Evidence That Major 78–44-kD Concanavalin A–binding Glycopolypeptides in Pig Epidermis Arise from the Degradation of Desmosomal Glycoproteins during Terminal Differentiation

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Abstract. The major concanavalin A (Con A)-binding component in urea/deoxycholate/mercaptoethanol extracts from pig ear epidermis had an apparent M_r of 78 kD. In indirect immunofluorescence affinity-purified polyclonal antibodies against this glycopolypeptide strongly stained the surface of suprabasal cells in the epidermis of pig and human skin. Immunocytochemical labeling with gold-labeled second antibody localized this staining to externally disposed, trypsin-sensitive components of desmosomes.

Western blotting showed that the 78-kD glycopolypeptide was immunologically related to several other Con A-binding components in pig epidermis. Immunoreactive components with M_r of 115 and 100 kD were membrane-bound, appeared to be susceptible to trypsin in intact epidermis, and were absent from the stratum corneum. Immunoreactive components of lower M_r (78–44 kD) were not membrane-bound, were resistant to trypsin in intact tissue, and were present predominantly in the keratinized layers of pig epidermis. The 115–44-kD glycopolypeptides were also recognized by antisera raised against desmoglein II/desmocollin glycoproteins isolated from bovine spinous layer

desmosomes. In addition, these antisera reacted with 120- and 105-kD bands that were apparently not recognized by the anti-78-kD glycopolypeptide antiserum in immunoblotting.

In immune precipitation the anti-78-kD glycopolypeptide and antidesmoglein II/desmocollin antisera precipitated comparable amounts of the radioiodinated 78–44-kD components. Both antisera also precipitated the 120- and 105-kD components although the anti-78-kD glycopolypeptide serum was less effective. Little reaction with the 115- and 105-kD components was observed in immune precipitation with either serum. Proteolytic peptide mapping confirmed that the various immunoreactive glycopolypeptides were biochemically as well as immunologically related.

The results suggest that terminal differentiation in pig epidermis is accompanied by the orderly degradation of desmoglein II/desmocollin glycoproteins resulting in the accumulation of 78–44-kD glycopolypeptides in the stratum corneum. These glycopolypeptides may represent functionally important nonmembranous domains of cell-adhesion molecules in desmosomes.

DESMOSOMES are specialized cell surface organelles involved in the adhesion of epithelial cells to one another (2, 10, 34). They are present in most epithelial tissues but are particularly abundant in keratinizing epithelia such as the epidermis. Ultrastructural studies have shown that the migration of epidermal cells out of the basal layer and through the spinous layer involves the breakdown and reformation of desmosomal junctions between adjacent cells (1, 31, 36). In the latter stages of keratinization, desmosomes are subject to considerable modification (1, 15, 30, 31, 36). Desmosome degradation starts in the granular layer and the subsequent loss of desmosome-mediated cell adhesion may be important in controlling the desquamation of cornified cells from the stratum corneum. The molecular changes associated with desmosome turnover and degradation have not yet been elucidated.

The glycoprotein components of desmosomes are of considerable interest because of their presumed role in epithelial cell-cell adhesion. Biochemical and immunologic characterization of desmosomal glycoproteins have been reported by several groups (9, 11–14, 19, 22). Most of these studies have used the spinous layer of bovine muzzle epidermis as starting material. In most cases the tissue was solubilized with 0.1 M citric acid, pH 2.6, as originally described by Skerrow and Matoltsy (32, 33). Gorbsky and Steinberg (19) introduced a modification that allowed the isolation of desmosomal "cores" that were depleted of plaque material and enriched in intercellular glycoproteins. Although there are differences in the reported M_r of individual components, there is general agreement that these cores contained at least two distinct glycoprotein species when analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-

PAGE). Desmoglein I, the larger desmosomal glycoprotein, had an M_r of 150 kD (9, 11, 14) or 164–175 kD (22). The other glycoprotein species, desmoglein II (3, 17) or desmocollin (12), comprised two bands of M_r 118 and 97 kD (9), 115 and 100 kD (11–13), or 130 and 115 kD (21) that were biochemically and immunologically related but were distinct from the larger glycoprotein. Studies on Madin-Darby bovine kidney cells have provided evidence that the desmoglein II/desmocollin glycoproteins may be directly involved in cell–cell adhesion (12).

We have been investigating the molecular basis for the changes in lectin-binding associated with keratinocyte differentiation (5, 26) and have devised procedures for isolating the major lectin-binding glycoproteins of pig epidermis (24). In this report we show that antibodies against a major 78-kD Con A-binding glycopolypeptide specifically stain desmosomes in pig epidermis. We present evidence that this apparently nonmembranous glycopolypeptide arises from the degradation of larger membrane-bound desmosomal glycoproteins during terminal epidermal differentiation.

Materials and Methods

Isolation of Con A-binding Glycoproteins

Con A-binding glycoproteins were isolated from the residue fraction of $CaCl_2$-separated pig ear epidermis as described previously (24) except that 1 mM phenylmethylsulfonyl fluoride (PMSF) was included in the extraction buffer. In some experiments epidermis was obtained by treating skin slices with 0.1% Dispase (Boehringer, Mannheim, Federal Republic of Germany) in phosphate-buffered saline (PBS), pH 7.4, for 30 min at 37°C, and the separated epidermis was washed several times with PBS. Briefly, tissue was extracted with 8 M urea/1% sodium deoxycholate/1% 2-mercaptoethanol/1 mM PMSF/10 mM Tris-HCl, pH 7.5, dialysed against 1% sodium deoxycholate/0.25 mM PMSF/10 mM Tris-HCl, pH 7.5, and extracts were incubated with Con A-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden). After extensive washing, glycoproteins were eluted with 5% α-methyl mannoside (Sigma Chemical Co., Poole, UK) in 1% sodium deoxycholate/0.25 mM PMSF/10 mM Tris-HCl, pH 7.5, precipitated with ethanol, and resuspended in H_2O. In some experiments sodium deoxycholate was omitted from the extraction and dialysis buffers.

Anti-78-kD Glycopolypeptide Antibodies

Con A-binding glycoproteins (1–2 mg) were separated on 6% polyacrylamide slab gels. The outer lanes were stained for protein to localize the 78-kD band which was excised. Initially guinea pigs were immunized with gel strips homogenized in complete Freund's adjuvant and boosted with gel in incomplete adjuvant. In later experiments the 78-kD glycopolypeptide was electrophoretically eluted (2 mA per tube, overnight) from unstained gel strips that had been transferred to 0.8 × 12-cm elution tubes and incorporated into 5% polyacrylamide. Eluates were dialysed against H_2O and precipitated with 10 vol of acetone at −40°C before injection into guinea pigs. The antisera produced by these two methods showed identical reactivity in immunoblotting and immunofluorescence. Guinea pigs were bled by cardiac puncture after two or three boosts.

Anti-78-kD glycopolypeptide antibodies were affinity-purified by absorption onto nitrocellulose strips containing the 78-kD glycopolypeptide (∼0.5 mg of protein/ml of serum) and elution as described previously for other epidermal glycoproteins (24). Absorbed controls were prepared by reabsorbing these affinity-purified antibodies twice with the 78-kD glycopolypeptide.

Antidesmoglein II/desmocollin antisera were kindly provided by Drs. E. Penn and T. McGee of the National Institute of Medical Research, Mill Hill, UK. These antisera were raised in guinea pigs against the 117/97-kD glycoproteins present in desmosomes isolated from the spinous layer of cow snout epidermis.

SDS-PAGE and Immunoblotting

Electrophoresis was usually carried out on 7% polyacrylamide slab gels

(25) which were stained with Coomassie Blue. SDS sample buffer contained 2% SDS, 10% glycerol, 0.01% bromophenol blue, 5% 2-mercaptoethanol, 0.05 M Tris-HCl, pH 6.8. Electrophoretic transfer to nitrocellulose (35), staining with amido black, and reaction with antibodies and ^{125}I-labeled protein A were essentially as described (24) except that 5% bovine serum albumin (BSA) was used for diluting antibodies and protein A, and 0.05% Tween 20 in PBS was used for washing. Two-dimensional gel electrophoresis was performed as described by O'Farrell et al. (27) using nonequilibrium pH gradient electrophoresis in the first dimension.

Whole Epidermal Extracts

Epidermis from pig ear and other body sites was obtained by scraping with a scalpel blade or by treating skin slices with either 1 M $CaCl_2$ or 0.1% Dispase. The tissue was chopped finely, boiled for 5 min with 10 vol of SDS sample buffer, and incubated overnight at 37°C. Large tissue fragments were removed by filtration through coarse nylon gauze and extracts were centrifuged at 100,000 g for 1 h.

Effect of Trypsin

Pig skin slices were treated with 0.1% Dispase for 30 min and washed in PBS. The epidermis was floated on 0.25% trypsin in PBS at 37°C for 30 min and vortexed to release epidermal cells, and the mixture was filtered through coarse nylon gauze. Trypsinization of the residual epidermis was repeated up to three times. The residual epidermis and pooled cell pellets were washed several times with PBS and solubilized with SDS sample buffer as above.

Tape Stripping

A marked area of pig skin was repeatedly stripped (30–40 times) with adhesive tape to remove the stratum corneum. The tape was boiled in 2% SDS/1% 2-mercaptoethanol for 5 min and incubated overnight at 37°C. The solubilized material was dialysed against several changes of 0.1% SDS, freeze-dried, redissolved in SDS sample buffer, and centrifuged at 100,000 g for 1 h.

Indirect Immunofluorescence

Cryostat sections of frozen tissue (24) were stained unfixed or after 10 min of treatment with freshly prepared 2% paraformaldehyde in PBS. Sections were incubated with normal or immune serum (up to 1:100 dilution) or with affinity-purified antibodies or the absorbed control (up to 1:10 dilution) for 30 min. After washing sections were incubated with fluorescein-conjugated swine anti-guinea pig IgG (1:50) for 30 min, washed, and mounted as before (24).

Immunocytochemical Labeling

1-mm cubes of tissue and cell pellets were fixed in 2% paraformaldehyde for 10 min. Samples were dehydrated and embedded in Lowicryl K4M resin (Agar Scientific, Stansted, UK) using a modification (16) of the method of Roth et al. (29). Immunolocalization was carried out on the surface of ultrathin sections mounted on copper grids coated with parlodion (Agar Scientific). Sections were preincubated with 1% BSA in PBS (30 min) before incubation with affinity-purified antibody (1 h). Control grids were incubated with PBS or with absorbed antibody. After washing with 1% BSA in PBS (three times for 5 min), sections were incubated for 1 h with goat anti-guinea pig immunoglobulin-coated gold (15 nm, Janssen Pharmaceutica Ltd, Wantage, UK). Washed grids were stained with aqueous uranyl acetate and lead citrate.

Immune Precipitation

Con A-binding glycoproteins (∼1 mg) were radiolabeled with 1 mCi of carrier-free $Na^{125}I$ (Amersham International, Little Chalfont, UK) using chloramine-T (21) with an efficiency of 40–50%, dissolved in 1% SDS, and dialysed against two changes of 1% SDS. The glycoprotein solution was diluted with 10 vol of immune precipitation buffer (1% NP-40, 1 mM EDTA, 1 mM PMSF, 0.15 M NaCl, 20 mM Tris-HCl, pH7.5). A sample was precipitated with 10 vol of acetone at −40°C and redissolved in SDS sample buffer. Other 1-ml aliquots were incubated with normal guinea pig serum (10 μl) for 1 h at 4°C and were then incubated for 1 h with 100 μl of protein A-agarose (Bio-Rad Laboratories, Watford, UK) that had been washed in immune-precipitation buffer. After removing the beads by low-speed cen-
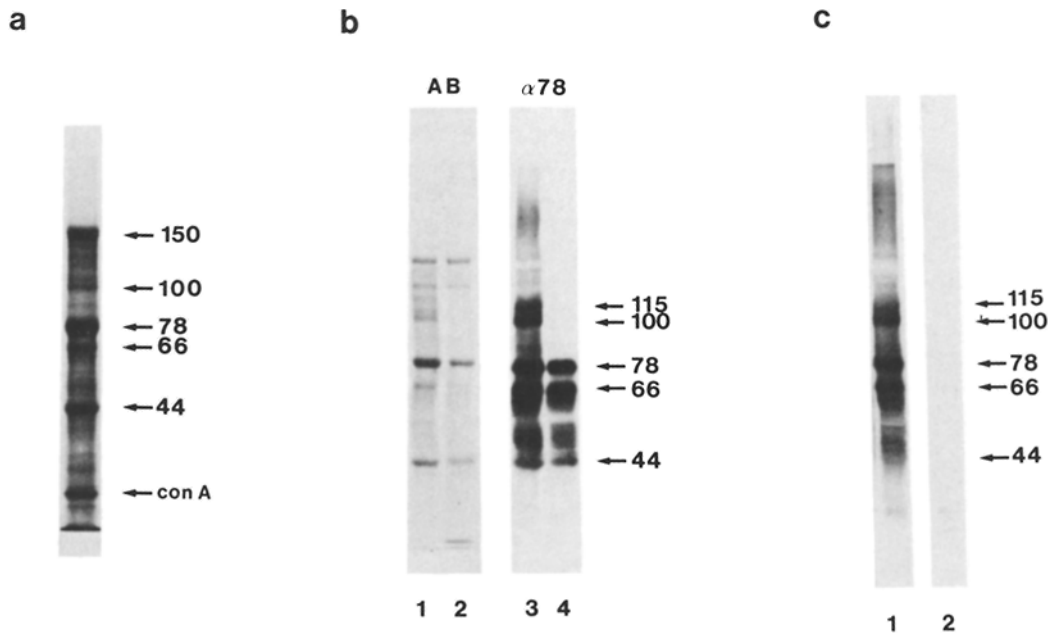
Figure 1. SDS-PAGE and immunoblotting of Con A-binding glycoproteins from $CaCl_2$-separated pig ear epidermis. (*a*) Con A-binding glycoproteins (100 µg) from urea/deoxycholate/mercaptoethanol extract of the epidermis stained with Coomassie Blue. Arrows indicate the major glycopolypeptide components. This gel was deliberately overloaded to reveal minor constituents. (*b*) Con A-binding glycoproteins (60 µg per lane) from urea/deoxycholate/mercaptoethanol extracts (lanes *1* and *3*) and urea/mercaptoethanol extracts (lanes *2* and *4*) of the epidermis were transferred to nitrocellulose and duplicate strips were stained with amido black (*AB*) or reacted with anti–78-kD glycopolypeptide antiserum (1:500) followed by ^{125}I-labeled protein A (α78). No reaction was seen with normal guinea pig serum. Arrows indicate that major immunoreactive components. (*c*) Immunoblot showing the reaction of affinity-purified anti–78-kD glycopolypeptide antibodies (1:20) with Con A-binding glycopolypeptides from urea/deoxycholate/mercaptoethanol extracts of the epidermis before (lane *1*) and after (lane *2*) absorption with the 78-kD glycopolypeptide. Note that absorption removes reactivity against all the immunoreactive components (*arrows*) in the preparation.

trifugation the supernatant was further incubated with 10 µl of antiserum and 100 µl of protein A-agarose. The beads were washed three times with immune-precipitation buffer, once with 20 mM Tris-HCl, pH 7.5, and immune complexes were eluted by boiling in SDS sample buffer.

### Proteolytic Peptide Mapping

Con A-binding glycoproteins were radioiodinated as above, dissolved in SDS sample buffer, and separated on a 6% polyacrylamide gel which was stained with Coomassie Blue and rapidly destained. Individual polypeptide bands were excised, equilibrated, and subjected to peptide mapping (8) on a 15% polyacrylamide gel using 0.1 µg per well of V8 protease from *Staphylococcus aureus*. Iodinated bands were detected by fluorography (4).

Results

Fig. 1 *a* shows the polypeptide composition of the Con A-binding material isolated from $CaCl_2$-separated pig epidermis. Similar patterns were seen in preparations from Dispase-separated epidermis. The 150-kD component consistently observed in these preparations has been identified as a glycosylated subunit of type VI collagen which is the major Con A-binding component in the dermal compartment of the skin (manuscript in preparation). It probably reflects incomplete separation of papillary dermis from the $CaCl_2$-separated epidermal sheets.

The other major Con A-binding component in pig epidermis had an apparent M_r of 78 kD. To characterize this glycopolypeptide it was isolated on preparative gels and the purified material that migrated as a single spot during re-electrophoresis on two-dimensional gels was used to immunize guinea pigs. Fig. 1 *b* shows the reactivity of the

resulting antisera when tested against isolated Con A-binding glycopolypeptides by immunoblotting. In addition to recognizing the 78-kD glycopolypeptide, the antisera consistently recognized Con A-binding components within a wide range of apparent M_r (115–44 kD). Immunoreactive components with M_r of 115 and 100 kD were present in urea/deoxycholate/mercaptoethanol extracts of the epidermis but were not extracted by urea/mercaptoethanol solutions containing no detergent. They are therefore likely to be membrane-bound components. The 115-kD band detected by immunoblotting did not comigrate precisely with either of the amido black-stained polypeptides (M_r 120 and 105-kD) detected in this region of the gel. This immunoreactive component is therefore likely to be a relatively minor component in our glycoprotein preparations. In contrast, the immunoreactive band and the amido black-stained band at 100 kD showed a much closer correspondence. The antisera also reacted with several major Con A-binding components in the M_r range 78–44 kD. In contrast to the high M_r immunoreactive components, the extraction of the 78-kD glycopolypeptide itself and of the lower M_r-immunoreactive components was little affected by the absence or presence of detergent (Fig. 1 *b*). Affinity-purified antibodies were prepared using nitrocellulose strips containing the isolated 78-kD glycopolypeptide as immunoabsorbent. In immunoblotting (Fig. 1 *c*) these antibodies showed identical reactivity to the original antisera recognizing the same Con A-binding components in the M_r range 115–44 kD. Immune reactivity against all these components was abolished by reabsorbing these antibodies with the

a

b

c
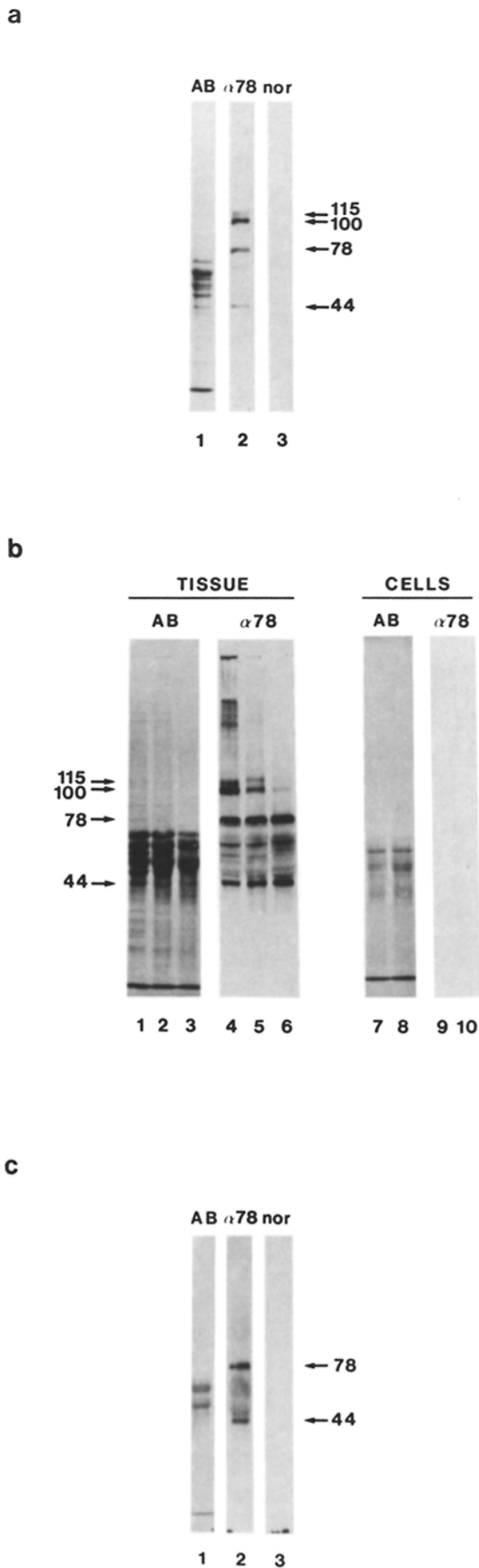
isolated 78-kD glycopolypeptide (Fig. 1 c), suggesting that the 115–44-kD Con A-binding components were closely related immunologically.

It was possible that these various immunoreactive components had arisen by proteolytic modification of larger glycoproteins during tissue extraction and dialysis prior to the isolation of Con A-binding material. To check this possibility immunoblotting was carried out on epidermis that had been solubilized directly in SDS sample buffer. As found when tested against isolated Con A-binding glycopolypeptides (Fig. 1), the antiserum reacted with several tissue components in the M_r range 115–44 kD. Fig. 2 a shows an immunoblot of SDS sample buffer-solubilized pig ear epidermis that had been obtained by scraping intact skin with a scalpel blade. Similar blots were obtained with $CaCl_2$- and Dispase-separated epidermis. Identical blotting patterns were observed in SDS-solubilized epidermis from diverse body sites including the back, belly, ear, snout, tail, and leg. The immunoreactive 115-, 100-, 78-, and 44-kD components seen in isolated glycoprotein preparations were clearly detected in whole epidermal extracts (Fig. 2 a). Immunoreactive components between 78 and 44 kD were present, but these bands were distorted by the large amounts of keratin polypeptides solubilized under these conditions. The 115- and 100-kD components often appeared to be more prominent in tissue solubilized with SDS sample buffer. Whether this difference reflected more efficient extraction of the higher-M_r components in the presence of SDS or less efficient extraction of the lower-M_r components in the absence of urea remains to be established. Fig. 2 a also shows an immunoblot obtained with normal guinea pig serum. Although a low level of antikeratin antibodies was seen, antibodies reacting with epidermal glycopolypeptides were not detected in normal serum.

To examine the distribution of these various immunoreactive components within the tissue, immunoblotting was per-

Figure 2. SDS-PAGE and immunoblotting of various fractions from pig ear epidermis solubilized in SDS sample buffer. (a) Whole epidermis scraped from intact skin was subjected to SDS-PAGE and transferred to nitrocellulose, and duplicate strips were either stained with amido black (AB, lane 1), or incubated with anti-78-kD glycopolypeptide antiserum diluted 1:100 (α78, lane 2) or normal guinea pig serum (nor, lane 3) followed by ^{125}I-labeled protein A. (b) Dispase-separated pig ear epidermis was trypsinized up to three times and the trypsin-released cells were collected by centrifugation. After washing, the residual epidermis and cell pellets were solubilized, subjected to SDS-PAGE, and transferred to nitrocellulose, and duplicate strips were stained with amido black (AB) or incubated with anti-78-kD glycopolypeptide serum (α78) and ^{125}I-labeled protein A. Lanes 1 and 4 show intact Dispase-separated epidermis. Lanes 2 and 5 and 7 and 9 show the residual epidermis and cells released after a single trypsinization. Lanes 3 and 6 and 8 and 10 show residual epidermis and cells released after three trypsin treatments. (c) Stratum corneum removed by tape stripping was solubilized, subjected to SDS PAGE, and transferred to nitrocellulose, and duplicate strips were stained with amido black (AB, lane 1) or incubated with anti-78-kD glycopolypeptide serum (α78, lane 2) or normal guinea pig serum (nor, lane 3) followed by ^{125}I-labeled protein A. Arrows indicate the major immunoreactive components.
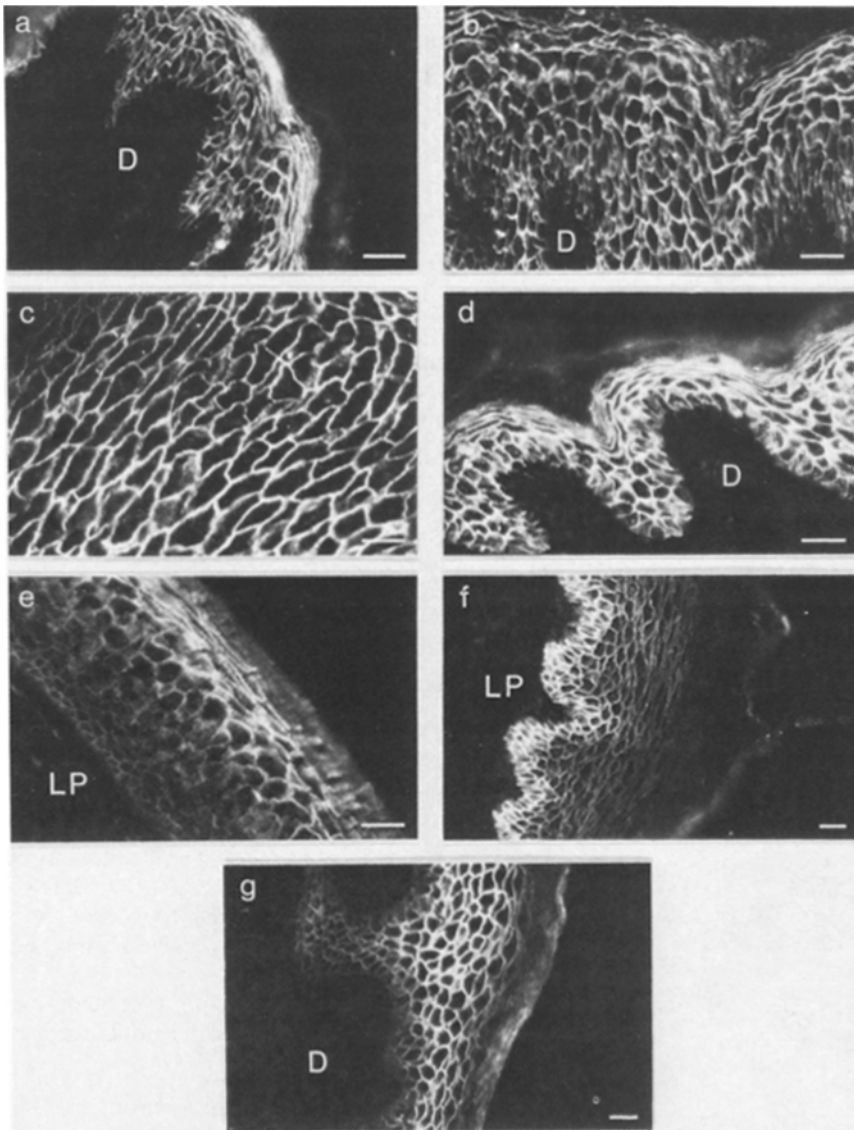
Figure 3. Indirect immunofluorescence of pig ear skin (*a*), pig back skin (*b*), pig snout skin (*c*), pig leg skin (*d*), pig cornea (*e*), pig esophagus (*f*), and human foreskin (*g*) showing specific labeling of the surface of cells in various stratified epithelia. Unfixed sections were stained with affinity-purified anti–78-kD glycopolypeptide antibodies diluted 1:10. Note the absence of staining in the dermis (*D*) or lamina propria (*LP*). Control sections incubated without primary antibody or with absorbed antibody were not stained. Bars, 20 μm.

formed on epidermis that had been subjected to varying degrees of trypsinization. Treatment with trypsin cleaves desmosomes between cells in the lower layers of the epidermis resulting in the dispersal and release of viable keratinocytes. Trypsin does not disrupt adhesion between the more keratinized cells in the stratum corneum. Fig. 2 *b* shows immunoblots comparing Dispase-separated epidermis before and after trypsin treatment. The 115- and 100-kD immunoreactive components were clearly susceptible to proteolysis in intact tissue because the level of these components was progressively reduced during trypsinization (Fig. 2 *b*, lanes *4–6*). They were not detected in those keratinocytes released during trypsin treatment (Fig. 2 *b*, lanes *9* and *10*). These components are therefore likely to be located at the surface of cells in the living layers of the epidermis where they are degraded to low-M_r peptides by trypsin. In contrast, the 78–44-kD immunoreactive components were resistant to proteolysis in intact tissue. These components were quantitatively recovered and even enriched in the residual epidermis remaining after extensive trypsinization (Fig. 2 *b*,

lanes *4–6*). They were not present in keratinocytes that were released by trypsin (Fig. 2 *b*, lanes *9* and *10*). These results suggested that the 78–44-kD components were present in those keratinized layers of the epidermis that were not disrupted by trypsinization. To confirm this, immunoblotting was performed on stratum corneum removed by stripping with adhesive tape (Fig. 2 *c*). The stratum corneum contained the lower-M_r immunoreactive components but the 115- and 100-kD components were never detected.

In indirect immunofluorescence of unfixed pig skin sections the anti–78-kD glycopolypeptide antibodies gave strong staining of the surface of cells throughout the living layers of the epidermis (Fig. 3). Essentially similar results were obtained with the original antiserum and affinity-purified antibodies. Similar cell surface staining was seen in pig epidermis from a variety of different body sites (Fig. 3, *a–d*). The cells in the basal layer were usually less strongly stained than those in the suprabasal layers. Staining was usually seen on the lateral surfaces of basal cells but the surfaces adjoining the basement membrane were never stained. Cell surface
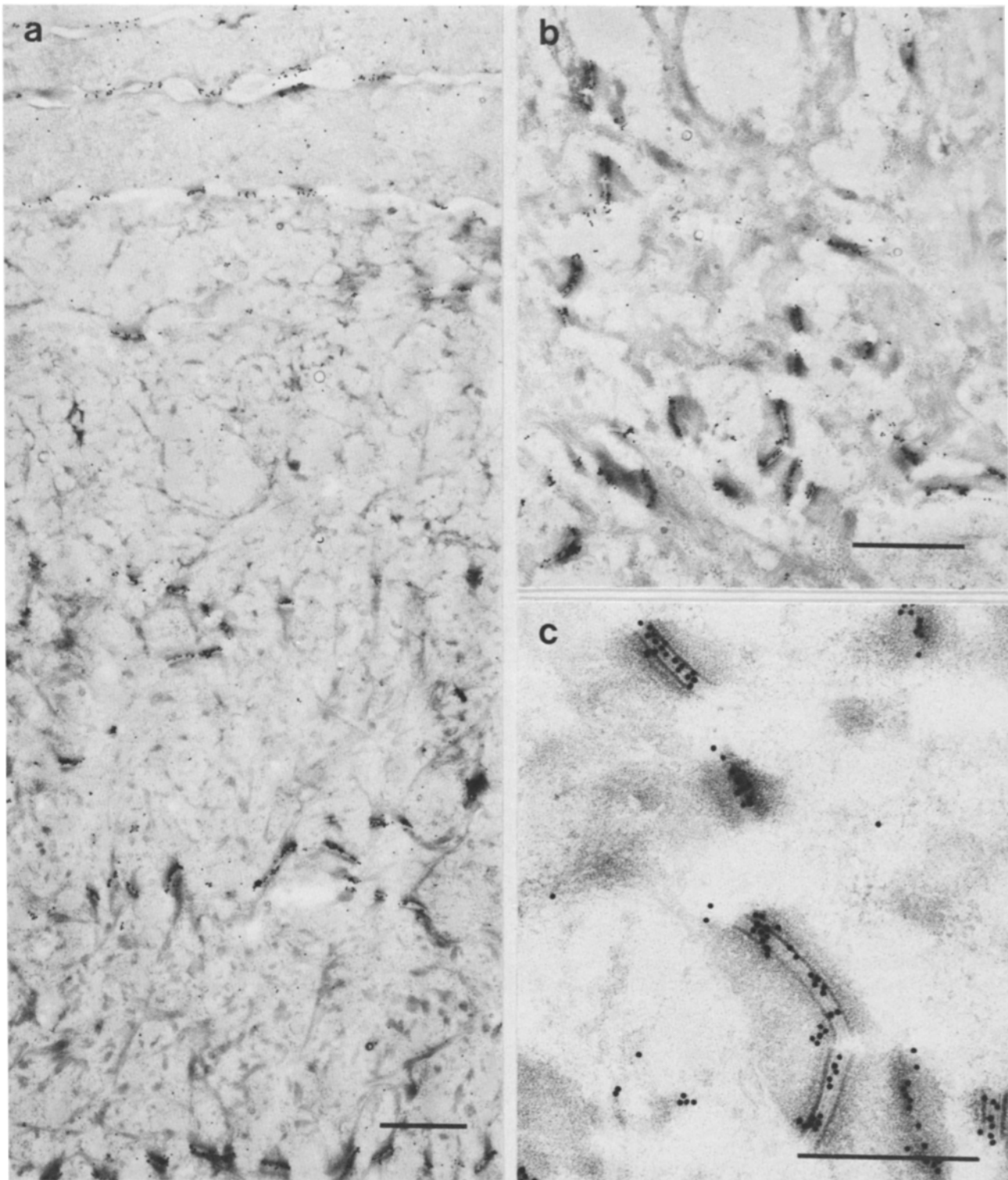
Figure 4. Immunogold labeling of pig ear epidermis showing the localization of gold particles in desmosomal junctions between cells in the spinous and granular layers. Briefly fixed sections were stained using affinity-purified anti–78-kD glycopolypeptide antibodies diluted 1:50. Control sections incubated without primary antibody or with absorbed antibody were not labeled. Bars, 1 μm (*a* and *b*) and 0.5 μm (*c*).

staining extended into the granular layer of the epidermis and the lower layers of the stratum corneum but was not observed in the more fully keratinized layers of the tissue. The dermis was not stained. Strong cell surface staining was also observed in the stratified epithelia lining pig cornea (Fig. 3 *e*) and oesophagus (Fig. 3 *f*). In esophagus, staining was more prominent in the lower cell layers whereas in cornea it was more prominent in the higher layers of the epithelium. The antibodies also stained the surface of cells in the stratum spinosum of human foreskin epidermis (Fig. 3 *g*) indicating that they were not species-specific. Similar though weaker patterns of staining were seen in paraformaldehyde-fixed sections of pig and human skin. However, in this case the cell surface staining was often "dotted" in appearance reminiscent
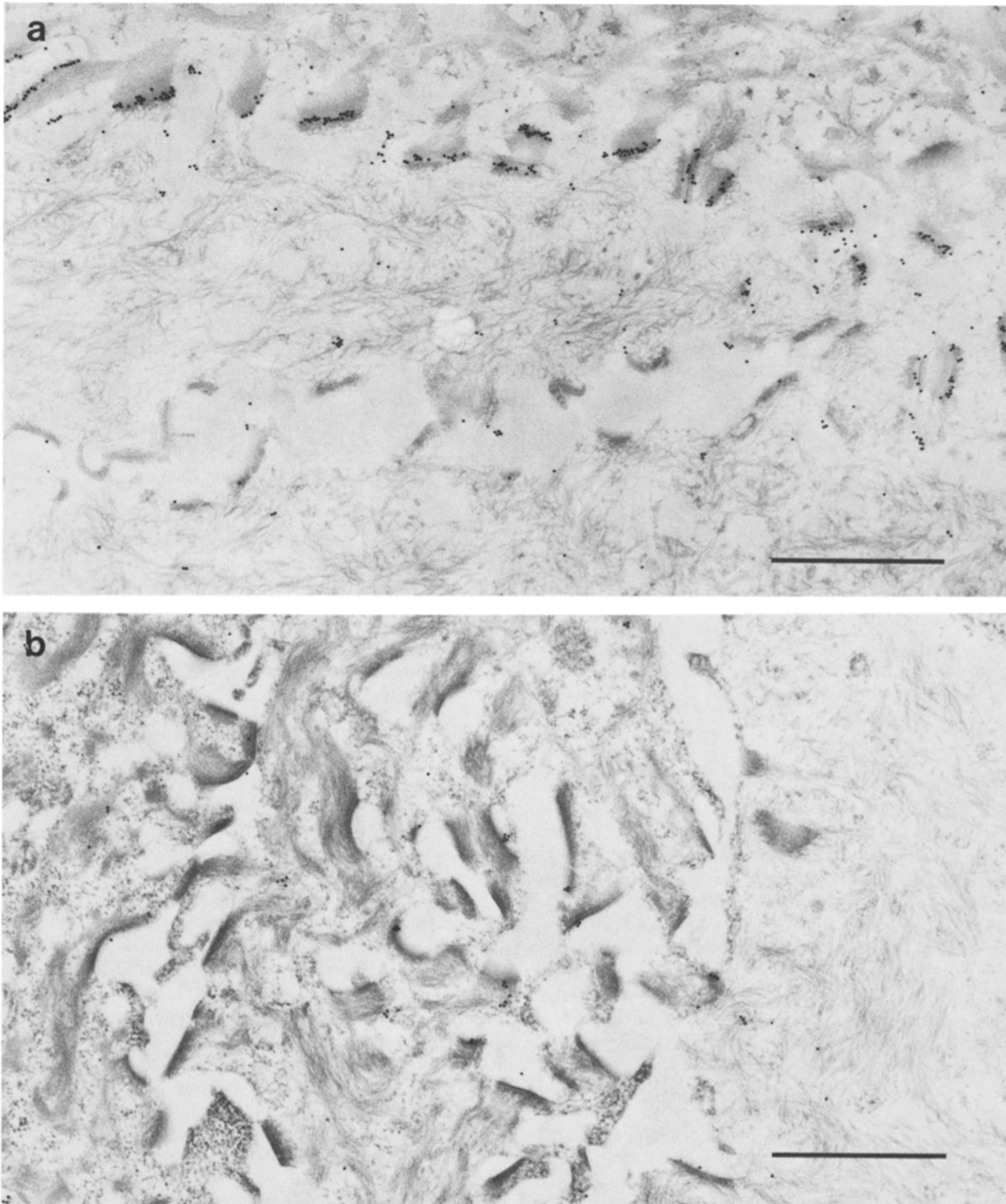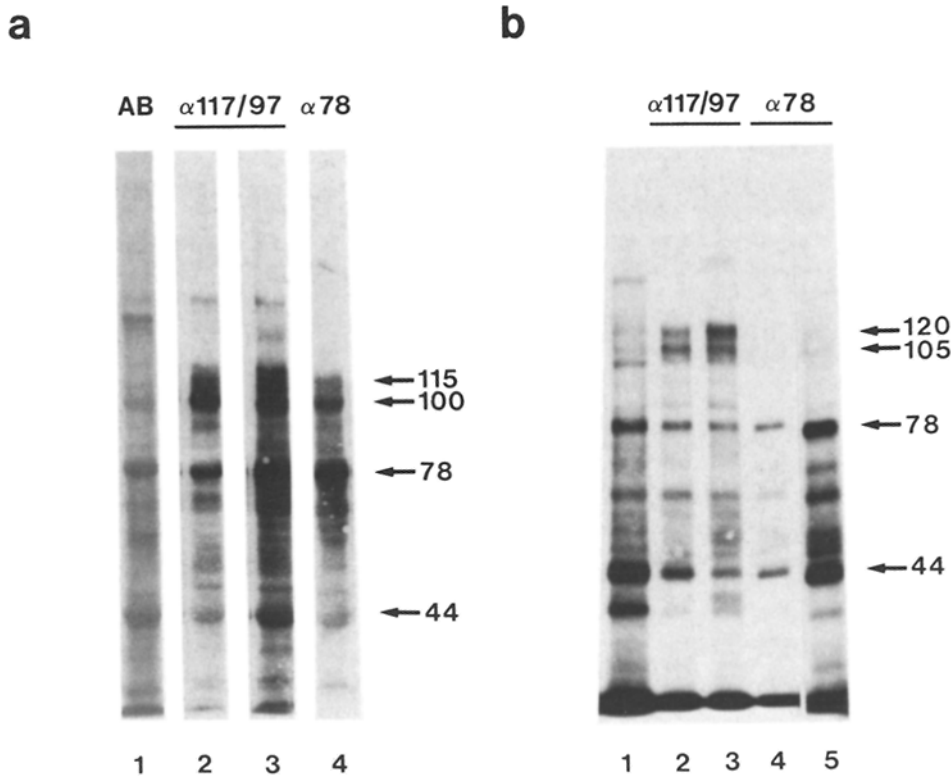
Figure 5. Immunogold labeling of trypsinized pig ear epidermis showing the trypsin sensitivity of desmosomal staining obtained with anti–78-kD glycopolypeptide antibodies (1:50). Tissue was incubated with trypsin (0.25%) for 30 min at 37°C and vortexed to release keratinocytes which were collected by low-speed centrifugation. In the residual epidermis (*a*) intact desmosomes (upper part of picture) are heavily labeled whereas cleaved desmosomes along the lower edge of the same cell are unlabeled. Cleaved desmosomes in the pellet of trypsin-dispersed keratinocytes (*b*) are not labeled. Bars, 1 μm.

of the punctate staining observed with antibodies against components isolated from bovine muzzle desmosomes (9, 11, 13, 17). No staining of pig or human skin sections was seen with affinity-purified antibodies that had been reabsorbed with the 78-kD glycopolypeptide.

The reaction of the anti–78-kD glycopolypeptide antibodies with epidermal cell surfaces was examined in more detail by immunocytochemical staining using gold-labeled second antibody. Low-power electron micrographs (Fig. 4 *a*) confirmed that the gold particles were associated predominantly

Figure 6. Reaction of antidesmoglein II/desmocollin antisera with Con A-binding glycoproteins in urea/deoxycholate/mercaptoethanol extracts of pig epidermis by immunoblotting and immunoprecipitation. (*a*) Epidermal glycoproteins were subjected to SDS-PAGE and transferred to nitrocellulose, and duplicate strips were either stained with amido black (*AB*, lane *1*) or incubated with a 1:1,000 dilution of two different antidesmoglein II/desmocollin antisera (α*117/97*, lanes *2* and *3*) or anti-78-kD glycopolypeptide antiserum (α*78*, lane *4*) followed by ^{125}I-labeled protein A. (*b*) Epidermal glycoproteins were radioiodinated, dissolved in 1% SDS, and diluted with 10 vol of immune-precipitation buffer containing 1% NP40. An aliquot was precipitated with 10 vol acetone (lane *1*). Other aliquots were pretreated with normal guinea pig serum/protein A-agarose before incubation with two different antidesmoglein II/desmocollin antisera (α*117/97*) or the anti-78-kD glycopolypeptide antiserum (α*78*, lanes *4* and *5*) and isolation of immune complexes on protein A-agarose. Iodinated bands were detected by fluorography (4). Lanes *1-4* were exposed for 48 h. Lane *5* is identical to lane *4* except that it was exposed for 144 h. Arrows indicate the major immunoreactive components.

with the surface of cells in the living layers of the tissue. This cell surface labeling was restricted almost entirely to desmosomes (Fig. 4, *b* and *c*). Precise localization of the labeling to ultrastructural features of individual desmosomes was not possible even at high magnification. However, more gold particles appeared to be associated with the intercellular region of the desmosome than with the plaque. The extracellular location of this desmosomal staining was confirmed by its susceptibility to trypsin in intact tissue. Fig. 5 compares the immunocytochemical staining of the residual epidermis and keratinocytes released from trypsinized tissue. In the residual epidermis most desmosomes resisted trypsin and these intact desmosomes were strongly labeled (Fig. 5 *a*). However, labeling was not seen on those few desmosomes that had been cleaved by trypsin treatment. Keratinocytes released from the epidermis by trypsin contained no intact desmosomes (Fig. 5 *b*). The half-desmosomes seen in these cells were never labeled by the anti-78-kD glycopolypeptide antibodies. Little intracellular labeling was seen in trypsin-dispersed cells or the residual epidermis.

To determine whether the immunoreactive 115-44-kD components in pig epidermis were related to previously identified desmosomal glycoproteins, blots were probed with antisera raised against the desmoglein II/desmocollin glycoproteins isolated from bovine snout desmosomes. As shown in Fig. 6 *a* these sera reacted with the same 115-44-kD glycopolypeptides recognized by our anti-78-kD glycopolypeptide antibodies. However the antidesmoglein II/desmocollin sera showed some additional reactivity against higher-M_r Con

A-binding components. In particular both antisera appeared to recognize the 120- and 105-kD amido black-stained components. Reaction of the anti-78-kD glycopolypeptide antibodies with these two components was not detected by immunoblotting.

Fig. 6 *b* shows the results obtained when Con A-binding glycopolypeptides from pig epidermis were radioiodinated and subjected to immunoprecipitation analysis. The two antidesmoglein II/desmocollin sera (Fig. 6 *b*, lanes *2* and *3*) and the anti-78-kD glycopolypeptide serum (Fig. 6 *b*, lanes *4* and *5*) all precipitated comparable amounts of the 78-44-kD Con A-binding components. These three antisera also immune-precipitated the 120- and 105-kD Con A-binding components although significant quantitative differences were observed. The anti-78-kD glycopolypeptide serum was considerably less effective in precipitating these higher-M_r components than the antidesmoglein II/desmocollin sera. Surprisingly all three antisera showed a much weaker reaction with the 100-kD component in immunoprecipitation experiments than was observed in immunoblotting.

To examine whether the various immunoreactive components in our preparations were related chemically, Con A-binding glycopolypeptides from pig epidermis were radioiodinated and the major Coomassie-stained components were compared by proteolytic peptide mapping (Fig. 7). It was not possible to map the 115-kD immunoreactive component because this band could not be detected by protein staining. The higher-M_r components, particularly that at 100 kD, were more resistant to digestion with V8 protease than the
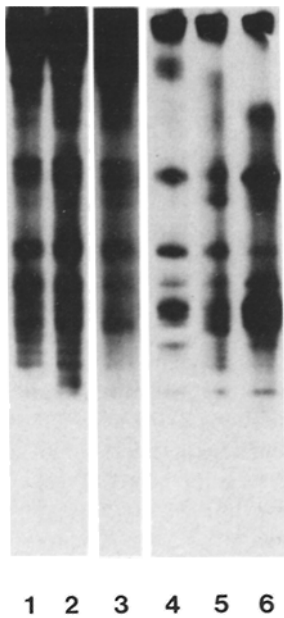
Figure 7. Peptide mapping of the 120-kD (lane *1*), 105-kD (lane *2*), 100-kD (lane *3*), 78-kD (lane *4*), 66-kD (lane *5*), and 44-kD (lane *6*) Con A-binding glycopolypeptides from CaCl$_2$-separated pig epidermis. Iodinated peptides were detected by fluorography (4). Lanes *1* and *2* were exposed for 20 h, lane *3* for 40 h, and lanes *4–6* for 10 h.

78–44-kD glycopolypeptides. Nevertheless, the peptide maps of the 120-, 105-, 100-, 66-, 77-, and 44-kD Con A-binding components revealed considerable homology among these immunoreactive glycopolypeptides (Fig. 7).

Discussion

The 78-kD polypeptide in pig epidermis was specifically eluted from Con A by α-methyl mannoside, suggesting that it is a mannose-containing glycopolypeptide. This glycopolypeptide was always a major component of the epidermis accounting for up to 30% of the total Con A-binding material. The large amounts of the 78-kD component recovered in our preparations suggest that it is unlikely to be a proteolytic artifact arising during the isolation of Con A-binding material. Several other observations support this view. First, the recovery of this glycopolypeptide was not affected by the inclusion of additional protease inhibitors such as *N*-ethylmaleimide to the extraction and dialysis buffers. PMSF was routinely present in all buffers. Secondly, the 78-kD glycopolypeptide was clearly present in whole epidermis extracted directly with SDS sample buffer. Finally, a major Con A-binding component of similar size was identified by Brysk et al. (6, 7) and Reano et al. (28), who overlaid gels containing detergent extracts of human epidermis with ^{125}I-labeled lectins. The 78-kD glycopolypeptide is therefore likely to be a genuine constituent of the epidermis.

The 78-kD glycopolypeptide is not an integral membrane component in that it does not require detergent for its solubilization. Although 2-mercaptoethanol was routinely included in extraction buffers, omission of this reagent did not greatly affect the recovery of the 78-kD component from the epidermis. It is therefore unlikely to be involved in intermolecular disulphide linkages with other epidermal constituents. Rather this glycopolypeptide appears to be a relatively soluble epidermal constituent that is released when the tissue is disrupted with chaotropic agents. The 78-kD glycopolypeptide was recovered from 8 M urea extracts that had been

dialysed twice against 10 vol of urea-free buffer and centrifuged at 100,000 *g*. Thus it remained soluble under conditions where the theoretical urea concentration was <0.1 M.

The 78-kD glycopolypeptide appears to be present predominantly in the keratinized layers of the epidermis. We cannot exclude the possibility that it may also be associated with cells in the living layers of the tissue. However, the immunoblotting results obtained with trypsinized epidermis suggest that this is unlikely. The 78-kD glycopolypeptide was not present in keratinocytes released by trypsin, suggesting that if it had been present in these cells it must have been a surface component that was degraded by the enzyme. However, this component was found to be enriched in extensively trypsinized epidermis when compared with intact tissue and was not depleted as would be expected if it had been subject to proteolytic degradation.

Western blotting showed that the 78-kD glycopolypeptide is immunologically related to several other Con A-binding components in the epidermis. These immunoreactive components comprise two distinct classes. The larger components (115 and 100 kD) require detergent for their solubilization, suggesting that they are true integral membrane-bound glycoproteins. They appear to be sensitive to trypsin in intact tissue and are not present in the stratum corneum, suggesting that they are plasma membrane-bound components of keratinocytes in the lower layers of the epidermis. The smaller components (78–44 kD) recognized by our antibody do not require detergent for their extraction. They are all resistant to trypsin in intact epidermis and they are all present in the more keratinized layers of the tissue. The 78-kD glycopolypeptide in our preparations can be clearly distinguished from the plaque proteins of similar size identified in bovine snout desmosomes. These desmoplakins do not contain carbohydrate and are not related to higher-M_r glycoproteins (11, 17, 18, 22). The 78–44-kD immunoreactive components are in a similar size range to keratins, the major protein species in the epidermis. However, we can exclude the possibility that these major Con A-binding components are keratins. First, these components were not recognized by a rabbit antiserum raised against pig prekeratin (23) or a commercially available anti–bovine keratin antiserum in immunoblotting analysis. (However, these antisera did consistently recognize a minor Con A-binding doublet M_r ~52 kD). Secondly, the anti–78-kD glycopolypeptide antibodies did not give intracellular or stratum corneum staining as would be expected if they were recognizing keratin polypeptides.

The series of glycopolypeptides recognized by our antibody appear to be related to the desmoglein II/desmocollin glycoproteins previously identified in isolated cow snout desmosomes. Independently raised antisera against the 78-kD glycopolypeptide and desmoglein II/desmocollin showed similar though not identical reactivity towards Con A-binding components in pig epidermis. The major difference was observed in their reactivity with the higher-M_r components. The anti–desmoglein II/desmocollin sera reacted with the 120- and 105-kD bands in both immunoblotting and immunoprecipitation. The anti–78-kD glycopolypeptide serum did not appear to recognize these components in immunoblotting but it did precipitate small amounts of these bands. Both antisera reacted strongly with 115- and 100-kD glycopolypeptides on blots. However, for reasons that are not yet clear, these components were not efficiently precipitated by either

serum. In contrast, both antisera displayed identical reactivity towards the 78–44-kD glycopolypeptides in both immunoblotting and immunoprecipitation.

The immunologic relatedness of the various Con A-binding components in pig epidermis is unlikely to reflect cross-reactivity because of the presence of a few common antigenic determinants. Rather the results from immunoblotting with affinity-purified and reabsorbed antibodies, together with those from immune precipitation and peptide mapping, suggest that the various immunoreactive components possess considerable antigenic and chemical similarity. Taken together the observations suggest that the apparently nonmembranous 78–44-kD glycopolypeptides present in stratum corneum are generated during terminal epidermal differentiation by the sequential modification of larger membrane-bound glycoproteins related to desmoglein II/desmocollin. The precise relationship of individual higher-M_r immunoreactive bands in our preparations to the desmoglein II/desmocollin glycoprotein species seen in isolated desmosomes remains to be established.

The immune precipitation data would suggest that the 120/105-kD rather than the 115/100-kD bands may represent the molecular form present in desmosomes in the spinous layer of the epidermis. The 115/100-kD glycopolypeptides, which were not detected in the stratum corneum, may represent early cleavage products of the 120/105-kD desmosomal glycoproteins and may arise in the granular layer of the tissue where irreversible desmosome degradation is thought to be initiated (1, 31). However, because of the discrepancy between the immunoblotting and immune precipitation results for these bands, we cannot entirely exclude the possibility that the 115/100-kD components are distinct but related (possibly underglycosylated) forms of desmoglein II/desmocollin.

The immunolocalization studies are consistent with the view that the 78–44-kD glycopolypeptides arise from the degradation of desmoglein II/desmocollin-like glycoproteins during terminal differentiation. Affinity-purified anti-78-kD glycopolypeptide antibodies gave strong cell surface staining in the light microscope and specific desmosomal staining at the electron microscope level. This staining was of comparable intensity and specificity to that observed with antibodies against glycoproteins isolated from bovine desmosomes (9, 11, 13, 17). Staining was absent in desmosomes of trypsinized keratinocytes and therefore reflected binding to components that were exposed at the cell surface. The specificity of the staining suggests that the various immunoreactive glycopolypeptides may all be present in desmosomes but that individual components may be associated with junctions in different layers of the epidermis. Desmosome staining was never seen beyond the first few layers of the stratum corneum suggesting that the lower-M_r glycopolypeptides may not persist beyond this level. However, we cannot exclude the possibility that these components become masked during the complex chemical and enzymic processes associated with terminal differentiation.

Several other observations support the hypothesis that the 78–44-kD glycopolypeptides arise from the in vivo degradation of larger desmosomal components during terminal differentiation. First, we have been unable to metabolically label these lower-M_r components with radiolabeled amino acids or sugars in explant cultures of pig epidermis or longer-term cultures of human foreskin keratinocytes. These com-

ponents are therefore unlikely to be primary gene products of epidermal cells. Cultured epidermal cells express many features of terminal differentiation (20) but they do not form a normal stratum corneum. It is probable therefore that the lower-M_r components arise during formation of the stratum corneum in vivo. In support of this we have observed immunoreactive Con A-binding components of similar size in normal human stratum corneum and have found differences in the levels of these components in the abnormal horny layer produced in disorders of keratinization such as psoriasis.

It is not yet clear whether the generation of the lower-M_r glycopolypeptides (presumably involving proteases and possibly glycosidases) occurs in the granular layer or in later stages of keratinization. However, these glycopolypeptides may comprise important nonmembranous domains of adhesive glycoproteins in desmosomes, and their generation may be associated with functionally important changes in adhesive interactions between terminally differentiating keratinocytes. In this respect desmosomal glycoproteins may be similar to other adhesive glycoproteins with characteristic degradation products reflecting functional domains of the native cell-adhesion molecules.

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