *2, 4,* and *6*) strips incubated similarly but in the presence of added purified desmoplakin I (lane *2,* 2 μg/ml), 37-kD protein (lane *4,* 2 μg/ml) or keratins (lane *6,* 2 μg/ml). Antibodies were: (lanes *1* and *2*) affinity-purified rabbit anti-desmoplakin I (0.1 μg/ml); (lanes *3* and *4*) rabbit antiserum to the 37-kD protein (1:1,000); lanes *5* and *6,* antiserum to keratin (1:1,000).

Fig. 5 (*c* and *d*), the tissue was cut slightly tangentially, so that the absence of staining of the basal layer by anti–37-kD antibody (Fig. 5 *c*) in comparison with staining by anti-desmoplakin (Fig. 5 *d*) was accentuated. Similarly, the basal cells contained desmoplakins but not the 37-kD protein in tongue, buccal mucosa, and hard palate (not shown). In addition, the protein was found to be present in snout and footpad epithelium (not shown). In epidermis from several area of the trunk, however, no staining for 37-kD protein was present in any layer (Fig. 5 *e*), but desmoplakin I/II staining was present throughout the epidermis (Fig. 5 *f*). Thus, the 37-kD protein appeared to have an unusual distribution for a junctional protein; it was present only in a limited distribution in epithelium, because it was present only in suprabasal cells and only in certain regions of stratified squamous epithelium. Confocal microscopy of the sections better demonstrated the morphology of the structures containing the 37-kD protein. In hard palate, for example (Fig. 6), the structures were limited to the periphery of the cell in punctate or globular shapes sometimes organized in double file at cell-cell junctions (Fig. 6 *b*), consistent with the appearance of junctions in adjacent cells.

There were small differences in the location of staining of the 37-kD protein and desmoplakin by immunofluorescence studies, but the differences were not easily resolved by light microscopy. However, by immunoelectron microscopy, the 37-kD protein was localized in the region of cell–cell junctions but was excluded totally from desmosomes (Fig. 7). These results were identical whether the tissue was incubated with antibody before (Fig. 7) or after embedding

(not shown). Although antibody might in theory fail to penetrate desmosomal structures when incubated with tissue blocks before embedding, the failure of antibody to the 37-kD protein to label desmosomes when incubated with cut surfaces of embedded tissue indicates that the protein is likely to be absent from desmosomes. The basal layer, which did not contain the 37-kD protein by immunofluorescence, also did not show labeling of the 37-kD protein on immuno-electron microscopy by either technique (not shown). Thus, the 37-kD protein appeared not to colocalize with desmosomes, even though it was isolated from a fraction generally considered to contain predominantly desmosomes.

To investigate the possibility that the 37-kD protein was a component of adherens junctions, we compared its distribution with that of uvomorulin, vinculin, and actin. Double immunostaining of oral tissues with anti-uvomorulin or anti-vinculin antibodies showed that uvomorulin and vinculin were present in the tissues where the 37-kD protein was found and appeared to colocalize with the 37-kD protein (Fig. 8). In tongue (Fig. 8 *a* and *b*) and upper esophageal epithelium (Fig. 8 *c* and *d*), the 37-kD protein (Fig. 8 *a* and *c*) colocalized with uvomorulin (Fig. 8 *b* and *d*), and, like the 37-kD protein, uvomorulin was not detected by immunofluorescence in basal cells. A similar result was obtained in other oral epithelia (not shown). Tongue epithelium (Fig. 8, *e* and *f*) stained with both anti-37 kD (Fig. 8 *e*) and antibody to vinculin (Fig. 8 *f*) also showed similar staining of both proteins, which appeared to be limited to suprabasal layers. In the epidermis, vinculin (Fig. 8 *g*) and uvomorulin (Fig. 8 *h*) were both detectable by immunofluorescence, indicating that
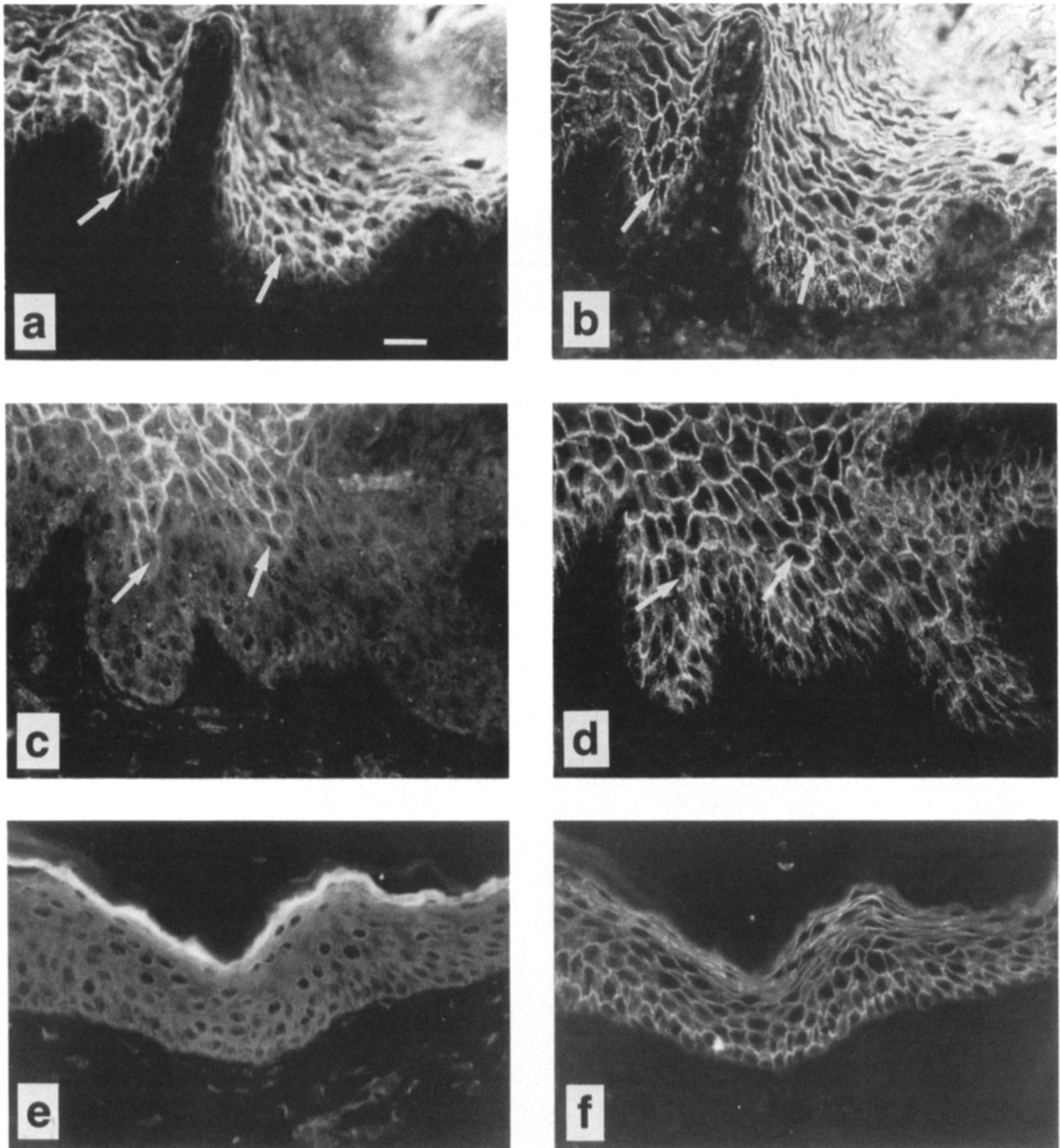
Figure 5. The 37-kD protein is limited to suprabasal cells. Sections of various pig tissues were processed for double immunofluorescence using antiserum (1:500) to the 37-kD protein (*a*, *c*, and *e*) or mouse mAb (0.5 μg/ml) to desmoplakin I (*b*, *d*, and *f*). Identical sections are shown of esophageal mucosa (*a* and *b*), gingiva (*c* and *d*), or abdominal skin (*e* and *f*). Arrows indicate similar locations in the corresponding panels. *e* is overexposed for comparison with *f* but showed no specific fluorescence. Desmoplakin antibody stains all cell layers and demonstrates the location of the basal cells of the epithelium; anti–37-kDa antibody does not stain this layer of cells. No 37-kD protein is demonstrated in epidermis; the staining of the stratum corneum is nonspecific. Bar, 25 μm.

adherens junctions are present in this tissue, a finding that we have confirmed in human epidermis as well in related studies of adherens junctions (Kaiser et al., 1993). As was noted above, the 37-kD protein was absent from the epi-

dermis of the trunk (Fig. 5 *e*). Immunoblotting with antibody to the 37-kD protein against extracts of tissues (obtained by freezing tissue in liquid nitrogen, pulverizing with mortar and pestle, and extracting with Fairbanks' sample buffer con-
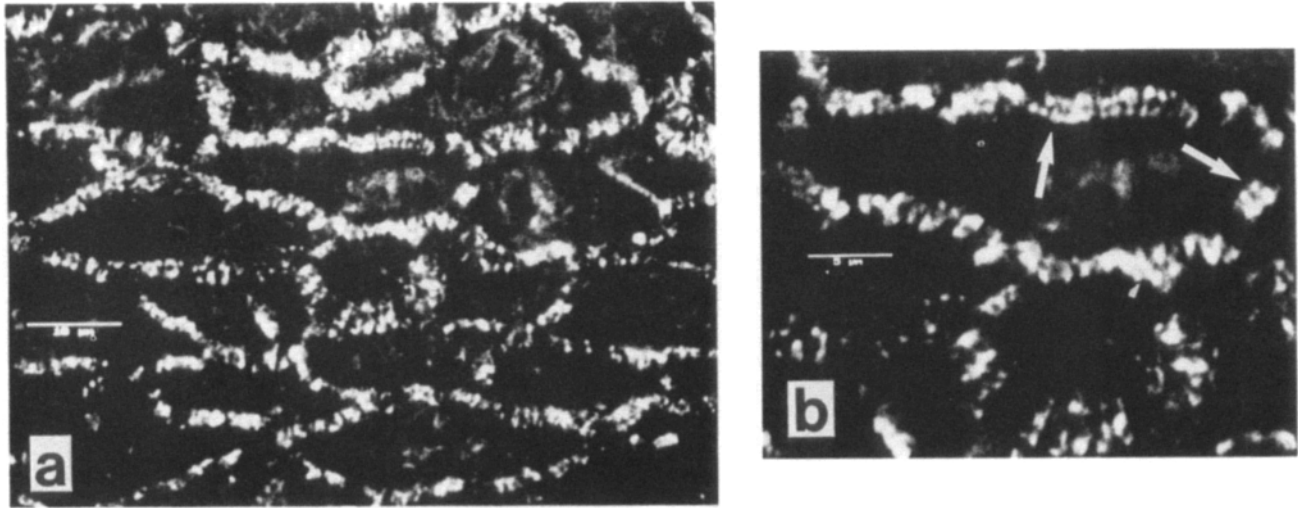
Figure 6. Confocal microscopy of hard palate epithelium showing localization of the 37-kD protein. Section stained with antibody to the 37-kD protein shows structures at cell periphery (*a*) and corresponding symmetrical structures in adjacent cells (*b*). Arrows in (*b*) indicate regions where symmetrical structures can be seen in apposed cells. Bars, (*a*) 10 μm; (*b*) 5 μm.

taining SDS and DTT) confirmed the presence of the 37-kD protein in epithelium from hard palate, esophageal mucosa, tongue, snout, and footpad and its absence from heart, liver, or epidermis from abdomen or back, thus confirming the immunofluorescence studies (data not shown).
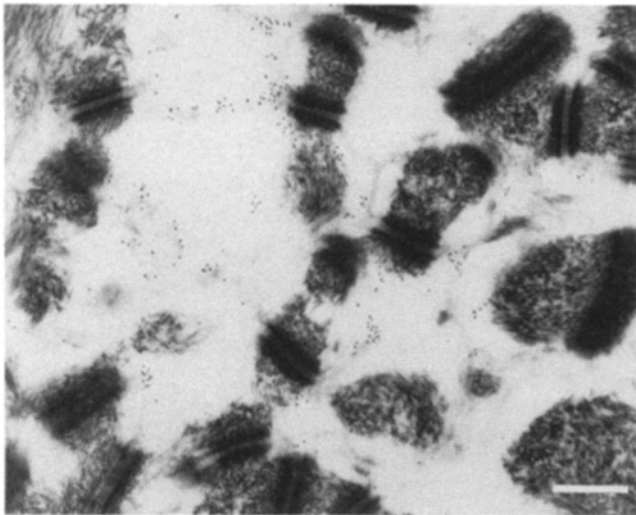

Figure 7. Immunoelectron microscopic localization of the 37-kD protein in pig snout. No desmosomes are labeled, and specific structures cannot be identified in the areas labeled, but the faint density associated with the labeling is compatible with a cell–cell junction. Bar, 0.25 μm.

Colocalization of the 37-kD protein with vinculin and uvomorulin (Boller et al., 1985) in the oral mucosa was consistent with an adherens junction–like distribution of the 37-kD protein. Although vinculin appeared to colocalize with the 37-kD protein in the oral epithelia, vinculin is well known to be present in other tissues containing adherens junctions such as the bile canaliculi of liver (e.g., Tsukita and Tsukita, 1989c), and is also present in epidermis (Fig. 8 *g*), where the 37-kD protein was not found by either immunofluorescence or immunoblotting, suggesting that, if the 37-kD protein is an adherens junction protein, it is present in only a subset of adherens junctions. The distribution of actin, another protein present in all known adherens junctions, was examined. Double immunofluorescence studies of actin and the 37-kD protein demonstrated that actin filaments were closely associated with the 37-kD protein (Fig. 9 *a* and *b*), further supporting the possibility that the 37-kD protein is a component of adherens junctions. As a control, we performed comparison studies of actin and desmoplakin I/II, which showed substantial lack of exact correspondence (Fig. 9 *c* and *d*), although both were located in the same region, consistent with proximity of the two distinct structures, adherens junctions and desmosomes, and consistent with previous investigations of these structures in epithelia (e.g., Geiger et al., 1983; Drenckhahn and Franz, 1986; O'Keefe et al., 1987).

A 37-kD protein has not been described in the literature in desmosome preparations, which are usually made from cow tissues. Rapidly frozen cow tongue epithelium did not

Figure 8. Colocalization of the 37-kD protein and uvomorulin or vinculin in oral epithelium. Sections of various pig tissues were processed for double immunofluorescence using antiserum (1:500) to the 37-kD protein (*a*, *c* and *e*) and mAb (1:50) to uvomorulin (*b*, *d*, and *h*) or vinculin (*f* and *g*). Identical sections are shown of tongue (*a* and *b*; *e* and *f*) or esophageal mucosa (*c* and *d*). Arrows in *e* and *f* indicate identical locations in corresponding panels; arrowheads in *c* and *d* and *e* and *f* also indicate location of the basement membrane. In *g* and *h*, arrows indicate basement membrane. Although there is nonspecific staining in *b*, *d*, and *f*, especially in subepidermal tissue, areas in epithelium specifically stained for uvomorulin or vinculin colocalize with staining for the 37-kD protein, and specific staining for uvomorulin and vinculin, like that for 37-kD, is absent in the basal layer. In epidermis (*g* and *h*), vinculin (*g*) and uvomorulin are present (*h*). Bar, 25 μm.
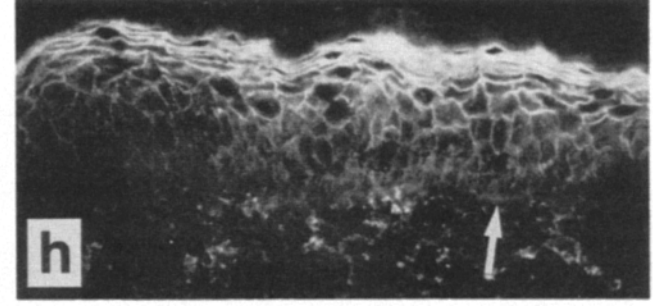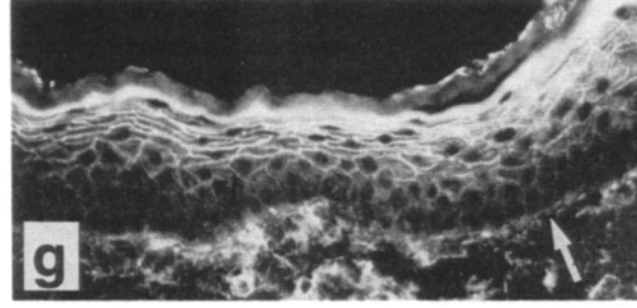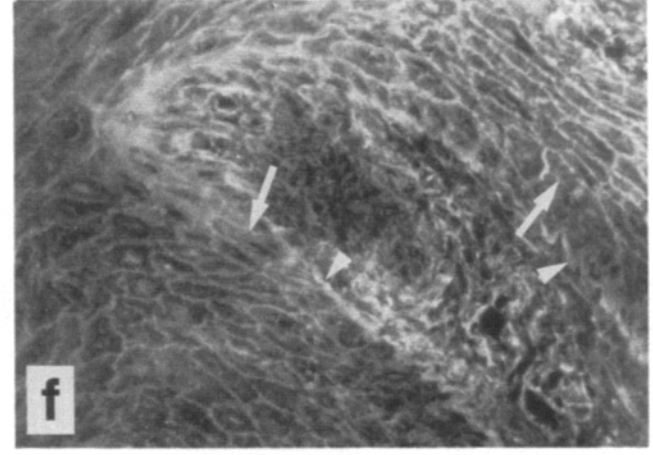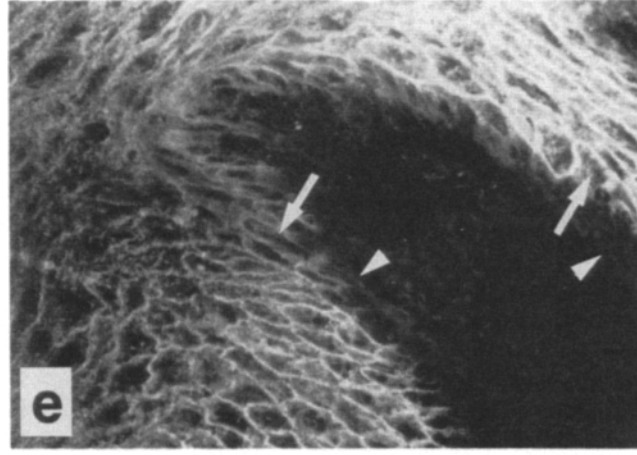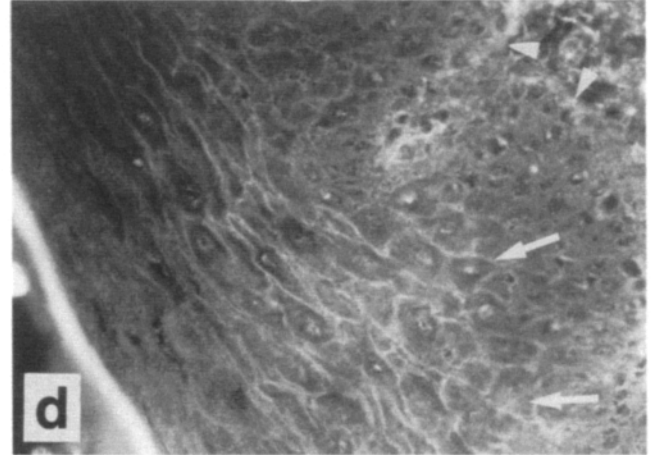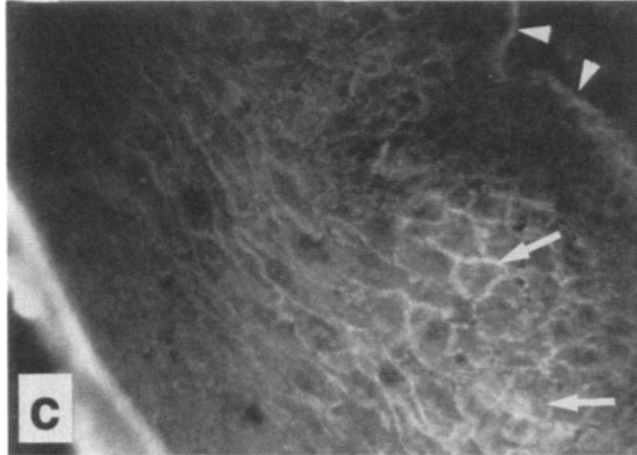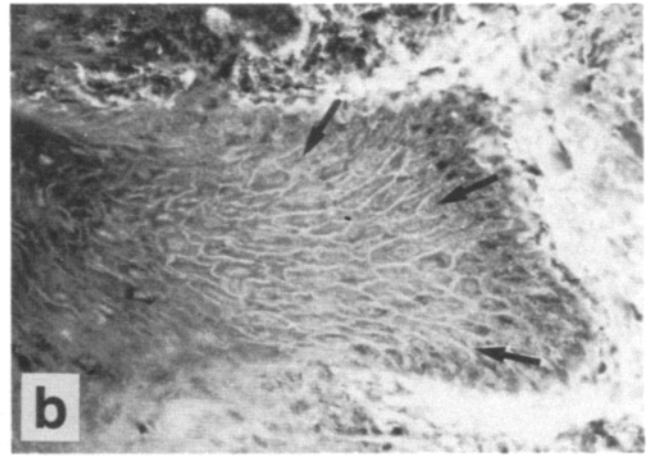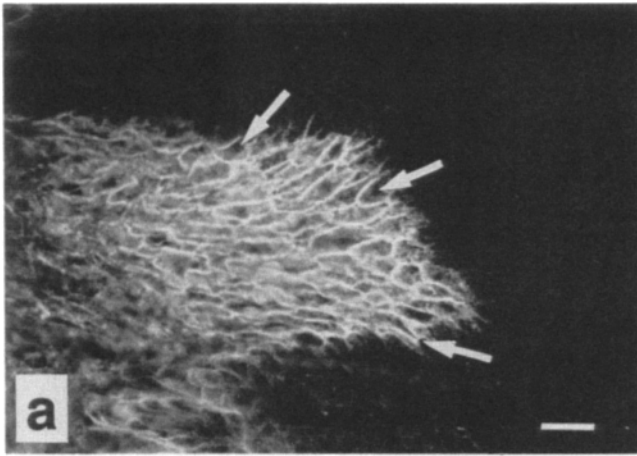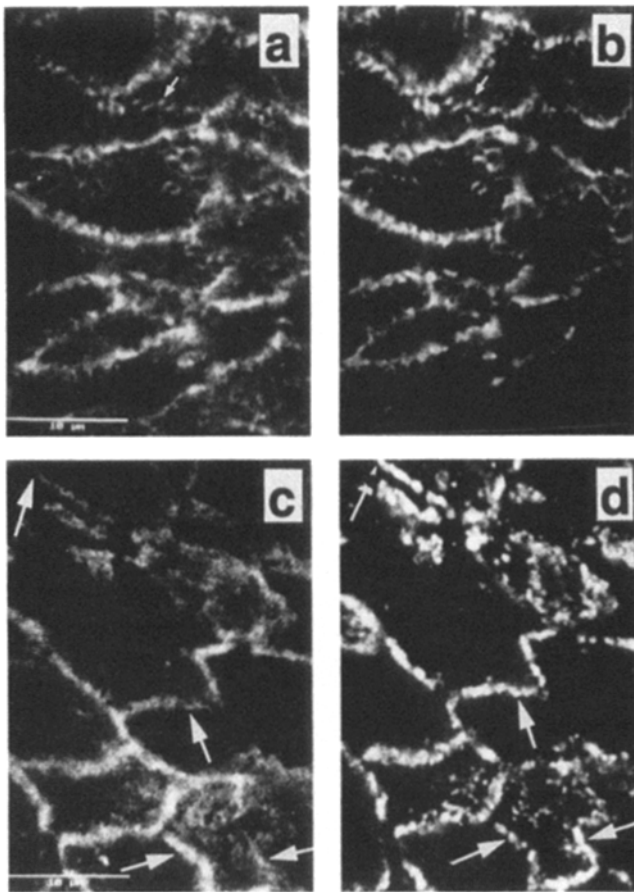
Figure 9. Colocalization of the 37-kD protein but not desmoplakin I/II with actin. Sections of hard palate were stained with rhodamine-phalloidin (*a* and *c*) and antibody to the 37-kD protein (*b*) or antibody to desmoplakin I/II (*d*) and examined by confocal microscopy. Arrow in (*a*) and (*b*) indicates region of correspondence of actin and 37-kD protein. Arrows in (*c*) and (*d*) indicate regions of noncorrespondence of actin and desmoplakin. Bar, 10 μm.

Figure 10. The 37-kD protein cannot be identified in bovine desmosomes. Coomassie blue (*CB*) stained SDS–polyacrylamide gel of desmosome preparation from pig (*P*) and cow (*C*) with purified 37-kD (*37K*) protein for comparison. Immunoblot (*IB*) of pig (*P*) and cow (*C*) desmosome preparation incubated with antibody to the 37-kD protein.

contain the 37 kD by immunoblotting (not shown). Furthermore, a cow tongue desmosome preparation did not show a significant band in the range of 37 kD (Fig. 10 *a*, lane *C*), and immunoblotting showed no cross-reacting band of any size (Fig. 10 *b*, lane *C*), suggesting that the function of the 37-kD protein, if a corresponding protein is present in bovine tissue, is served by a noncross-reacting protein probably of different molecular weight.

Discussion

Possible Origin of the 37-kD Protein

The 37-kD protein appears to be a junctional protein. Like desmosomes and also like gap junctions, the structure of which it is a component was very insoluble, requiring 9 M urea for solubilization from the citric acid-insoluble pellet. Furthermore, SDS was required to dissociate it from other solubilized proteins, because even in 9 M urea the 37-kD protein ran in the void volume of a Fractogel column unless SDS was added, in which case it was included in the gel. The
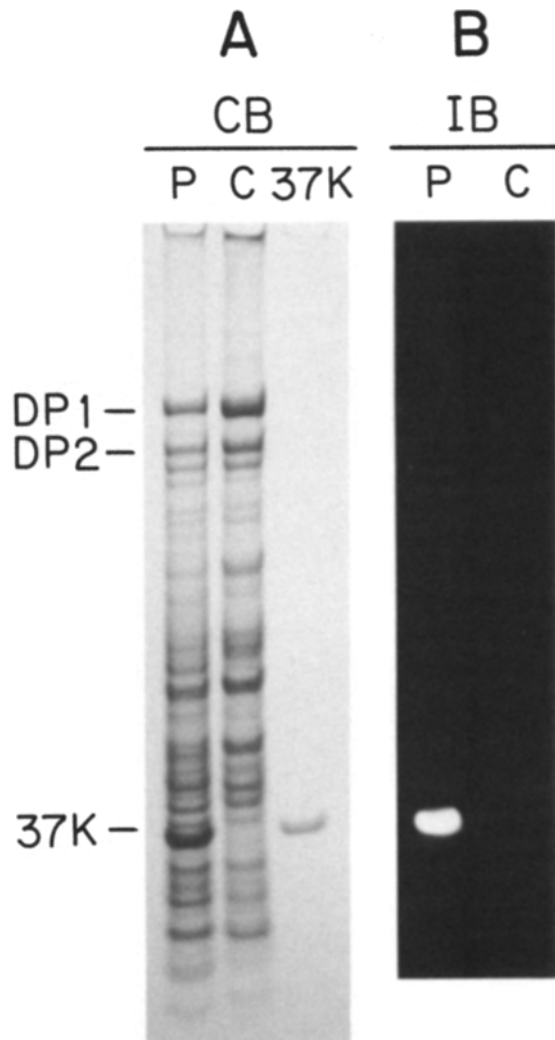
immunofluorescence staining pattern was consistent with that of an adherens junction protein. By confocal microscopy antibody to the 37-kD protein decorated cell–cell junctions, was discontinuous, and was symmetrical in some sections in adjacent cells, and colocalized with adherens junction proteins actin, vinculin, and uvomorulin.

Although the 37-kD protein was isolated from a fraction rich in desmosomes, it is probably not a desmosome protein. The 37-kD protein was absent from the basal layer of epithelial cells, whereas desmoplakin I/II was present in both basal and suprabasal cells, suggesting that the two proteins are in different structures. Localization of a desmosomal protein to limited layers of the epithelium has not been described; desmoplakin II, once thought to be absent from simple epithelia and some cell lines, has recently been shown to be present in these tissues (Angst et al., 1990). This would not exclude the possibility, however, that the 37-kD protein is an acces-

sory protein present only in suprabasal desmosomes. The most compelling evidence against the possibility that the 37-kD protein is a desmosome protein comes from immuno-electron microscopy, which shows that the immunolabeled areas are excluded from desmosomes.

The electron micrographs of immunolabeled tissue did not show detail of any structure with which the 37-kD protein might be associated, although the label did appear to be localized at cell–cell junctions interspersed with desmosomes. In immunofluorescence studies, the limitation of the staining of uvomorulin and vinculin to the suprabasal layers of the oral epithelium corresponded to the identical or very similar limitation of the 37-kD protein and suggested that the protein has a distribution similar to that of adherens junctions, and colocalization with actin strongly supported this possibility. Adherens junctions have been shown to be present in keratinocytes in vitro (Green et al., 1987; O'Keefe et al., 1987) and in some epithelia in vivo (Drenckhahn and Franz, 1986; Zieske et al., 1989).

It is not surprising that preparations containing desmosomes from epithelium might also contain other types of junctions, because the tissue is insoluble and therefore difficult to fractionate. The recent purification of adherens junctions (Tsukita and Tsukita, 1989c) also yielded structures in the initial purification step (bile canaliculi) containing desmosomes as well as adherens junctions, although desmosomes were substantially removed in later steps. The insolubility of the junctional protein complexes may well result in the enrichment of several types of junction by procedures that remove most soluble proteins. Extensive attempts to separate the junctions containing the 37-kD protein from desmosomes by differential centrifugation, using the 37-kD protein and desmoplakins as markers, produced only modest enrichment as judged by intensity of Coomassie blue staining of desmoplakins I and II and the 37-kD protein on SDS polyacrylamide gels (data not shown). The desmosome-rich junction preparation was found by immunoblotting to contain vinculin (not shown), further supporting the idea that the preparation contains both desmosomes and adherens junctions.

Proteins associated with the 37-kD protein in the purification procedure may provide clues about the nature of the relevant junction, because, as noted above, the 37-kD protein in the 9 M urea extract behaved as a macromolecular complex in the absence of SDS; proteins of the complex could be purified and studied. Also, overlays (Fig. 4) indicated that the 37-kD protein binds to a distinctive group of proteins in the citric acid–insoluble pellet used as starting material; these proteins were not identified by binding of keratin filaments or purified desmoplakin I and may be specifically associated with the 37-kD protein. Efforts to determine whether one of these proteins is uvomorulin, which has been shown to be present in desmosome preparations (Jones, 1988), have been inconclusive because of apparent failure of polyclonal and mAb to react with pig uvomorulin on western blots. Antibody to vinculin, however, identified a 130-kD band on blots of immobilized 9 M urea extract; whether vinculin is one of the 37-kD protein-binding proteins is under investigation. As mentioned above, attempts to immunoprecipitate the 37-kD protein to examine associated proteins were unsuccessful because of the insolubility of the junctions.

Unusual Restricted Distribution of the 37-kD Protein

The distribution of the 37-kD protein is unusual if not unique for known junction proteins. First, it is present in oral stratified epithelia but not in many regions of another stratified epithelium, the epidermis. Second, it is present only in suprabasal layers of the oral epithelium. This type of distribution was also found for vinculin and uvomorulin in our studies and suggests that the 37-kD protein is an adherens junction protein. Actin, which is more widely distributed in the epithelium than the 37-kD protein and is also present in other tissues, colocalized with the 37-kD protein in cells staining for this protein. The presence of the 37-kD protein in adherens junctions in oral epithelia but not in other tissues such as liver implies a heterogeneity of the component proteins of adherens junctions in different tissues. Heterogeneity of proteins has been described for adherens junctions of lens and a corresponding structure of muscle, the myotendinous junction, by Geiger et al. (1985). Lens adherens junctions were found to contain a 135-kD protein that was absent in the muscle junction; conversely, adherens junctions in lens lacked talin, but talin was present in muscle. The heterogeneity we describe between regions and layers of stratified squamous epithelium, however, has not been documented previously with respect to either adherens junctions or desmosomes.

The restricted distribution to specific layers and tissues within stratified squamous epithelium found for the 37-kD protein has not been demonstrated previously for a junction protein. The apparent restriction of uvomorulin and vinculin staining in our studies suggests that adherens junctions may be limited to suprabasal layers in some stratified epithelia, but our data do not establish this with certainty; more detailed studies with additional antibodies, preferably polyclonal and raised against protein of the same species, would be required. A different kind of restriction in distribution has been described for the desmosomal band 6 protein, an accessory protein of desmosomes, which was found to be restricted to certain epithelia and was absent in the basal layer of the epithelium (Kapprell et al., 1988). Possible limitation of adherens junctions to suprabasal epithelial cells is also suggested by the figures of Drenckhahn and Franz (1986) but has not been studied in detail.

Although the molecular weight of the 37-kD protein is in the range of that of the major gap junction proteins, the connexins, amino acid sequences of tryptic digests as well as preliminary cDNA sequence data indicate no homology with connexins (data not shown). Although the amino terminus of the 37-kD protein appears to be blocked, tryptic digests of the protein yielded sequences which were unique when compared with sequences in the GenBank and EMBL databases with the University of Wisconsin Genetics Computer Group software.

Even though our antibodies do not identify a protein homologous to the 37-kD protein in other species tested, it is likely that the function subserved by the protein in the pig requires a homologous protein in the oral epithelia of other species, because the 37-kD protein is a major protein of the oral epithelium. Species-specific isotypes having limited antibody cross-reactivity have been described for other structural proteins, e.g., ankyrin (Bennett, 1979). Because of the unique distribution of this major protein of oral junctional

preparations, it will be important to determine whether there is a homologous protein in other species. Whether the protein shows homologies with other junction proteins in the pig or other species as well as other important clues to its structure and identity may be determined by molecular cloning studies now under way. Purification of proteins in the junction preparation associated with the 37-kD protein may also yield important information.

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