A type VI collagen-related glycopolypeptide is the major concanavalin A-binding component in pig skin

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The major concanavalin A-binding component in urea/deoxycholate/mercaptoethanol extracts of pig skin was a collagenous disulphide-cross-linked glycopolypeptide with an apparent molecular mass of 150 kDa and a pI of 5.5. Antiserum against the electrophoretically purified glycopolypeptide gave strong dermal staining similar to that seen with fluorescent concanavalin A. Immunocytochemical labelling showed prominent labelling of 3–4 nm dermal microfilaments, particularly those associated with dermal blood vessels and mast cells. Immunoblotting with authentic antiserum indicated that the major skin glycopolypeptide was probably identical with collagen-like glycoprotein, the tissue form of the $\alpha 1/\alpha 2$ subunits of type VI collagen. This was confirmed by immunoblotting of authentic type VI collagen from pepsin-treated pig skin. Immunoblotting, metabolic labelling with [^3H]glucosamine and immune precipitation showed that an immunoreactive collagenous glycopolypeptide was synthesized and secreted by cultured pig skin fibroblasts. The results suggest that type VI collagen is the major concanavalin A-binding component in pig skin.

INTRODUCTION

Several studies have shown that the differentiation of epidermal cells (keratinization) is accompanied by changes in their lectin-binding properties [1–6]. However, the molecular basis for this differential lectin binding is not yet clear. To identify differentiation-associated glycoproteins in the epidermis we have developed procedures for isolating the major lectin-binding glycoproteins from pig epidermis [7]. We have raised antisera against various electrophoretically purified glycopolypeptides and have used such antibodies to localize a 135 kDa component to the surface of basal cells [7] and 78 to 44 kDa components to the surface of suprabasal cells in pig epidermis [8].

Antisera were also raised against a ConA-binding component with an apparent molecular mass of 150 kDa that was present in pig epidermal extracts. However, these antisera reacted strongly with the dermis in indirect immunofluorescence and did not stain the epidermis. This observation prompted us to compare ConA-binding components in the epidermis and dermis of pig skin. We show here that this 150 kDa glycopolypeptide is collagenous and is present predominantly in the dermis, where it accounts for over 90% of the total ConA-binding material. It is associated with 3–4 nm dermal microfilaments and is closely related to the 140 kDa subunits of type VI collagen recently identified in various bovine, human and avian tissues [9–14]. The results suggest that type VI collagen is the major ConA-binding component in pig skin.

EXPERIMENTAL

Materials

Sephacrose-bound ConA and Protein A were from Pharmacia Fine Chemicals, Hounslow, Middx., U.K. Purified bacterial collagenase (form III) was from Advance Bio Factors Corporation, Lynbrook, NY,

U.S.A. D-[6- ^3H]Glucosamine hydrochloride (30 Ci/mmol) was from NEN Research Products, Boston, MA, U.S.A. FITC-conjugated ConA was from BDH Chemicals, Poole, Dorset, U.K. Pig anti-(rabbit IgG) antibody and its FITC conjugate were from Dakopatts, Glostrup, Denmark. Gold (15 nm)-conjugated goat anti-(rabbit IgG) antibody was from Janssen Pharmaceuticals, Wantage, Oxon, U.K. Dulbecco's modified Eagle's medium was from Flow Laboratories, Rickmansworth, Herts., U.K., and foetal-calf serum was from Biological Industries, Haemek, Israel.

Isolation of ConA-binding material from pig skin

Skin slices were separated with the use of 1 M-CaCl₂ [7], and the epidermis and dermis were homogenized in 10 vol. of 1 mM-PMSF/10 mM-Tris/HCl buffer, pH 7.5, and filtered through nylon mesh (0.13 mm hole size). The bulk of the homogenate from both tissues was retained on the mesh, and this fraction was extracted with 8 M-urea/1% (w/v) sodium deoxycholate/1% (v/v) 2-mercaptoethanol/1 mM-PMSF/10 mM-Tris/HCl buffer, pH 7.5. Extracts were dialysed against 1% (w/v) sodium deoxycholate/0.25 mM-PMSF/10 mM-Tris/HCl buffer, pH 7.5, and centrifuged, and ConA-binding material was isolated as described previously [7,8].

Collagenase digestions

Samples containing 1 mg of protein/ml or less were treated with 10 or 100 units/ml of enzyme from *Clostridium histolyticum* in 10 mM-CaCl₂/10 mM-Tris/HCl buffer, pH 7.5, at 20 °C for 20 h or 2 h respectively. Samples were dialysed against water at 4 °C and freeze-dried.

SDS/polyacrylamide-gel electrophoresis and immunoblotting

Electrophoresis was performed on polyacrylamide gels [15], which were then stained with Coomassie Blue or

Abbreviations used: ConA, concanavalin A; FITC, fluorescein isothiocyanate; PMSF, phenylmethanesulphonyl fluoride; CLGP, collagen-like glycoprotein.

processed for fluorography [16]. Molecular masses were estimated relative to globular standards. Isoelectric focusing was performed on 2.5 mm × 90 mm gels [17] with 0.8% pH 4–6, 0.8% pH 6–8 and 0.4% pH 3–10 or 1.6% pH 4–6, 0.4% pH 3–10 Ampholines. Gels were either stained with Coomassie Blue and sliced into 0.5 cm sections that were eluted with water before pH measurement or were subjected to SDS/polyacrylamide-gel electrophoresis in the second dimension [17]. Proteins were electrophoretically transferred to nitrocellulose [18], which was either stained with Amido Black or blocked with 5% (w/v) bovine serum albumin before overlay with antibodies and ¹²⁵I-labelled Protein A [8].

Production of antisera

ConA-binding material from pig dermis was run on a preparative (3 mm thick) 5%-polyacrylamide slab, and the major band was located by staining the edges. The 150 kDa band was electrophoretically eluted from unstained gel strips, dialysed against 0.1% SDS and precipitated with acetone [8]. Rabbits were immunized subcutaneously with about 200 µg of protein in complete Freund's adjuvant and were boosted monthly with 100 µg in incomplete Freund's adjuvant. Serum was obtained after the third boost. Affinity-purified antibodies were prepared by absorption on to nitrocellulose strips containing the dermal glycopolyptide followed by elution with 0.5 M-glycine, pH 2.8 [19]. As a control these antibodies were re-absorbed twice with the major dermal glycopolyptide.

Fluorescence microscopy

Frozen sections of tissues taken from 6–8-week-old piglets [7] were stained unfixed, after 10 min methanol treatment at 4 °C or after fixation with freshly prepared paraformaldehyde (2%) for 15 min. FITC-conjugated ConA was used at 25 µg/ml in phosphate-buffered saline (137 mM-NaCl/2.7 mM-KCl/8.1 mM-Na₂HPO₄/1.5 mM-KH₂PO₄, pH 7.4) with or without 5% (w/v) α-methyl mannoside. Affinity-purified antibodies or re-absorbed antibodies were used at 1:10 dilution in phosphate-buffered saline for 1 h. After being washed, sections were incubated with FITC-conjugated pig anti-(rabbit IgG) antibody diluted 1:50. Fibroblasts grown on eight-chamber glass tissue-culture slides were fixed with methanol at 4 °C for 10 min before indirect staining.

Immunogold labelling

Pieces of pig skin were fixed in 2% (w/v) paraformaldehyde or 0.1% (v/v) glutaraldehyde/2% (w/v) paraformaldehyde, dehydrated and embedded as described previously [8,20]. Sections were stained with various dilutions of the affinity-purified antibody and re-absorbed control for 1 h. After being washed with 1% (w/v) bovine serum albumin in phosphate-buffered saline, sections were incubated for 1 h with Protein A-coated colloidal gold (20 nm diameter) or goat-anti-(rabbit IgG)-antibody-coated gold (15 nm diameter). Washed grids were stained with aqueous uranyl acetate and lead citrate. Measurement of filament diameters was made with a catalase crystal as internal standard.

Isolation of pepsin-treated type VI collagen

Type VI collagen was isolated from pepsin-treated guanidinium chloride extracts of pig dermis, and was

purified by gel filtration on Sephacryl S-400 in SDS as described by Trüeb *et al.* [21] for other tissues.

Biosynthetic studies

Pig fibroblasts were propagated from skin explants in Dulbecco's modified Eagle's medium containing 8% (v/v) foetal-calf serum, penicillin/streptomycin (100 units/ml), non-essential amino acids and ascorbic acid (25 µg/ml). Confluent or 1-week post-confluent cells were labelled with [³H]glucosamine (20 µCi/ml) for 18 h in serum-free medium. Secreted glycoproteins were precipitated either with 5 vol. of ethanol (18 h at –40 °C) or with (NH₄)₂SO₄ at 80% saturation. Precipitates from the medium and cell layers were dissolved directly in SDS sample buffer or were extracted with 8 M-urea/1% (w/v) sodium deoxycholate/1 mM-PMSF/1% (v/v) 2-mercaptoethanol/10 mM-Tris/HCl buffer, pH 7.5, dialysed and incubated with ConA-Sepharose as described for the tissue extracts.

For immune precipitation untreated medium was incubated with antiserum (10 µl/ml) or normal rabbit serum for 2 h at 20 °C followed by pig anti-(rabbit IgG) antibody (100 µl/ml) for 18 h. Immune precipitates were washed three times with 1% (v/v) Nonidet P40 in phosphate-buffered saline and once with water, and were dissolved in SDS sample buffer.

Fig. 1. Effect of 2-mercaptoethanol on isolation of ConA-binding glycoproteins from pig skin

Dermis (lanes a and b) and epidermis (lanes c and d) were extracted with 8 M-urea/1% (w/v) sodium deoxycholate/1 mM-PMSF/10 mM-Tris/HCl buffer, pH 7.5, containing (lanes a and c) no reducing agent or (lanes b and d) 1% (v/v) 2-mercaptoethanol. After dialysis, ConA-binding glycoproteins were isolated and analysed by electrophoresis on 7% polyacrylamide gel. Small arrows indicate the position of globular-molecular-mass markers. The large arrow on the right indicates the glycopolyptide of apparent molecular mass 150 kDa.

Fig. 2. Collagenase-sensitivity of ConA-binding glycoproteins from pig skin

The ConA-binding material from (lanes a and b) dermis and (lanes c and d) epidermis was incubated (lanes a and c) without enzyme or (lanes b and d) with purified bacterial collagenase as described in the Experimental section and analysed by electrophoresis on 10 % polyacrylamide gel. Arrows are as in Fig. 1.

RESULTS

Skin

The total recoveries of ConA-binding glycoprotein from urea/deoxycholate/mercaptoethanol extracts of CaCl_2 -separated pig dermis and epidermis were $360 \pm 93 \mu\text{g}$ and $120 \pm 38 \mu\text{g/g}$ wet wt. of tissue respectively.

In the dermal extract (Fig. 1, lane b) a single broad band with an apparent molecular mass of 150 kDa comprised over 90 % of the total ConA-binding material. This major dermal component was not extracted in the absence of 2-mercaptoethanol (Fig. 1, lane a), indicating that it was probably disulphide-linked within the tissue. A disulphide-cross-linked glycopolypeptide of similar size was also present in the epidermal extract (Fig. 1, lane d), although in this tissue it comprised a smaller and more variable proportion of the total ConA-binding protein. The 150 kDa ConA-binding component in reduced dermal (Fig. 2, lane b) and epidermal (Fig. 2, lane d) extracts was completely degraded by treatment with bacterial collagenase. The absence of any residual Coomassie Blue-stained material in this region of the gel after digestion indicated that this band did not contain comigrating non-collagenous contaminants. Collagenase treatment produced several fragments in the molecular-mass range 64 kDa to 30 kDa (Fig. 2, lane b). Collagenase-sensitivity was lost on prolonged storage at -20°C , probably reflecting re-oxidation of thiol groups and aggregation of the collagenous glycopolypeptide. On isoelectric-focusing gels the major dermal glycopolypeptide migrated with a pI of about 5.5 (Fig. 3b). On two-dimensional gels (Fig. 3c) it appeared as one major spot, although some streaking was usually seen in the

Fig. 3. Isoelectric focusing and two-dimensional gel electrophoresis of ConA-binding glycoproteins from pig dermis

Isoelectric focusing was performed as described in the Experimental section. Gels were either (a) eluted with water for measurement of the pH gradient or (b) stained for protein with Coomassie Brilliant Blue (CBB). Duplicate gels were subjected to electrophoresis in the second dimension on 7 % polyacrylamide gel and were (c) stained for protein or (d) transferred to nitrocellulose and probed with antiserum (diluted 1:500) against the dermal glycopolypeptide of apparent molecular mass 150 kDa followed by ^{125}I -labelled Protein A ($\alpha 150$). Only the upper halves of the two-dimensional gels are shown. The lower portion did not contain any significant protein-stained or immunoreactive spots.

isoelectric-focusing dimension, suggesting possible isoelectric variation.

Antiserum raised against the major dermal glycopolypeptide reacted strongly with the collagenous glycopolypeptide in both dermal (Fig. 4, lane c) and epidermal (Fig. 4, lane d) glycoprotein preparations. Identical immunoreactivity was observed with antibodies that had been affinity-purified. No reaction was seen with normal rabbit serum or re-absorbed antibodies. On two-dimensional immunoblots (Fig. 3d) the antiserum reacted with the major pI 5.5 spot. The antiserum also reacted with several minor, lower-molecular-mass, ConA-binding components which probably reflect degradation of the collagenous glycopolypeptide *in vivo*.

Fig. 5 compares the distribution of ConA-binding sites and the collagenous glycopolypeptide in pig skin by fluorescence microscopy. ConA and the affinity-purified antibodies both gave a similar, strong, diffuse staining of the dermis. However, the epidermis, which was clearly stained by ConA (Fig. 5a), did not react with the

Fig. 4. Immunoblotting analysis of ConA-binding glycoproteins from pig skin

ConA-binding material from (lanes a and c) dermis and (lanes b and d) epidermis was fractionated by electrophoresis on 7% polyacrylamide gel and transferred to nitrocellulose. Duplicate strips were (lanes a and b) stained with Amido Black (AB) or (lanes c and d) incubated with antiserum (diluted 1:500) raised against the dermal glycopolyptide of apparent molecular mass 150 kDa followed by ^{125}I -labelled Protein A ($\alpha 150$). Arrows are as in Fig. 1.

antibodies (Fig. 5*b*). Antibodies against the dermal glycopolyptide also produced strong diffuse staining of the lamina propria in pig oesophagus, tongue, vagina, ureter and bladder, indicating a wide distribution in connective-tissue matrices.

The localization of the collagenous glycopolyptide in pig skin was examined in more detail by immunocytochemical labelling with gold-conjugated Protein A or second antibody. Gold particles were distributed throughout the dermis, but the epidermis was not significantly labelled. Within the dermis there was little labelling of amorphous elastin or elastin-associated microfibrils. Relatively sparse labelling was seen over areas of striated collagen (Fig. 6*a*). Here the gold particles appeared to be present between rather than over individual collagen fibrils. Stronger labelling was seen in areas rich in microfilamentous structures. These gold-decorated microfilaments (mean diameter 3.14 ± 0.44 nm) were particularly prominent around dermal blood vessels (Fig. 6*b*), and were also observed around nerves (Fig. 6*c*). Dermal mast cells were not preserved in tissue fixed with para-formaldehyde alone. However, they were observed when the fixative was supplemented with 0.1% (v/v) glutaraldehyde. These mast cells were consistently strongly labelled (Fig. 6*d*) with gold particles clustered over intracellular granules as well as extracellular microfilaments (mean diameter 3.7 ± 1.08 nm).

Fig. 5. Immunofluorescent staining of pig skin and cultured pig fibroblasts

(a) and (b) Cryostat sections of pig skin stained with (a) FITC-conjugated ConA and (b) affinity-purified antibodies against the dermal glycopolyptide followed by FITC-conjugated anti-(rabbit IgG) antibody. (c) Cultured pig skin fibroblasts indirectly stained with affinity-purified antibodies followed by FITC-conjugated anti-(rabbit IgG) antibody. The bar is 20 μm .

Type VI collagen

The properties of the collagenous glycopolyptide in pig skin were very similar to those reported for collagen-like glycoprotein (CLGP) recently isolated from bovine aorta and ligament [9–11] and identified as the native form of the $\alpha 1/\alpha 2$ chains of pepsin-treated type VI collagen [22]. We have found that a collagenous immunoreactive polypeptide that co-migrates with the dermal glycopolyptide is also the major ConA-binding component in reduced guanidinium chloride extracts of pig aorta (results not shown).

Fig. 6. Immunogold localization of the dermal ConA-binding glycopolypeptide to 3–4 nm microfilaments in pig skin

(a) An area of sparsely labelled collagen fibrils (C) with more heavily labelled microfilaments in the upper part of the picture. (b) A dermal capillary with adjacent fibroblast (F). Microfilaments in the sub-basement-membrane area of the endothelial cell (E) are heavily labelled. (c) A Schwann cell (S) surrounded by heavily labelled microfilaments. (d) A mast cell (M) with intracellular labelling of secretory granules and extracellular labelling of microfilaments. Tissue was fixed with 2% (w/v) paraformaldehyde in (a)–(c) and 0.1% (v/v) glutaraldehyde/2% (w/v) paraformaldehyde in (d). Sections were stained with affinity-purified antibodies followed by (a) gold-conjugated Protein A and [(b)–(d)] gold-conjugated anti-(rabbit IgG) antibody. The scale bar is 1 μ m.

Further evidence that the ConA-binding glycopoly-peptide in pig skin is related to CLGP was obtained by immunoblotting analysis with an authentic anti-(bovine CLGP) serum. This antiserum reacted strongly with the dermal glycopolypeptide (Fig. 7, lane c), suggesting that it was immunologically related to type VI collagen. Confirmation of this was provided by immunoblotting analysis of purified type VI collagen isolated from pepsin-treated extracts of pig dermis. Reduced type VI collagen migrated as three bands with apparent molecular masses of 68 kDa, 55 kDa and 46 kDa (Fig. 7, lane e), in common with preparations from other tissues [21]. Anti-serum against the dermal glycopolypeptide reacted strongly with the $\alpha 1$ and $\alpha 2$ chains but failed to recognize the $\alpha 3$ chain (Fig. 7, lane f).

Cultured skin fibroblasts

Indirect immunofluorescence of cultured pig skin fibro-

blasts produced strong, granular, perinuclear staining (Fig. 5c). Similar staining was seen in early (first) and late (sixth) subcultures. Little staining of extracellular matrix was detected, even in post-confluent cultures, where matrix-associated fibronectin could be readily observed.

The biosynthesis of the collagenous glycopolypeptide in culture was examined by metabolic labelling with [3 H]glucosamine and immunoblotting. Fig. 8 compares the cell layer and medium from fibroblasts that had been labelled for 18 h in serum-free medium. Antiserum against the collagenous glycopolypeptide recognized [3 H]-glucosamine-labelled bands of about 145 kDa in the cell layer (Fig. 8, lanes a and d) and 155 kDa in the medium (Fig. 8, lanes b and e). About 60% of the total immuno-reactive material in these cultures was recovered in the medium during an 18 h incubation, and the immuno-reactive 155 kDa band was the major secreted [3 H]-glucosamine-labelled component. It was susceptible to

Fig. 7. Relationship of the dermal ConA-binding glycopolypeptide to CLGP and type VI collagen

The left-hand panel shows immunoblotting analysis (7% acrylamide) of dermal ConA-binding glycopolypeptides with (lane b) antiserum against the 150 kDa component, (lane c) anti-(bovine CLGP) serum and (lane d) normal rabbit serum. The right-hand panel shows immunoblotting analysis (8% acrylamide) of authentic type VI collagen from pepsin-treated pig dermis with (lane f) antiserum against the 150 kDa component and (lane g) normal rabbit serum. All sera were used at a dilution of 1:500. Lanes a and e show the relevant transfers stained for protein (AB). The constituent chains of type VI collagen are designated $\alpha 1$, $\alpha 2$ and $\alpha 3$ in accordance with ref. [21].

bacterial collagenase without prior reduction (Fig. 8, lanes c and f), although we were unable to detect any [^3H]glucosamine-labelled or immunoreactive fragments under these conditions. During fractional precipitation it remained soluble in $(\text{NH}_4)_2\text{SO}_4$ at 30% saturation, but it could be precipitated by raising the $(\text{NH}_4)_2\text{SO}_4$ concentration to 80% saturation (results not shown). A close relationship between the collagenous glycopolypeptide secreted in culture and that isolated from skin was further suggested by immune precipitation and binding to ConA. The major [^3H]glucosamine-labelled band in fibroblasts-conditioned medium (Fig. 8, lane g) was specifically precipitated by antiserum against the dermal glycopolypeptide (Fig. 8, lane i). It was also bound by ConA (Fig. 8, lanes l and o). However, we were unable to isolate the lower-molecular-mass immunoreactive species present in fibroblast cell layers by affinity chromatography on ConA.

DISCUSSION

The collagenous ConA-binding glycopolypeptide in pig skin was originally observed in epidermal glycoprotein preparations [7]. However, this component could not be demonstrated in the epidermis or in other epithelial tissues by indirect immunofluorescence. We cannot exclude the possibilities that this component is a minor component of the epidermis or that it is masked by other epidermal constituents. However, it is more likely to

reflect contamination of CaCl_2 -separated epidermal sheets with fragments of papillary dermis trapped within their undulating undersurfaces.

Type VI collagen was first detected in pepsin digests of aortic intima [23], and was subsequently isolated from bovine and human placentas after pepsin digestion [24–27]. It consisted of high-molecular-mass aggregates, which on reduction were shown to comprise polypeptide chains with molecular masses between 70 kDa and 40 kDa [22]. Several reports identified a collagenous glycoprotein that appeared to represent the undegraded tissue form of type VI collagen [9–11, 28]. CLGP, a 140 kDa glycoprotein isolated from bovine ligament and aorta [9, 11], was originally thought to comprise the three distinct but co-migrating subunits of type VI collagen. However, recent studies suggest that type VI collagen contains only two 140 kDa subunits ($\alpha 1$ and $\alpha 2$) together with one 200 kDa subunit [12]. The $\alpha 1$ and $\alpha 2$ subunits of native type VI collagen in mammalian tissues appear to have very similar physical properties and have not been resolved by either electrophoretic or chromatographic techniques. However, they can be distinguished immunologically [12], and they have recently been shown to be the products of separate genes [29]. In avian tissues the $\alpha 1$ and $\alpha 2$ subunits have apparent molecular masses of 140 kDa and 130 kDa [14] or 150 kDa and 140 kDa [30] and can be resolved by electrophoresis on analytical SDS/polyacrylamide gels.

The collagenous glycopolypeptide in pig skin is bio-

Fig. 8. Biosynthesis of collagenous glycopolypeptides by cultured pig skin fibroblasts

Confluent cells were labelled with [^3H]glucosamine in serum-free medium for 18 h. The left-hand panel compares [^3H]glucosamine-labelled material in the cell layer and medium by fluorography (^3H) and immunoblotting with antiserum (diluted 1:250) followed by ^{125}I -labelled Protein A ($\alpha 150$). Cell layers (lanes a and d) were solubilized in SDS sample buffer, and the medium was precipitated with 80 % saturated $(\text{NH}_4)_2\text{SO}_4$, dialysed against 0.25 mM-PMSF/10 mM-Tris/HCl buffer, pH 7.5, and incubated without (lanes b and e) or with (lanes c and f) purified bacterial collagenase. Arrows indicate the two immunoreactive forms in the cell layer and medium. In the middle panel conditioned medium from [^3H]glucosamine-labelled fibroblasts (lane g) was immune-precipitated with normal rabbit serum (lane h) or antiserum against the dermal glycopolypeptide (lane i) followed by pig anti-(rabbit IgG) antibody. The arrow shows the immune-precipitated glycopolypeptide. The right-hand panel compares ConA-binding material in fibroblast-conditioned medium by fluorography (^3H) and immunoblotting ($\alpha 150$). Medium from [^3H]glucosamine-labelled cells was incubated without (lanes j and m) or with (lanes k and n) bacterial collagenase to identify the collagenous glycopolypeptide (arrow). ConA-binding material (lanes l and o) was isolated from the medium as described in the Experimental section.

chemically and immunologically related to CLGP. Both components are bound by carbohydrate affinity columns (Fig. 1 and ref. [11]), both are extensively disulphide-linked *in situ* (Fig. 1 and refs. [11,28,31]) and both produce similar-sized fragments with bacterial collagenase (Fig. 2 and ref. [11]). Since the skin glycopolypeptide could not be isolated from non-reduced extracts on ConA columns (Fig. 1), we could not test directly whether the non-reduced form was resistant to bacterial collagenase, as reported for CLGP [11]. However, the observation that collagenase-sensitivity was lost when the skin glycopolypeptide re-aggregated during storage would suggest that only the reduced form was susceptible to cleavage with this enzyme. The small differences in apparent molecular mass may be due to different gel procedures or could reflect species-to-species variation [21].

The immunoblotting analyses suggested that the dermal glycopolypeptide was closely related to CLGP and type VI collagen, and the immune localization studies confirmed this close relationship. The 150 kDa glycopolypeptide and type VI collagen had a similar widespread distribution in connective-tissue matrices [13,32]. Both components were associated with fine microfilaments

[13,33], particularly those in a perivascular location. The strong labelling of mast-cell-associated microfilaments was surprising and has not been noted in previous studies with type VI collagen antibodies. However, mast cells are relatively labile cellular components of the skin and are only observed under certain fixation conditions.

Although our antiserum recognized both the $\alpha 1$ and $\alpha 2$ chains of pepsin-treated type VI collagen (Fig. 7), it is still not entirely clear whether the glycopolypeptide bound by ConA represents the parent form of both chains, which are known to co-migrate on electrophoresis on SDS/polyacrylamide gels [12], or just one of these polypeptides. The skin glycopolypeptide always migrated as one major band and was never resolved into two components. However, it would be difficult to accommodate non-collagenous fragments of the size seen in collagenase digests, together with the collagenous domain, within a single 150 kDa chain. We have been unable to prepare large pepsin-resistant fragments from the isolated glycopolypeptide. Pepsin treatment produced extensive degradation, presumably because of the denaturation and reduction of tissue extracts before lectin affinity chromatography. Nevertheless this glycopolypeptide was the major ConA-binding component in the skin, accounting

for up to 90% of the total ConA-binding material. It probably accounts for most of the strong dermal staining seen when skin slices are stained with fluorescent ConA (Fig. 5). Binding to ConA could therefore provide the basis for a simple and rapid method for isolating type VI collagen chains from small samples of normal and diseased human skin.

Fibroblasts cultured from pig skin synthesized and secreted a major collagenous glycopolyptide that was recognized by antiserum against the skin glycopolyptide. However, we were unable to demonstrate a matrix-associated disulphide-cross-linked form either by indirect immunofluorescence or by differential extraction with and without reducing agent. The immunoreactive material in the cell layer was completely extracted by 8 M-urea in the absence of 2-mercaptoethanol (I. A. King, unpublished work). This cell-associated material was not bound by ConA, and may be an underglycosylated precursor of the secreted glycopolyptide that was both larger and bound by ConA. The secreted component was sensitive to bacterial collagenase without prior reduction, which contrasts with some reports of secreted type-VI-collagen-related glycoproteins [31,34]. However, other authors claim that secreted collagenous glycoproteins of similar size are unaffected by reducing agent [35] or contain little intermolecular disulphide bonding [36]. This may reflect species and/or tissue differences in the ability of fibroblasts to incorporate these components into their extracellular matrix. We have found that human skin fibroblasts, unlike those from pig, do incorporate type VI collagen-related glycoproteins into a disulphide-cross-linked matrix (I. A. King, unpublished work). Cutis-laxa fibroblasts, which had a depleted extracellular matrix in culture, were reported to secrete increased amounts of a collagenous glycoprotein-GP140-related glycoprotein that was not disulphide-cross-linked [35]. Finally, embryonic human lung fibroblasts that had been virally transformed and did not deposit glycoprotein-GP140 into their extracellular matrix were found to secrete a larger, mainly monomeric, form of this glycoprotein into the medium [36]. Further studies are required to clarify the possible relationship between the secretion of different type-VI-collagen-related glycoproteins and the ability of different cell types to assemble an extracellular matrix in culture.

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