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Isolation and characterization of a collagen-binding glycoprotein from chondrocyte membranes

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A collagen-binding glycoprotein was isolated from purified chick chondrocyte surface membranes by affinity chromatography on type II collagen-Sepharose. The purified glycoprotein has an apparent mol. wt. of 31 000 and binds to native chick collagen types I, II, III, V and M. Although it contains 30% carbohydrates, the majority of which is fucose, it is hydrophobic and soluble only in detergents. The integral membrane protein character of the 31-K protein became apparent from its ability to insert into lecithin vesicles. Liposome-inserted 31-K protein binds ^{125}I-labelled type II collagen in the presence of 0.5 M NaCl, while detergent-solubilized 31-K protein is dissociated from type II collagen by 0.05 – 0.1 M NaCl. Electron microscopic studies employing the rotary shadowing technique indicate that 31-K protein particles bind to the ends of collagen molecules. We propose that this glycoprotein serves as anchorage site for extracellular collagen to the chondrocyte membrane and thus may be involved in cell-matrix interactions in cartilage.

Key words: chondrocyte/membrane/glycoprotein/collagen

Introduction

The question of how connective tissue cells interact with the extracellular collagen matrix has gained increasing interest in recent years. The discovery of the collagen-binding glycoprotein fibronectin (for reviews, see Vaheri and Mosher, 1978; Yamada and Olden, 1978) and other cell attachment factors such as laminin (Timpl and Martin, 1981) and chondronectin (Hewitt *et al.*, 1980, 1982) has initiated numerous studies which point to the conclusion that interactions of cells with the extracellular matrix are generally mediated or enhanced by such adhesion molecules (for review, see Kleinmann *et al.*, 1981; Hynes *et al.*, 1981; Ruoslahti *et al.*, 1981). On the other hand, alternative adhesion mechanisms independent from serum- or matrix-derived attachment factors have also been proposed for the adhesion of fibroblasts to native collagen (Grinnell and Minter, 1978; Linsenmayer *et al.*, 1978), or of hepatocytes to native collagen or collagen peptides (Rubin *et al.*, 1981).

The existence of collagen receptors on the cell surface was first suggested several years ago from binding studies of radiolabeled collagen molecules and their peptides to both platelets (Chiang *et al.*, 1977) and fibroblasts (Chiang *et al.*, 1978; Goldberg, 1979). Models for fibroblast membrane glycoproteins with matrix-binding properties have been described concomitantly by Lehto *et al.* (1980), Harper and Juliano (1981) and Hughes *et al.* (1981).

In this study we provide evidence for direct cell-collagen interactions by describing a novel collagen-binding glycoprotein from chondrocyte surface membranes. In hyaline cartilage, chondrocytes are imbedded in an extracellular matrix consisting predominantly of chondroitin sulfate proteoglycan, type II collagen and a number of minor collagens (for review, see Mayne and von der Mark, 1982). The macromolecules of the extracellular matrix have been shown to play an important role not only in the metabolism and expression of the cartilage phenotype of chondrocytes, but also in chondrogenic differentiation (Kosher and Church, 1975; Lash and Vasan, 1978; von der Mark, 1978).

Close association of chondrocytes with type II collagen was demonstrated by immunofluorescence studies on isolated chondrocytes (Gay *et al.*, 1976; von der Mark *et al.*, 1977; Dessau *et al.*, 1978): antibodies to type II collagen located a pericellular glycocalyx on the chondrocyte surface which is composed of strands of type II collagen extending from the cell surface into the extracellular environment. Flattening and spreading of chondrocytes on the culture dish occurred concomitantly with the loss of the pericellular type II collagen matrix (Dessau *et al.*, 1978). We interpreted these observations as a loss of collagen receptors from the chondrocyte surface or their redistribution to sites of focal contacts with the culture dish.

Here we describe the isolation of the collagen-binding protein from chondrocyte surface membranes by affinity chromatography on type II collagen-Sepharose. The hydrophobic protein binds to collagen in the absence of attachment factors or serum and is likely to serve as anchorage site for extracellular collagen to the chondrocyte surface.

Results

Purification of chondrocyte membranes

Freshly isolated chondrocytes from embryonic chick xyphoid cartilage were allowed to recover from trypsin treatment in Ham's F 12 medium, and then were homogenized thoroughly. In later experiments, whole xyphoid cartilage from 10-week-old chicken was homogenized and used as source of chondrocyte surface membranes.

Membranes were purified by several steps of centrifugation as described in Materials and methods and finally collected at the 17/40% by weight sucrose interface of a discontinuous sucrose gradient (Cates and Holland, 1978). Analysis of the membrane fraction by SDS-electrophoresis on acrylamide gels revealed a complex protein pattern in the mol. wt. range 20 000 – 100 000. A collagenase-sensitive, pepsin-resistant band of apparent mol. wt. 130 000 (by globular standards) (Figure 1C, arrow) probably represented the α1 chain of type II collagen. Comparison of chondrocyte membrane proteins (Figure 1c) with those of chick tendon fibroblasts (Figure 1b) or other chick cells (Figure 1a) demonstrated considerable differences in the protein pattern among different cell types.

Affinity chromatography on type II collagen-Sepharose

When chondrocyte membranes were solubilized in 0.1% Nonidet P40 (NP40)-0.001 M triethanolamine buffer and passed over a native type II collagen-Sepharose column,

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>90% of the applied protein did not bind to the column, as determined by protein analysis (Lowry *et al.*, 1951). Approximately 5% of the sample was eluted with a linear salt gradient (Figure 2) at ~50–100 mM sodium chloride; this fraction consisted predominantly of a protein with an apparent mol. wt. of 31 000, together with some other material (Figure 3b). Repeated absorption of this fraction on type II collagen-Sepharose in 0.1% deoxycholate-triethanolamine buffer, and elution with the same salt gradient resulted in a >98% enrichment of the 31-K protein (Figure 3c). From 60 chick sterna we obtained up to 5 mg of pure 31-K protein. This protein was recognized as a major component also in the SDS-electrophoresis of whole chondrocyte membranes (Figure 1c, arrow heads), but it was absent from membranes of non-chondrogenic cells. Small amounts of the 31-K protein were found in membranes of 3-day embryonic chick limb bud cells, (J.Mollenhauer, in preparation) suggesting that this protein may be present also in chondrocyte precursor cells.

The purity of the 31-K protein was verified by two-dimensional electrophoresis according to O'Farrell (1975) yielding a single spot with an isoelectric point of 6.1 (result not shown).

Characterization of the 31-K protein

The 31-K protein was soluble in detergents (NP40, Triton X-100, deoxycholate), but aggregated and precipitated in aqueous buffers. The amino acid analysis (Table I) did not, however, explain the hydrophobic character. The protein contained nine residues per thousand of an amino acid which eluted in the position of hydroxylysine; no hydroxyproline was found in the protein.

Carbohydrate analysis revealed an unusually high content (20% w/w) of fucose (Table II) and considerable proportions of glucosamine and galactosamine.

Binding of detergent-solubilized 31-K protein to collagen

The binding of the 31-K protein to various collagen types and non-collagenous proteins was studied in a time course experiment by reacting aliquots of ^{125}I-labelled 31-K protein with Sepharose beads coated with various collagen types and bovine serum albumin (BSA). The binding to type II collagen beads occurred very rapidly (Figure 4); after 10 min, ~80% of the radiolabelled 31-K protein was bound by type II collagen; maximally 90% of the applied radioactivity bound after 30 min. The binding to native collagen types I, III, V,

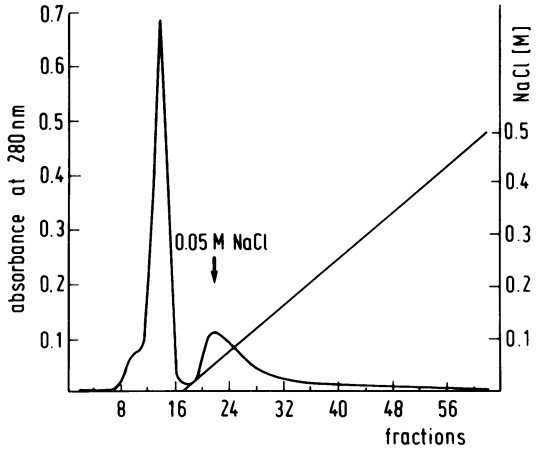

Fig. 2. Purification of the 31-K protein by affinity chromatography of chondrocyte membranes on type II collagen-Sepharose. Plasma membranes were solubilized in 0.1% NP40 in 1 mM triethanolamine buffer, pH 7.4 and passed over type II collagen coupled to Sepharose 4B. Bound proteins were eluted with a linear 0–1 M NaCl gradient over a total volume of 500 ml. The 31-K protein is eluted at 0.05–0.1 M NaCl.

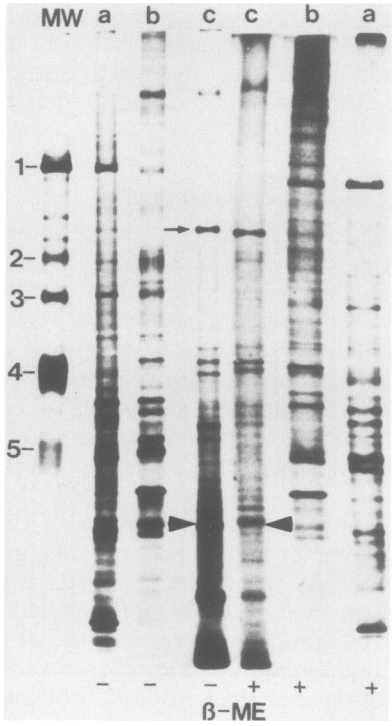

Fig. 1. Comparison of plasma membranes from chick chondrocytes **(c)**, tendon fibroblasts **(b)** and miscellaneous membranes (blood cells, liver, brain, muscle, skin, bone) **(a)** by SDS-gel electrophoresis on 5–18% polyacrylamide gradient gels (silver stain). Right three lanes: reduced with mercaptoethanol; left four lanes: unreduced. The arrow heads in **lane c** indicate the position of the 31-K protein, the arrow the position of the pro α1(II) chain. Mol. wt. standards are: 200 000 (1); 116 000 (2); 92 000 (3); 67 000 (4); 43 000 (5).

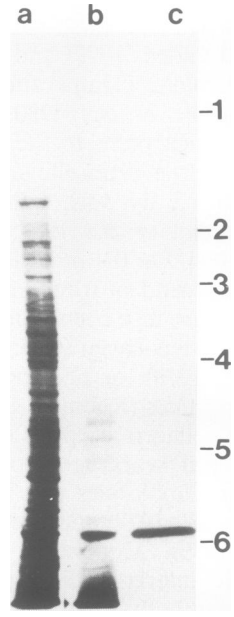

Fig. 3. SDS-polyacrylamide gel electrophoresis of the unbound fraction of chondrocyte membranes **(a)** and the fraction which eluted at 50–100 mM NaCl **(b)** from the type II collagen Sepharose (Figure 2). **(c)** 31-K protein, purified by a second chromatographic step on type II collagen-Sepharose in 0.1% DOC-triethanolamine buffer. Electrophoretic conditions and silver staining as in Figure 1. Mol. wt. standards 1–5: as in Figure 1; 6: 30 000.

M, (1α, 2α, 3α), all from chick, followed similar kinetics. Less than 1% of the applied [^{125}I]31-K protein bound to beads coated with BSA, or to uncoated Sepharose.

Rotary shadowing analysis of the 31-K protein-collagen complex

Solutions of native type II collagen in acetic acid were mixed with 31-K protein taken up in 0.1% NP40 and the complexes which formed were analyzed in the electron microscope by the rotary shadowing technique. In the presence of detergent, the 31-K protein forms aggregates hence the size of the 31-K particles was larger than expected on the basis of mol. wt. (Figure 5f). Most collagen molecules were bound through their ends to the 31-K protein particles (Figure 5a–e).

Table I. Amino acid composition of the 31-K protein

Amino acid	Residues/1000
Asx	81.2
Thr	40.3
Ser	88.4
Glx	107.5
Pro	48.2
Gly	183.5
Ala	70.8
Val	57.8
Met	18.0
Ile	43.9
Leu	86.9
Tyr	27.0
Phe	43.9
His	19.0
Hylys	8.7
Lys	26.9
Arg	44.8
Total	996.8

Values given are mean values of five separate analyses which, with the exception of serine, have not been corrected for possible losses during hydrolysis.

Integration into liposomes

To support the integral membrane protein character of the 31-K protein, liposomes were prepared from lecithin according to Racker *et al.* (1979) and mixed with ^{125}I-labelled 31-K protein, solubilized in 0.1% NP40. The mixture was separated by sucrose density gradient centrifugation, and the distribution of the 31-K protein was followed by counting the radioactivity in the gradient fractions (Figure 6). About half of the applied [^{125}I]31-K protein banded in the liposome fraction at the 8/17% sucrose interface, while after a similar gradient centrifugation without liposomes the 31-K protein collected at the top of the gradient. This suggested that the 31-K

Table II. Carbohydrate analysis of the 31-K protein

Carbohydrate	mg/mg protein	% of total weight
Fucose	0.265	20.9
Mannose	0.004	0.4
Glucose + Galactose	0.003	0.3
Glucosamine	0.101	9.0
Galactosamine	0.036	3.5
Total	0.409	34.1

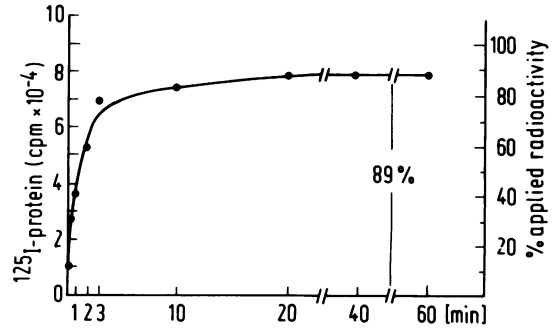

Fig. 4. Time course study of the binding of ^{125}I-labelled 31-K protein to type II collagen, linked to Sepharose beads. Aliquots of type II collagen beads were incubated with [^{125}I]31-K protein in 0.1% NP40 TEA buffer, washed and counted in a γ counter.

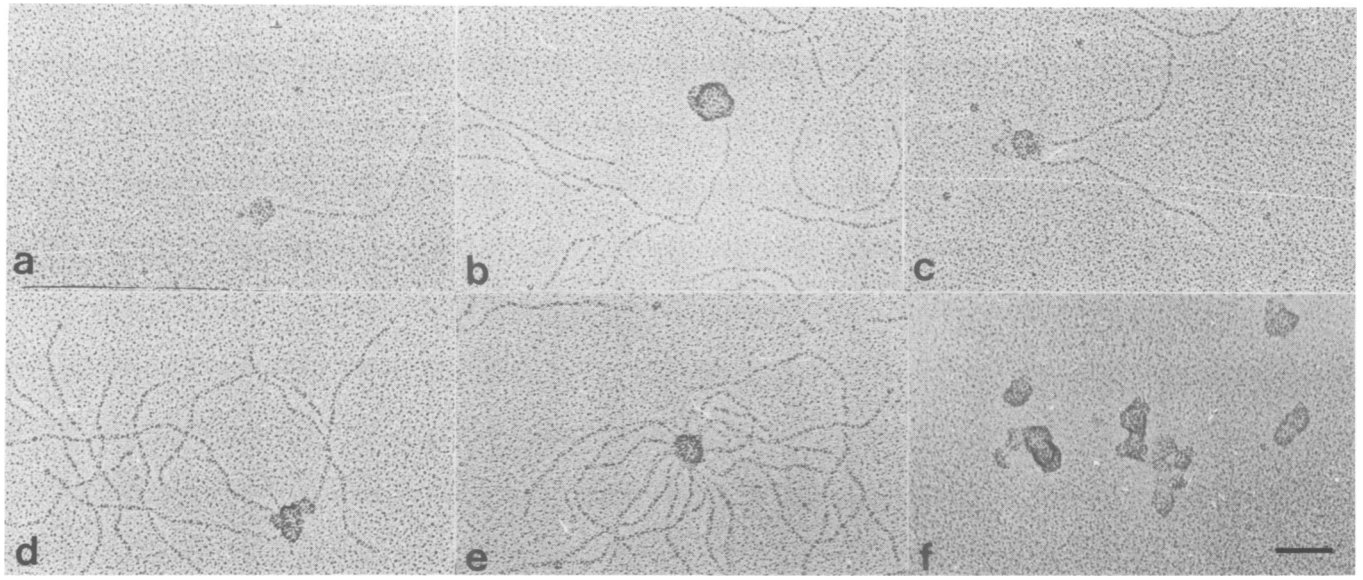

Fig. 5. Interaction between 31-K protein-detergent particles and type II collagen molecules, visualized by electron microscopy after rotary shadowing of the complexes (**a–e**). **f:** 31-K particles in the absence of collagen. Note that most collagen molecules bind to the particles with their ends. Bar: 100 nm; electron-optic magnification: x 30 000.

protein contains a hydrophobic portion which facilitates its insertion into the lipid bilayer of liposomes.

Binding of liposome-bound 31-K protein to collagen

Unlabelled 31-K protein was inserted into liposomes as above, and the liposomes were collected by density gradient centrifugation and used to study binding to ^{125}I-labelled type II collagen.

When 31-K liposomes were incubated with ^{125}I-labelled collagen in TEA-buffer, the collagen-bound radioactivity banded in the liposome fraction at the 8/17% sucrose interface after sucrose gradient centrifugation (Figure 7). When ^{125}I-labelled collagen was reacted with protein-free liposomes and centrifuged, the applied radioactivity migrated to the 17/40% interface, and only a small amount was found in the liposome fraction.

While in the presence of detergents the 31-K protein was dissociated from the collagen adsorbent at salt concentrations below 0.1 M NaCl (Figure 2), liposome-inserted 31-K protein also bound type II collagen at 0.5 M NaCl in the absence of detergents (Figure 7).

Discussion

By affinity chromatography on a type II collagen adsorbent we have isolated a 31-K glycoprotein from chondrocyte surface membranes with two distinct properties: (1) it is a chondrocyte-specific protein; and (2) it has a specific affinity for native collagen molecules. One- and two-dimensional SDS-gel electrophoresis revealed that the 31-K protein is only present in membranes of chondrocytes and limb bud mesenchymal cells. In accordance with this observation are results of immunofluorescent studies on chick tissues with an antiserum against 31-K protein, which revealed a positive reaction only in cartilage (Mollenhauer *et al.*, in preparation). This opens the possibility that this protein may be used as a specific marker for chondrogenic differentiation.

The purified 31-K glycoprotein has a specific affinity to collagen molecules in comparison to non-collagenous proteins, but *in vitro* it does not bind specifically to a distinct collagen type. The binding to collagen types occurs rapidly with similar time curves. In the presence of detergents, the 31-K protein is dissociated from the collagen adsorbent at ionic strength of 0.05 – 0.1 M NaCl which is below physiological conditions. However, in the absence of detergents, when inserted into lipid vesicles, the 31-K protein binds to collagen at salt concentrations between 0 and 0.5 M NaCl. Whether the 31-K protein also binds to procollagen, denatured collagen chains or collagen fragments is under investigation.

The membrane protein character of the 31-K protein is underlined by its hydrophobic properties and the ability to insert into liposomes. Although some of the 31-K protein may be included inside the lipid vesicles, at least some of the 31-K protein must be inserted right side-out in the lipid bilayer since the 31-K liposomes bound collagen, while protein-free liposomes did not.

Our rotary shadowing experiments suggest that 31-K protein-detergent micelles bind to the ends of the collagen molecules. This type of binding is in accordance with the radial arrangement of collagen fibres on the chondrocyte surface observed by both immunofluorescence (von der Mark *et al.*, 1977; Dessau *et al.*, 1978) and electron microscopy (Dessau *et al.* 1981). Whether the glycoprotein described here also serves as anchorage site for collagen on the chondrocyte surface *in situ* remains to be solved. Preliminary evidence for the involvement of the 31-K protein in the chondrocyte-collagen interaction was obtained from cell attachment studies: (1) the attachment of freshly isolated chick chondrocytes to type II collagen can be inhibited with Fab' fragments prepared from an antiserum against chick chondrocyte membranes; (2) chondrocyte attachment to type II collagen was inhibited by Fab' fragments from an antiserum directed against the purified 31-K protein. In both cases, the purified 31-K protein was able to neutralize this inhibition (Mollenhauer *et al.*, in preparation).

The finding of a collagen-binding membrane protein on chondrocytes is not at variance with the existence of serum factors such as the cartilage-specific attachment factor chondronectin (Hewitt *et al.*, 1980, 1982) which enhances binding

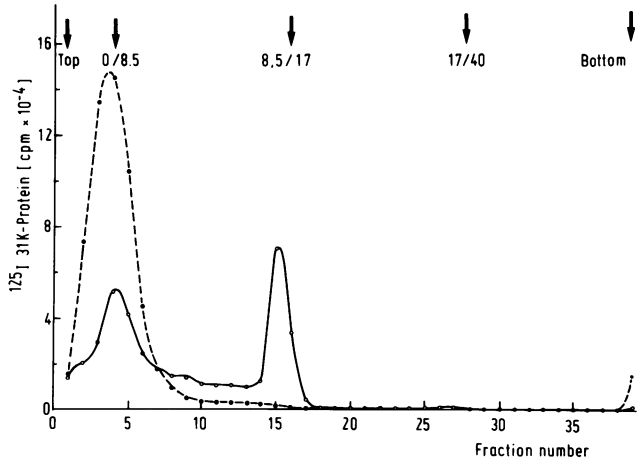

Fig. 6. Insertion of [^{125}I]31-K protein into liposomes. 1 ml of lecithin vesicles was mixed with 5 μl of [^{125}I]31-K protein (solubilized in 0.1% NP40), and separated by density gradient centrifugation on a stepwise sucrose gradient at 200 000 *g* for 4 h (solid line). The liposomes banded at the 8.5/17% sucrose interface. Dotted line: [^{125}I]31-K protein on the same sucrose gradient, without liposomes.

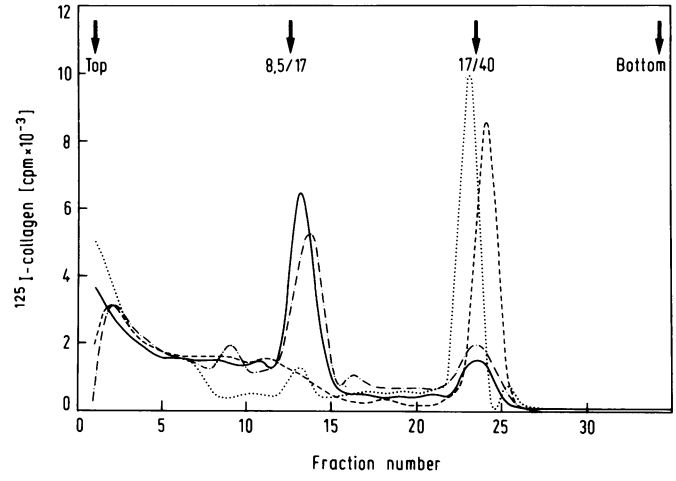

Fig. 7; Binding of 31-K protein-liposomes to ^{125}I-labelled type II collagen. Unlabelled 31-K protein was inserted into lecithin vesicles and isolated on a sucrose gradient as shown in Figure 6. ^{125}I-labelled collagen type II was incubated with 31-K protein-liposomes in TEA-buffer (—.—.—.—) or TEA buffer containing 0.5 M NaCl (——), or with protein-free liposomes (.....) for 1 h and liposome-bound and free [^{125}I]collagen were separated on a sucrose gradient. In the absence of liposomes (– – – –) or with protein-free liposomes [^{125}I]collagen banded at the 17/40% sucrose interface; incubation with 31 K-liposomes caused a shift to the liposome fraction at the 8.5/17% sucrose interface.

of chondrocytes to collagen-proteoglycan substrates. Independent mechanisms for chondronectin-mediated and 31-K protein-mediated chondrocyte-collagen interactions may exist, but it is also possible that the binding between chondrocytes and collagen involves both chondronectin and 31-K protein. Similar multi-component reactions have been reported for the interaction between hyaluronic acid and chondroitin sulfate proteoglycan (Hardingham, 1979), or the enhancement of binding between collagen and fibronectin by heparin (Jilek and Hörmann, 1979).

The existence of collagen-binding membrane proteins has been reported for two non-connective tissue cell types: Koehler *et al.* (1980) described a collagen-binding membrane glycoprotein of mol. wt. 40 000 in the acrosomal region of rabbit spermatozoa. Recently, Chiang *et al.* (1982) reported the isolation of a collagen-binding membrane protein of mol. wt. 65 000 from platelets. In our laboratory we have isolated a laminin-binding protein from muscle cell membranes with an apparent mol. wt. of 68 000 whose properties are similar to those of the 31-K glycoprotein. These studies and the observation reported here indicate that many cell types possess integral membrane glycoproteins which serve as anchorage sites for extracellular collagen. They are distinct from collagen-binding attachment factors such as fibronectin or chondronectin in that they are integrally associated with the plasma membrane. Owing to the hydrophobic character of these proteins, they can be solubilized in the presence of the detergent, and thus differ from hydrophilic, serum- or matrix-derived factors.

Materials and methods

Preparation of membranes

Chondrocytes were released with from 16-day embryonic chick sternae with collagenase and trypsin according to Dehm and Prockop (1973) with modifications as described previously (Dessau *et al.*, 1978). Embryonic chick tendon fibroblasts were prepared as described by Herrmann *et al.* (1980). Cells were allowed to recover from exposure to enzyme for 1 h in Ham's F12 medium (Seromed, Munich, FRG) containing 1% fetal calf serum, washed and preswollen for 30 min in 8.5% sucrose-TEA buffer (1 mM triethanolamine hydrochloride buffer pH 7.4, containing 0.1 mM phenylmethane sulfonylfluoride and 0.1 mM N-ethylmaleimide as protease inhibitors). The swollen cells were homogenized in an ice bath with 10 strokes of a glass-teflon homogenizer. The homogenate was centrifuged at 1500 *g* for 10 min, and the supernatant was pelleted by centrifugation at 20 000 *g* for 1 h. The resultant pellet was resuspended in 1 ml of 8.5% sucrose-TEA buffer and separated by density gradient centrifugation at 200 000 *g* for 1.5 h in a Beckman SW41 rotor according to Cates and Holland (1978), using a stepwise gradient of 8.5, 17 and 40% sucrose. Plasma membranes were enriched at the 17/40% interface.

Plasma membranes were also prepared from whole sternal cartilage of 10-week-old chicken. Sterna were dissected free of perichondrium and homogenized in 8.5% sucrose-TEA buffer for 20 min in an Ultraturrax homogenizer, and from the homogenate membranes were isolated as described above.

Affinity chromatography

Pepsin-extracted, native type II collagen from chick sternal cartilage was coupled to Sepharose 4B by CNBr as described previously (von der Mark *et al.*, 1976). Plasma membranes were solubilized in 0.1% NP40 in TEA-buffer and homogenized by sonification with a Branson-Sonifier (70–100 mW). 100–200 ml membrane solution (0.1 mg protein/ml) were applied to a column containing 25 ml of native type II collagen-Sepharose at room temperature. The column was washed with 0.1% NP40 in TEA-buffer, and then eluted with a linear salt gradient between 0 and 1 M NaCl over a total volume of 500 ml. The fraction eluting at 50–100 mM NaCl was dialyzed against 0.1% sodium-deoxycholate (DOC) in TEA-buffer and rechromatographed on type II collagen-Sepharose as described above, but in DOC-TEA buffer. The sample was finally concentrated by ultrafiltration on an Amicon YM 10 membrane and stored at −20°C.

Electrophoresis

One-dimensional slab-gel electrophoresis in 0.1% SDS was carried out on 5–18% polyacrylamide gradient gels in Tris-glycine buffer (Laemmli, 1970). Two-dimensional gel electrophoresis was performed by first isoelectric focussing and subsequent SDS-electrophoresis on 5–18% polyacrylamide gradient gels according to O'Farrell (1975). One- and two-dimensional gels were stained by the silver procedure as described by Oakley *et al.* (1980).

Protein iodination

Membrane proteins or whole membranes as well as type II collagen were iodinated with ^{125}I by the chloramine-T method according to Greenwood *et al.* (1963) to a specific radioactivity of $\sim 10^6$ c.p.m./μg protein.

Amino acid and carbohydrate analysis

0.5 mg 31-K protein was hydrolyzed in 6 N HCl for 24 h at 108°C under nitrogen and analyzed in an automatic Durrum amino acid analyzer.

For carbohydrate analysis, 1 mg 31-K protein was hydrolyzed in 0.5 N HCl, reduced with $NaBH_4$ and acetylated according to Schrager and Oates (1968). Xylose was added as internal standard. The acetylated sugars were analyzed by gas chromatography (Sawardeker *et al.*, 1965; Niedermeier and Tomana, 1974).

Insertion of 31K-protein into lipid vesicles

30 mg lecithin (Sigman Chem. Co.) were sonified in 1 ml TEA buffer for 10 min at 35 mW in an ice bath (Racker *et al.*, 1979). 5 μl ^{125}I-labelled 31-K protein (5 x 10^5 c.p.m.), solubilized in 0.1% NP40, were added and the mixture was applied to a sucrose gradient and centrifuged for 4 h as described above. Liposomes banded predominantly at the 8.5/17% interface. The gradient was eluted dropwise and counted in a γ counter. For binding studies with type II collagen, liposomes with inserted unlabelled 31-K protein were incubated for 1 h with 35 000 c.p.m. of ^{125}I-labelled type II collagen at room temperature, and centrifuged for 4 h on a sucrose gradient as above.

Rotary shadowing

Equal volumes of chick type II collagen (1 mg/ml) in 0.05 M ammonium bicarbonate, pH 7.8 and 31-K protein (1 mg/ml) in TEA buffer-0.1% NP40 were mixed and incubated for 30 min at room temperature. Aliquots were then diluted to 35 μg/ml with TEA-buffer, sprayed on mica-disks and shadow casted (Shotton *et al.*, 1979; Kühn *et al.*, 1981). Specimens were viewed under a Siemens Elmiskop 102 electron microscope.

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