A lectin-binding glycoprotein of M_r 135000 associated with basal keratinocytes in pig epidermis

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Pig epidermis separated by 1 M-CaCl₂ treatment was homogenized and separated into three fractions by filtration through nylon mesh and high-speed centrifugation. Lectin-binding glycoproteins were isolated from urea/deoxycholate/mercaptoethanol extracts of the residue fraction that resisted filtration, from deoxycholate extracts of the particulate material in the filtrate and from the soluble fraction. Concanavalin A, *Ricinus communis* (castor bean) agglutinin 1, peanut (*Arachis hypogaea*) agglutinin and *Ulex europaeus* (gorse) agglutinin-binding glycoproteins in the three epidermal fractions were analysed by SDS/polyacrylamide-gel electrophoresis. A major neuraminidase-sensitive glycoprotein component of the particulate fraction of M_r 135000 was strongly bound by concanavalin A and *Ricinus communis* agglutinin 1, but only weakly by peanut and *Ulex europaeus* agglutinins. This glycoprotein was not detected in the residue or soluble fractions of the epidermis, indicating that it had only a limited distribution within the tissue. The 135000- M_r glycoprotein was one of two major glycoprotein antigens in the particulate fraction. Rabbits immunized with total particulate glycoproteins produced antibodies directed mainly against 135000- and 110000- M_r components. Monospecific antibodies were obtained from guinea pigs immunized with the 135000- M_r glycoprotein band excised from polyacrylamide gels. Indirect immunofluorescence with the use of affinity-purified antibodies showed that the 135000- M_r glycoprotein was present at the surface of cells in the basal layer of the epidermis as well as at that of other stratified epithelia. It was not present on differentiating cells in the suprabasal layers of the epithelium, suggesting an important role in the attachment or proliferative functions of basal cells in stratified epithelia. Metabolic labelling studies with skin explants cultured in the presence of D-[³H]glucosamine showed that this basal-cell glycoprotein was synthesized by cultured tissue. The major D-[³H]glucosamine-labelled glycoprotein component in the residue and particulate fractions of cultured epidermis had an M_r of 135000, was immunoprecipitated by rabbit antisera raised against particulate epidermal glycoproteins and was bound by concanavalin A. The labelling of this glycoprotein with D-[³H]glucosamine was sensitive to tunicamycin, indicating that the basal-cell glycoprotein contained N-glycosidically linked oligosaccharides.

INTRODUCTION

During keratinization, epidermal cells follow an orderly differentiation programme involving proliferation in the basal layer, formation of tonofilaments and desmosomes in the suprabasal layers and finally degradation of intracellular organelles and the production of a covalently cross-linked cell envelope in the stratum corneum.

Most studies of the molecular changes accompanying epidermal differentiation have been concerned with the polypeptide components of keratin, the major protein species in the epidermis (Baden & Lee, 1978; Banks-Schlegel *et al.*, 1981; Bowden & Cunliffe, 1981; Skerrow & Skerrow, 1983). However, a number of recent reports have suggested that keratinization also involves specific changes in the structure and/or expression of carbohydrates at the epidermal-cell surface. When sections of rodent or human skin were incubated with fluorescent lectins, the various strata of the epidermis showed differential staining. Lectins such as *Bandeirea simplicifolia*

1-B₄ stained basal and lower spinous cells in newborn-rat epidermis (Brabec *et al.*, 1980), whereas PNA and UEA specifically stained cells in the suprabasal layers (Holt *et al.*, 1979; Reano *et al.*, 1982; Nemanic *et al.*, 1983). Other lectins, such as Con A and RCA₁₂₀, stained cells throughout the living layers of the epidermis. However, it was not clear from these largely histochemical studies whether altered lectin binding during differentiation reflected altered glycosylation of glycoproteins, synthesis of new glycoproteins or whether glycolipids were responsible.

To study the contribution of glycoproteins to the lectin-binding patterns seen *in situ*, Brysk & Snider (1982a,b) and Reano *et al.* (1984) used an analytical procedure involving the overlay of SDS/polyacrylamide gels containing epidermal extracts with ¹²⁵I-labelled lectins. This technique identified numerous Con A- and RCA₁₂₀-binding glycoproteins in rat and human epidermis. PNA showed binding to a restricted number of components, but no UEA-binding glycoproteins could be detected by this technique. In a different approach we

Abbreviations used: Con A, concanavalin A; RCA₁₂₀, *Ricinus communis* agglutinin 1; PNA, peanut agglutinin; UEA, *Ulex europaeus* agglutinin; Con A-4B etc., concanavalin A-Sepharose 4B etc.; NP40, Nonidet P40; e.l.i.s.a., enzyme-linked immunoabsorbent assay; FITC, fluorescein isothiocyanate; PMSF, phenylmethanesulphonyl fluoride; BSA, bovine serum albumin; phosphate-buffered saline, 8.1 mM-Na₂HPO₄/1.5 mM-KH₂PO₄/137 mM-NaCl/2.7 mM-KCl, pH 7.4.

used crossed immunoelectrophoresis to examine the interaction of Con A-binding glycoproteins from a plasma-membrane-enriched fraction of pig skin (King & Tabiowo, 1982) with various other lectins (King *et al.*, 1984). However, these analytical approaches were only of limited value in determining whether particular glycoproteins are associated with specific stages of epidermal differentiation.

In the present study we have used lectins as preparative, rather than analytical, tools, in order to isolate the major lectin-binding glycoproteins from pig epidermis. We have identified a novel lectin-binding glycoprotein of M_r 135000 that is present at the surface of basal cells in the epidermis and other stratified epithelia, but which is not present on differentiating cells in the suprabasal layers. The localization of this glycoprotein implies an important role in basal-cell function. It should also prove a valuable addition to other markers for basal cells such as bullous pemphigoid antigen (Stanley *et al.*, 1981) and less-well-characterized structures defined by monoclonal antibodies (Oseroff *et al.*, 1985).

EXPERIMENTAL

All lectins, FITC-lectins and Sepharose 4B-bound lectins were from BDH Poole, Dorset, U.K., except Con A and Con A-4B, which, together with Protein A, were obtained from Pharmacia Fine Chemicals, Hounslow, Middx., U.K. Neuraminidase from *Vibrio comma* was from Behringwerke, Marburg, Germany. D-[6- 3 H]-Glucosamine hydrochloride and Na 125 I were from Amersham International. Sodium deoxycholate was obtained from Koch-Light, Haverhill, Suffolk, U.K. Tunicamycin was obtained as described previously (King & Tabiowo, 1981). Nitrocellulose sheets (pore size 0.45 μ m) were from Schleicher and Schüll, Dassel, Germany. Heat-killed formalin-fixed *Staphylococcus aureus* Cowan 1 strain was from Miles Laboratories, Stoke Poges, Slough, Berks., U.K. Trasylol was from Bayer (U.K.), Haywards Heath, West Sussex, U.K. FITC-conjugated rabbit anti-(guinea-pig) immunoglobins were from DAKOPATTS, Glostrup, Denmark.

Subcellular fractionation of pig epidermis

Slices of pig ear skin (0.2–0.3 mm thick), taken with a Davies dermatome, were treated with 1 M-CaCl $_2$ at 37 °C for 1–2 h and the separated epidermis was collected in 10 mM-Tris/HCl, pH 7.5, containing 10 mM-EDTA. Excess buffer was removed and the epidermal sheets were stored at –20 °C. The thawed epidermis was finely minced and homogenized in 10 vol. of 10 mM-Tris/HCl, pH 7.5, with a Polytron PT20 homogenizer at maximum speed. The homogenate was filtered through nylon mesh (pore size 0.13 $\times$ 0.13 mm) and the material retained by the filter (residue fraction) was removed. The filtered homogenate was centrifuged at 115000 g_{av} and 4 °C for 1 h and the supernatant (soluble fraction) was removed. The pellet (particulate fraction) was washed once in 10 mM-Tris/HCl, pH 7.5.

Isolation of lectin-binding glycoproteins from epidermal fractions

The residue fraction was stirred in 10 vol. of 8 M-urea/1% sodium deoxycholate/1% 2-mercaptoethanol/10 mM-Tris/HCl, pH 7.5, at 4 °C for 18–24 h,

filtered through nylon mesh, centrifuged at 115000 g_{av} for 1 h at 4 °C, dialysed against 10–20 vol. of 1% sodium deoxycholate/10 mM-Tris/HCl, pH 7.5, overnight at 4 °C, and centrifuged at 115000 g_{av} . Dialysis and centrifugation were repeated once. The particulate fraction was extracted directly with 1% sodium deoxycholate/0.25 mM-PMSF/10 mM-Tris/HCl, pH 7.5, for 1 h at 4 °C and was centrifuged at 115000 g_{av} for 1 h at 4 °C. Extracts of the residue and particulate fractions were added to Sepharose 4B-bound lectins (usually 10 ml/ml of lectin) that had been washed in 10 mM-Tris/HCl, pH 7.5, and three times in 1% sodium deoxycholate/0.25 mM-PMSF/10 mM-Tris/HCl, pH 7.5. The soluble fraction was added to lectin that had been washed in 10 mM-Tris/HCl, pH 7.5. After overnight incubation at 4 °C on a rotary mixer, the lectin was washed four times with 1% sodium deoxycholate/0.25 mM-PMSF/10 mM-Tris/HCl, pH 7.5, or with 10 mM-Tris/HCl, pH 7.5, by low-speed centrifugation. Bound glycoproteins were eluted by 30 min incubation with 5% (w/v) α -methyl mannoside (Con A), galactose (RCA $_{120}$ and PNA) or L-fucose (UEA) in the appropriate buffer, were precipitated with ethanol (King & Tabiowo, 1982), and resuspended in water.

Antibodies against particulate glycoproteins

Antisera were produced by two methods. Rabbits received subcutaneous injections of the Con A-binding material from particulate epidermal fractions (0.5 mg of protein) emulsified in complete Freund's adjuvant and were boosted with monthly injections of 0.25 mg of protein in incomplete adjuvant. Antisera obtained by bleeding from the ear vein after the third boost were tested for antibodies by an e.i.s.a. assay (King *et al.*, 1984) using microtitre plates coated with particulate Con A-binding glycoproteins. In some experiments the antiserum was absorbed with Con A-binding glycoproteins from the soluble fraction that had been coupled to Sepharose 4B and then with Con A-4B. In addition, guinea pigs were immunized with the purified 135000- M_r glycoprotein band excised from polyacrylamide gels. A 2–5 mg portion of particulate Con A-binding glycoproteins was run on a slab gel. The edges of the gel were stained for protein, whereas the rest of the gel was frozen on solid CO $_2$ and stored frozen at –70 °C. The 135000- M_r glycoprotein band was cut out, homogenized in complete Freund's adjuvant and injected subcutaneously into guinea pigs. The animals received two booster injections of the purified glycoprotein homogenized in incomplete adjuvant and were bled by cardiac puncture. The purity of the 135000- M_r glycoprotein used as antigen was confirmed by re-electrophoresis of the excised band.

SDS/polyacrylamide-gel electrophoresis and immunoblotting

Electrophoresis was performed on 7% (w/v)-polyacrylamide slab gels (Laemmli, 1970). In some experiments glycoprotein fractions were treated with neuraminidase as described previously (King *et al.*, 1984). Gels were stained with Coomassie Blue or by the silver-staining procedure of Morrissey (1981). Proteins were transferred to nitrocellulose as described by Towbin *et al.* (1979). Strips of the nitrocellulose were stained with 0.1% Amido Black in 45% (v/v) methanol/10% (v/v) acetic acid or were quenched with 5% (w/v) BSA/0.05% NaN $_3$ /phosphate-buffered saline. Strips were overlaid with antiserum diluted in 5% BSA/0.05% NaN $_3$ /phosphate-

buffered saline for 2 h, washed, and then overlaid with ^{125}I -labelled Protein A (Greenwood *et al.*, 1963) (2×10^6 c.p.m./ml) diluted in 10% (v/v) foetal-calf serum/5% BSA/0.5% NaN_3 /phosphate-buffered saline for 2 h at room temperature. After extensive washing with phosphate-buffered saline, strips were air-dried and exposed to Singul-X RP X-ray film. Gels containing metabolically labelled glycoproteins were fixed in propan-2-ol/acetic acid/water (5:2:13, by vol.) before impregnation with diphenyloxazole and fluorography (Bonner & Laskey, 1974; Laskey & Mills, 1975).

Affinity purification of antibodies

Particulate Con A-binding glycoproteins (approx. 5 mg) were run on a slab gel and were electrophoretically transferred to nitrocellulose. The edges were stained with Amido Black and the rest of the transfer was quenched with BSA as described above. The strip of nitrocellulose containing the 135000- M_r glycoprotein was cut out and incubated with 1 ml of antiserum diluted with 1 ml of 5% BSA/0.05% NaN_3 /phosphate-buffered saline for 30 min on a rocking platform. After extensive washing with phosphate-buffered saline, bound antibodies were eluted with 0.5 M-glycine, pH 2.8 (Lehto & Virtanen, 1983). The eluate was immediately adjusted to neutral pH with strong Tris base and was dialysed against phosphate-buffered saline.

Metabolic labelling of epidermal glycoproteins

Slices (1 cm square) of pig ear skin (0.2–0.3 mm thick) were cultured in the presence of D-[6- ^3H]glucosamine (25 $\mu\text{Ci}/\text{ml}$) for 18 h as described previously (King *et al.*, 1980). In some experiments, 25 mM-NaOH (1 $\mu\text{l}/\text{ml}$) or tunicamycin (2 mg/ml in 25 mM-NaOH) were included in the culture medium (King & Tabiowo, 1981). After washing them with phosphate-buffered saline, skin slices

were separated with 1 M- CaCl_2 and fractionated as described above.

Immunoprecipitation

Fractions from D-[^3H]glucosamine-labelled epidermis were extracted with 0.5% NP40/0.2 M-NaCl/1 mM-PMSF/Trasylol (1000 units/ml)/50 mM-Tris/HCl, pH 7.5, at room temperature for 30 min and were centrifuged at 115000 g_{av} . Extracts were incubated with 10 μl of pre-immune rabbit serum or rabbit antiserum that had been raised against particulate epidermal glycoproteins and then absorbed with soluble epidermal glycoproteins and Con A. After 4 h at 4 °C the extract was incubated with 0.2 ml of a 10% (w/v) suspension of *Staphylococcus aureus* and incubated at 4 °C overnight. After four washes with 0.1% NP40/0.2 M-NaCl/1 mM-PMSF/Trasylol (1000 units/ml)/50 mM-Tris/HCl, pH 7.5, and one water wash, immune complexes were eluted by boiling in SDS sample buffer.

FITC-lectin staining and indirect immunofluorescence

Tissues obtained from freshly killed pigs were mounted in Tissue-Tek II OCT compound, frozen in isopentane cooled in liquid N_2 and cryostat sections (5 μm) were stored at -70 °C. For FITC-lectin staining, air-dried sections were fixed with 2% (w/v) paraformaldehyde for 15 min and incubated with FITC-lectins (50–100 $\mu\text{g}/\text{ml}$) for 30 min. Controls for non-specific binding contained 5% α -methyl mannoside (Con A), galactose (RCA_{120} and PNA) or L-fucose (UEA). Sections were mounted in glycerol/phosphate-buffered saline (9:1 v/v) containing 2.5% (w/v) 1,4-diazobicyclo[2.2.2]octane (Johnson *et al.*, 1982). For indirect immunofluorescence, unfixed sections were incubated with affinity-purified antibodies for 30 min, then with FITC-conjugated rabbit anti-(guinea-pig) immunoglobulins (antibodies/buffer, 1:50, v/v) for

Fig. 1. FITC-lectin staining of pig skin

Cryostat sections of pig skin were fixed with paraformaldehyde and washed with phosphate-buffered saline before incubation with FITC-Con A (a), FITC- RCA_{120} (b), FITC-PNA (c) and FITC-UEA (d). The bar represents 20 μm . D and E indicate the dermal and epidermal side of each section respectively.

30 min, and mounted as described above. Control sections were incubated with phosphate-buffered saline instead of first antibody.

Measurements of protein and radioactivity

Protein was measured by the Hartree (1972) method. ^3H was measured by liquid-scintillation counting, whereas ^{125}I was determined on a Nuclear Enterprises NE 1600 γ -radiation counter.

RESULTS

Binding of FITC-lectins to pig epidermis

Fig. 1 shows the staining of cryostat sections of pig epidermis with FITC-Con A, FITC-RCA₁₂₀, FITC-PNA and FITC-UEA. With all four lectins, staining at the stratum-corneum/granular-layer junction and in the stratum corneum was not blocked by the appropriate sugar inhibitor. Staining in these regions is therefore probably non-specific, although it may reflect high-affinity binding of the lectins. By contrast, the staining of cells in the living layers of the epidermis was specific, since it was completely blocked in the presence of the relevant sugar. Con A (Fig. 1a), RCA₁₂₀ (Fig. 1b) and PNA (Fig. 1c) specifically stained cell-surface components in all the living layers of the epidermis. Con A and RCA₁₂₀ also stained the dermis strongly. Species differences were noted with PNA. This lectin stained the surface of cells

throughout the living layers of pig epidermis (Fig. 1c), in contrast with human tissue, where only suprabasal cells were positive (results not shown). With UEA, specific staining was restricted to the surface of cells in the upper spinous and granular layers of pig epidermis (Fig. 1d).

Isolation of lectin-binding glycoproteins

To isolate epidermal glycoproteins involved in FITC-lectin binding, pig skin slices were separated by 1 M-CaCl₂ treatment and were subjected to vigorous Polytron

Table 1. Isolation of lectin-binding glycoproteins from sub-cellular fractions of pig epidermis

CaCl₂-separated pig epidermis was fractionated, and lectin-binding glycoproteins were isolated as described in the Experimental section. The results obtained in a typical experiment are shown.

Lectin	Fraction ...	Recovery of glycoprotein ($\mu\text{g/g}$ of epidermis)		
		Residue	Particulate	Soluble
Con A		115	24	46
RCA ₁₂₀		104	18	26
PNA		20	4	12
UEA		28	3	12

Fig. 2. SDS/polyacrylamide-gel electrophoresis of Con A-, RCA₁₂₀-, PNA- and UEA-binding glycoproteins from pig epidermis

Subcellular fractions from CaCl₂-separated epidermis were stored frozen at -20°C . After thawing the soluble fraction was clarified by re-centrifugation at $115000g_{\text{av}}$ for 1 h. Con A (a,e,i-), RCA₁₂₀ (b,f,j-), PNA (c,g,k-) and UEA (d,h,l-) binding glycoproteins were isolated from the residue (a-d), particulate (e-h) and soluble (i-l) fractions as described in the Experimental section. The Figure shows three separate 7%-(w/v)-polyacrylamide gels containing about $5\mu\text{g}$ of lectin-binding glycoprotein per track. Gels were stained with silver. Comparison with gels of authentic lectins showed that the bands in the $30000-M_r$ region common to all three fractions represented lectin that had leaked from the Sepharose 4B. The small arrows ($\rightarrow$) indicate the position of M_r markers myosin (200000), β -galactosidase (130000), phosphorylase a (96000), BSA (68000), ovalbumin (43000) and of residual Con A (27000) that leaked from the Sepharose 4B. The large arrow ($\blacktriangleleft$) indicates the position of the $135000-M_r$ glycoprotein present in the particulate fraction.

homogenization. The bulk of the tissue, presumably derived from the upper keratinized layers of the epidermis, resisted disruption and was retained during filtration of the homogenate on nylon mesh. This material (referred to as the 'residue fraction') was extracted with urea/deoxycholate/mercaptoethanol. The urea was removed by dialysis and deoxycholate-soluble glycoproteins were isolated on Sepharose 4B-bound lectins. The material that passed through the nylon mesh was separated into particulate and soluble fractions by high-speed centrifugation. Particulate lectin-binding glycoproteins were isolated from the pellet after extraction with deoxycholate, whereas soluble lectin-binding glycoproteins were isolated from the high-speed supernatant.

Table 1 shows the result of a typical experiment comparing the amounts of protein bound by Con A-4B, RCA₁₂₀-4B, PNA-4B and UEA-4B in the residue, particulate and soluble fractions of pig epidermis. Precise comparison of glycoprotein yields was not possible because of variable leakage of the different lectins from Sepharose 4B. However, PNA and UEA always bound considerably less glycoprotein than did Con A or RCA₁₂₀. Fig. 2 shows three gels in which approximately equal amounts of lectin-bound protein from each fraction were examined by SDS/polyacrylamide-gel electrophoresis and silver staining. In general, Con A and RCA₁₂₀ were found to bind similar glycoproteins in all epidermal fractions. The major exception was a Con A-binding glycoprotein of M_r 78000 in the residue (Fig. 2a), which was not bound by RCA₁₂₀ (Fig. 2b). In the particulate fraction RCA₁₂₀ only bound small amounts of a 110000- M_r Con A-binding glycoprotein, but considerably more of a glycoprotein of M_r 85000 (Fig. 2f). In all three fractions, PNA and UEA bound little or none of the major Con A-binding glycoproteins. The predominant glycoproteins bound by PNA in all fractions migrated as a broad diffuse band in the M_r range 80000–70000 (Figs. 2c, 2g and 2k). These components were also bound, albeit less strongly, by UEA (Figs. 2d, 2h and 2l).

To compare the glycoprotein composition of the three subcellular fractions directly, the Con A-binding material from the residue, particulate and soluble fractions of pig epidermis were analysed on the same gel (Fig. 3). Because of the greater yield of glycoprotein with Con A (Table 1), these gels could be stained with Coomassie Blue. Some minor differences in relative staining intensity were noted when Coomassie Blue-stained gels (Fig. 3) were compared with silver-stained ones (Fig. 2). To determine whether individual Con A-binding glycoproteins were sialylated, the effect of neuraminidase on their electrophoretic mobility was also examined (results not shown).

Neuraminidase-sensitive glycoproteins of M_r 165000, 150000 and 130000 were present in both the residue (Fig. 3a) and particulate (Fig. 3b) fractions of the epidermis, but were not detected in the soluble fraction (Fig. 3c). These glycoproteins are therefore likely to be structural, rather than cytosolic, components. A neuraminidase-sensitive glycoprotein of M_r 140000 in the residue (Fig. 3a) could be distinguished from a neuraminidase-sensitive component of M_r 135000 in the particulate fraction (Fig. 3b), since the latter glycoprotein always migrated more quickly. Neither of these components was present in a soluble form (Fig. 3c). Neuraminidase-resistant components of M_r 120000, 110000 and 105000

Fig. 3. SDS/polyacrylamide-gel electrophoresis of Con A-binding glycoproteins from pig epidermis

Con A-binding glycoproteins were isolated from freshly prepared fractions of CaCl_2 -separated pig epidermis as described in the Experimental section. About 75 μg of protein from the residue (a), particulate (b) and soluble (c) fractions was run on a 7% polyacrylamide gel, which was stained with Coomassie Blue. Arrows are as for Fig. 2.

present predominantly in the residue (Fig. 3a) were distinct from the broad particulate band of M_r 110000 (Fig. 3b), since the latter component was sensitive to neuraminidase. Major Con A-binding glycoprotein components in the residue, of M_r 78000 and 46000 (Fig. 3a), were resistant to neuraminidase and were only trace components of the particulate fraction. These components could be distinguished from bands of similar size in the soluble fraction, since the mobility of all soluble Con A-binding glycoproteins was consistently increased by neuraminidase treatment.

Antibodies to particulate Con A-binding glycoproteins

To determine whether any of the lectin-binding glycoproteins identified in Figs. 2 and 3 were differentially expressed at the surface of epidermal cells during keratinization, antisera were raised against isolated epidermal glycoproteins. Particulate Con A-binding glycoproteins were used for these studies, since (1) it was reasoned that the particulate fraction would be more likely to contain cell-surface-associated components than the residue or soluble fractions of the epidermis, and (2) Con A bound a wider range of particulate glycoproteins than did the other lectins.

Fig. 4. Immunoblotting analysis of antisera raised against particulate Con A-binding glycoproteins

The first panel shows the reactivity of rabbit antiserum raised against the total Con A-binding material from particulate fractions of CaCl_2 -separated pig epidermis. Particulate Con A-binding glycoproteins (about $50 \mu\text{g}$ per track) were separated on a 7%-polyacrylamide gel and were transferred to nitrocellulose. Strips of the transfer were stained with Amido Black (a) or were incubated with rabbit antiserum (diluted 1:500), followed by ^{125}I -labelled Protein A (b). The second panel shows the reactivity of guinea-pig antiserum raised against the $135000\text{-}M_r$ particulate glycoprotein excised from polyacrylamide gels. About $50 \mu\text{g}$ of Con A-binding glycoproteins from the residue (c,f), particulate (d,g) and soluble (e,h) fractions of pig epidermis were separated on a 7%-polyacrylamide gel and were transferred to nitrocellulose. Strips were stained with Amido Black (c,d,e) or incubated with guinea-pig antiserum (diluted 1:100) and ^{125}I -labelled Protein A (f,g,h). No reaction was observed when pre-immune rabbit or guinea-pig sera were used. Arrows are as for Fig. 2.

Initially rabbits were immunized with the total Con A-binding material isolated from particulate epidermal fractions. The resulting antisera reacted predominantly with 135000- and $110000\text{-}M_r$ particulate glycoproteins and with Con A in immunoblotting (Fig. 4b). Absorption of this antiserum with soluble epidermal glycoproteins coupled to Sepharose 4B and with Con A-4B removed antibodies against Con A but did not affect reactivity against the 135000- and $110000\text{-}M_r$ glycoproteins (results not shown). To obtain more specific antibodies against particulate Con A-binding glycoproteins, guinea pigs were immunized with individual Con A-binding glycoproteins excised from unfixed polyacrylamide gels. Positive sera were obtained from guinea pigs treated with the $135000\text{-}M_r$ glycoprotein band. In immunoblotting these antisera reacted strongly with the $135000\text{-}M_r$ glycoprotein in the particulate fraction (Fig. 4g), but did not recognize Con A-binding glycoproteins in the residue (Fig. 4f) or soluble (Fig. 4h) fractions of the epidermis. For indirect immunofluorescent localization of this glycoprotein, antibodies were affinity-purified by using nitrocellulose transfers containing the $135000\text{-}M_r$ glycoprotein as immunoabsorbent. These affinity-purified antibodies specifically stained the surface of basal

keratinocytes in unfixed sections of pig skin (Fig. 5a). No basal-cell staining was seen in control sections incubated with phosphate-buffered saline instead of the affinity-purified antibody (Fig. 5b). Staining was not observed in skin sections that had been treated with paraformaldehyde or organic solvents, indicating that the antigenic sites on the $135000\text{-}M_r$ glycoprotein were fixation-sensitive. Affinity-purified antibodies against the $135000\text{-}M_r$ glycoprotein also reacted with other stratified epithelial tissues (results not shown). Basal cells in the stratified epithelium lining pig oesophagus were positive. Although the non-keratinized epithelia lining vagina and ureter were poorly preserved in unfixed sections, staining was consistently observed in the basal cell area of both tissues.

Expression of the $135000\text{-}M_r$ glycoprotein in culture

To determine whether the basal-cell glycoprotein was expressed by cultured tissue, skin slices were subjected to short-term explant culture in the presence of D- ^3H glucosamine. A D- ^3H glucosamine-labelled glycoprotein of M_r 135000 was a major component of the residue (Fig. 6a) and particulate (Fig. 6b) fractions prepared from cultured epidermis and solubilized with SDS. This band was not detected in the soluble fraction

Fig. 5. Indirect immunofluorescent staining of pig skin with affinity-purified antibodies against the 135000- M_r glycoprotein

Cryostat sections of pig ear skin were washed in phosphate-buffered saline and incubated with affinity-purified antibodies against the 135000- M_r Con A-binding glycoprotein from particulate fractions of pig epidermis (a) or with phosphate-buffered saline (b). After washing, sections were incubated with FITC-conjugated rabbit anti-(guinea pig) immunoglobulins diluted 1:50. The bar represents 20 μ m. D and E indicate the dermal and epidermal side of each section respectively.

from metabolically labelled epidermis (Fig. 6c). To check whether the heavily labelled glycoprotein of M_r 135000 was identical with the basal-cell glycoprotein found in fresh tissue, fractions from cultured epidermis were extracted with NP40. Detergent extracts were incubated with the rabbit antiserum raised against particulate Con A-binding glycoproteins followed by protein A-bearing staphylococci. The D-[3 H]glucosamine-labelled band of M_r 135000 was precipitated from the residue (Fig. 7e) and particulate (Fig. 7g) fractions by the rabbit antiserum. Small amounts of a D-[3 H]glucosamine-labelled band of M_r 165000 were also precipitated. No labelled material was precipitated from the soluble fraction (Fig. 7i). The specificity of the immune precipitations was shown by control incubations with pre-immune rabbit sera, which did not precipitate any D-[3 H]glucosamine-labelled material (Figs. 7d, 7f and 7h).

Further evidence that the D-[3 H]glucosamine-labelled

Fig. 6. SDS/polyacrylamide-gel electrophoresis of D-[3 H]glucosamine-labelled fractions from epidermis of cultured pig skin slices

Skin slices were labelled with D-[3 H]glucosamine for 18 h, separated with 1 M-CaCl₂, and the epidermis was fractionated as described in the Experimental section. Residue (a), particulate (b) and soluble (c) fractions were solubilized with SDS sample buffer and samples, containing approx. 5×10^4 c.p.m., were separated on 7% -polyacrylamide gels, which were processed for fluorography. The heavily labelled material in the stacking gel is hyaluronic acid (King, 1981). Arrows are as for Fig. 2.

glycoprotein of M_r 135000 was identical with the basal-cell glycoprotein found in fresh tissue was obtained by homogenizing metabolically labelled epidermis with an excess of fresh non-cultured tissue and isolating Con A-binding glycoproteins from deoxycholate extracts of the particulate fraction. SDS/polyacrylamide gels of particulate Con A-binding glycoproteins were stained for protein and the stained gels were processed for fluorography. Comparison of the protein staining and labelling patterns in the same gel confirmed that the D-[3 H]glucosamine-labelled glycoprotein of M_r 135000 (Fig. 8a) co-migrated precisely with the Con A-binding

Fig. 7. Immune precipitation from NP40 extracts of D-[^3H]glucosamine-labelled epidermal fractions

Residue, particulate and soluble fractions of D-[^3H]glucosamine-labelled pig epidermis were extracted with 0.5% NP40/0.2 M-NaCl/1 mM-PMSF/Trasyol(1000 units/ml)/50 mM-Tris/HCl, pH 7.5. Extracts containing approximately equal amounts of ^3H were incubated with pre-immune rabbit serum or with rabbit antiserum raised against total particulate Con A-binding glycoproteins and with Protein A-bearing staphylococci as described in the Experimental section. The first panel shows a fluorogram prepared from a 7% -polyacrylamide gel containing NP40 extracts of residue (a), particulate (b) and soluble (c) fractions from D-[^3H]glucosamine-labelled epidermis. The second panel shows the labelled glycoproteins precipitated from NP40 extracts of the residue (d,e) particulate (f,g) and soluble (h,i) fractions by preimmune serum (d,f,h) and the rabbit antiserum (e,g,i). Arrows are as for Fig. 2.

glycoprotein of the same size. Similar experiments were carried out with epidermis that had been labelled in the presence of the glycosylation inhibitor tunicamycin (King & Tabiowo, 1981). The labelling of 135000- M_r glycoprotein with D-[^3H]glucosamine and its subsequent binding to Con A were prevented by tunicamycin treatment (Fig. 8b).

DISCUSSION

Isolation and characterization of epidermal cell-surface glycoproteins is a prerequisite for understanding the role of these components in epidermal differentiation (keratinization). In a previous report (King & Tabiowo, 1982), we isolated the Con A-binding glycoproteins from a vesicular-plasma-membrane-enriched fraction thought to be derived from cells in the lower layer of the epidermis (Gray *et al.*, 1980). However, this membrane fraction may have been unrepresentative of the total plasma membrane

Fig. 8. SDS/polyacrylamide gel electrophoresis of Con A-binding glycoproteins from D-[^3H]glucosamine-labelled particulate fractions of pig epidermis

Skin slices (ten, each 1 cm²) were labelled with D-[^3H]glucosamine for 18 h in the absence (a) or presence (b) of tunicamycin (2 $\mu\text{g}/\text{ml}$). After CaCl_2 separation, the epidermis was mixed with 1 g of unlabelled epidermis, and Con A-binding glycoproteins were isolated from the particulate fraction as described in the Experimental section. Samples containing 50 μg of protein were separated on a 7% -polyacrylamide gel, which was stained with Coomassie Blue and then processed for fluorography. The Coomassie Blue-stained glycoprotein band with an M_r of 135000 (large arrow) and the D-[^3H]glucosamine-labelled band of similar size co-migrated exactly.

in the epidermis, since (1) it was only obtained in very low yield, and (2) it did not contain specialized plasma-membrane areas such as desmosomes or the thickened plasma membranes of differentiated cells. Further, although the glycoproteins isolated from these membrane vesicles were strongly bound by RCA_{120} , they only interacted weakly with other lectins (King *et al.*, 1984). To identify other lectin-binding glycoproteins in the epidermis we have now examined the glycoproteins bound by Con A, RCA_{120} , PNA and UEA in all three fractions of Polytron-homogenized tissue. The lectin-binding glycoproteins identified in Figs. 2 and 3 should

therefore comprise all the major glycoprotein components present in the epidermis.

Having identified the major lectin-binding glycoproteins in the epidermis, we next sought to determine whether particular components were associated with specific stages of epidermal differentiation. In the present study we have prepared monospecific antibodies against a membrane-bound sialoglycoprotein of M_r 135000 and have shown that this glycoprotein is present at the surface of basal, but not suprabasal, keratinocytes in the epidermis (Fig. 5a). The immunofluorescent localization of this glycoprotein is strongly supported by the finding that it had a limited distribution within the various subcellular fractions of the epidermis. It was a major component of particulate fractions of the tissue, but was not present in the residue (derived mainly from keratinized layers) or in the soluble fraction. This glycoprotein is likely to be fairly heavily glycosylated, since (1) it always migrated as a diffuse rather than a sharp band, (2) its electrophoretic mobility was increased by neuraminidase treatment, (3) it was bound by all four lectins used in the present study, and (4) it was heavily labelled with D-[3H]glucosamine in culture. At least some of the carbohydrate in the 135000- M_r glycoprotein is *N*-glycosidically linked to asparagine, since tunicamycin prevents both its labelling with D-[3H]glucosamine and its binding to Con A (Fig. 8b).

The 135000- M_r glycoprotein appears to be strongly antigenic, possibly because of its high carbohydrate content. It is one of two major glycoprotein antigens in particulate fractions of the epidermis (Fig. 4b), and in our view is likely to be a major antigen of basal keratinocytes. The antigenicity of the 135000- M_r glycoprotein suggests that it may be important as an autoantigen in autoimmune blistering diseases affecting the skin. Indeed we have found that about one-third of patients with such diseases (both pemphigus and bullous pemphigoid) have circulating antibodies that bind to particulate epidermal glycoproteins in e.l.i.s.a. (I. A. King, unpublished work). In immunoblotting the highest-titre pemphigus serum showed binding to the 165000- and 135000- M_r glycoproteins, whereas the strongest pemphigoid serum reacted with the 135000- and 110000- M_r glycoproteins. Our 135000- M_r glycoprotein may therefore be identical with the 130000- M_r glycoprotein previously described by Stanley *et al.* (1982). This cell-surface molecule was precipitated from metabolically labelled extracts of cultured mouse epithelial cells and human keratinocytes by some but not all pemphigus sera.

The M_r -135000 glycoprotein was present only in particulate fractions of fresh epidermis. However, the D-[3H]glucosamine-labelled glycoprotein was present in both the residue and particulate fractions from cultured tissue (Fig. 6), indicating that the 135000- M_r glycoprotein may be expressed by suprabasal, as well as by basal, keratinocytes in tissue culture. Previous studies on the synthesis of keratin polypeptides (Fuchs & Green, 1978; Sun & Green, 1978) suggest that epidermal cells may preferentially express a basal-cell phenotype in culture. Keratinocytes grown in 3T3 feeder layers synthesized the lower- M_r keratins typical of basal cells, but not the higher- M_r polypeptides found in the more differentiated layers of the epidermis. However, we have not yet been able to demonstrate suprabasal expression of the 135000- M_r glycoprotein by indirect immunofluorescence of cultured tissue.

The present results emphasize the need for caution in interpreting the staining of tissue sections with FITC-lectins. The differential staining of cells in the various layers of the epidermis is often discussed in terms of the addition of sugars, particularly fucose (Zieske & Bernstein, 1982), to incomplete carbohydrate chains during the process of keratinization. Although this may be the case for some glycoconjugates, our results show that such conclusions should not be applied generally. Despite the apparent absence of UEA-reactive components in basal cells of pig epidermis revealed by FITC-UEA staining (Fig. 1d), we have isolated a glycoprotein that is both present in basal cells (Fig. 5a) and is bound (albeit weakly) by UEA-4B (Fig. 3h).

The 135000- M_r glycoprotein was not unique to the epidermis, since it was also found in three other stratified epithelial tissues (both keratinizing and non-keratinizing). It was not detected in simple epithelia. Its function is not yet known. Nevertheless, the localization and distribution of this glycoprotein suggest an important role in one of the two major functions of basal cells in stratified epithelia. It may be involved in the attachment of the epithelium to underlying connective tissue via the basement membrane. Alternatively it may play a role in the proliferative activity of basal cells.

REFERENCES

- Baden, H. P. & Lee, L. D. (1978) *J. Invest. Dermatol.* **71**, 148–151
- Banks-Schlegel, S. P., Schlegel, R. & Purkiss, S. (1981) *Exp. Cell Res.* **136**, 465–469
- Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83–88
- Bowden, P. E. & Cunliffe, W. J. (1981) *Biochem. J.* **199**, 145–154
- Brabec, R. K., Peters, B. P., Bernstein, I. A., Gray, R. H. & Goldstein, I. J. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 477–479
- Brysk, M. M. & Snider, J. M. (1982a) *J. Invest. Dermatol.* **78**, 366–370
- Brysk, M. M. & Snider, J. M. (1982b) *J. Invest. Dermatol.* **79**, 193–197
- Fuchs, E. & Green, H. (1978) *Cell* (Cambridge, Mass.) **15**, 887–897
- Gray, G. M., King, I. A. & Yardley, H. J. (1980) *Br. J. Dermatol.* **103**, 505–515
- Greenwood, E. C., Hunter, W. M. & Glover, J. S. (1963) *Biochem. J.* **89**, 114–123
- Hartree, E. F. (1972) *Anal. Biochem.* **48**, 422–427
- Holt, P. J. A., Anglin, J. H., Jr. & Nordquist, R. E. (1979) *Br. J. Dermatol.* **100**, 237–245
- Johnson, G. D., Davidson, R. S., McNamee, K. C., Russell, G., Goodwin, D. & Holborow, E. J. (1982) *J. Immunol. Methods* **55**, 231–242
- King, I. A. (1981) *Biochim. Biophys. Acta* **674**, 87–95
- King, I. A. & Tabiowo, A. (1981) *Biochem. J.* **198**, 331–338
- King, I. A. & Tabiowo, A. (1982) *Biochem. J.* **201**, 287–295
- King, I. A., Tabiowo, A. & Williams, R. J. (1980) *Biochem. J.* **190**, 65–77
- King, I. A., Tabiowo, A. & Pope, F. M. (1984) *J. Invest. Dermatol.* **83**, 42–47
- Laemmli, U. K. (1970) *Nature* (London) **227**, 680–685
- Laskey, R. A. & Mills, A. D. (1975) *Eur. J. Biochem.* **56**, 335–341
- Lehto, V. P. & Virtanen, I. (1983) *J. Cell Biol.* **96**, 703–716
- Morrissey, J. H. (1981) *Anal. Biochem.* **117**, 307–310
- Nemanic, M. K., Whitehead, J. S. & Elias, P. M. (1983) *J. Histochem. Cytochem.* **31**, 887–897

- Oseroff, A. R., Pfendt, E. A., Dicicco, L. & Morhenn, V. (1985) *J. Invest. Dermatol.* **84**, 257–261
- Reano, A., Faure, M., Jacques, Y. & Reichert, U. (1982) *Br. J. Dermatol.* **107**, Suppl. **23**, 143–146
- Reano, A., Ledger, P. W., Bonnefoy, J.-Y. & Thivolet, J. (1984) *J. Invest. Dermatol.* **83**, 202–205
- Skerrow, D. & Skerrow, C. J. (1983) *Exp. Cell Res.* **143**, 27–35
- Stanley, J. R., Hawley-Nelson, P., Yuspa, S. H., Shevach, E. M. & Katz, S. I. (1981) *Cell (Cambridge, Mass.)* **24**, 897–903
- Stanley, J. R., Yaar, M., Hawley-Nelson, P. & Katz, S. I. (1982) *J. Clin. Invest.* **70**, 281–288
- Sun, T.-T. & Green, H. (1978) *J. Biol. Chem.* **253**, 2053–2060
- Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- Zieske, J. D. & Bernstein, I. A. (1982) *J. Cell Biol.* **95**, 626–631

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