Type VI collagen and glycoprotein MFPI are distinct components of the extracellular matrix

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Two collagenous glycoproteins, M_r 140000 and M_r 150000, are synthesized and secreted into the medium of cultured fibroblasts. The glycoprotein of M_r 140000 is identical with the 140K(VI) component of type VI collagen by both immunological and physicochemical criteria. The glycoprotein of M_r 150000 is immunologically distinct and exhibits the physicochemical characteristics of the putative elastic microfibrillar glycoprotein MFPI.

INTRODUCTION

Evidence is accumulating for the existence of discrete glycoproteins of specific M_r and composition that, although minor components of the extracellular matrix, may have important morphogenetic and structural functions [1, 2]. In general, these glycoproteins can only be isolated from tissues with the use of highly dissociative conditions, but when a biosynthetic approach has been adopted it has been possible to identify and isolate the native precursors. Thus biosynthetic studies on foetal bovine nuchal-ligament fibroblasts led to the identification of a glycoprotein of M_r 150000 that could be immunoprecipitated from culture medium by an antiserum known to stain the microfibrils at the periphery of elastic fibres [3]. Accordingly this glycoprotein, designated MFPI, was believed to be a major constituent of these microfibrils [4, 5]. Glycoprotein MFPI was also identified in fibroblast cultures from human skin [6], bovine tendon [7], dental pulp [8] and periodontal ligament [9], and was unusual in that it was partially collagenous [5].

More-recent studies have described the extraction and purification of another collagenous glycoprotein (M_r 140000) from a number of tissues varying markedly in their elastin contents [10–13]. This glycoprotein represents a reduced subunit of the intact form of type VI collagen and is designated 140K(VI) component [12]. Type VI collagen is not, however, a constituent of elastin-associated microfibrils [12, 14, 15].

The obvious similarity between these newly synthesized and tissue-extracted glycoproteins led, not surprisingly, to proposals that they were probably identical [10, 14, 16–18]. However, we have now identified the newly synthesized form of type VI collagen in fibroblast culture medium and provide evidence that this collagenous protein is immunologically and chemically distinct from glycoprotein MFPI.

MATERIALS AND METHODS

Culture and labelling of cells

Fibroblasts from foetal bovine nuchal ligament and tendon were propagated from freshly excised tissue and

subcultured in Dulbecco's modification of Eagle's minimum essential medium supplemented with 10% (v/v) foetal-calf serum [5, 7]. Confluent cultures were preincubated for 24 h with growth medium and then labelled for 24 h with either L-[5- 3 H]proline (10 μ Ci/ml) or L-[6- 3 H]fucose (20 μ Ci/ml) in fresh medium without serum. Medium was routinely supplemented with ascorbate (50 μ g/ml) except where otherwise stated. Labelled medium was removed and supplemented with proteinase inhibitors [5]. The results obtained with the two cell types were essentially the same.

Salt precipitation of macromolecules secreted into the medium

Proteins secreted into the culture medium were concentrated by sequential precipitation with $(\text{NH}_4)_2\text{SO}_4$ at 30% and 80% saturation at 4 °C. The 0–30%-satn.- and 30–80%-satn.- $(\text{NH}_4)_2\text{SO}_4$ precipitates were dissolved in 0.5 M-NaCl/50 mM-Tris/HCl buffer, pH 7.4, containing EDTA (8 mM) and phenylmethanesulphonyl fluoride (1 mM) and dialysed against the same buffer before immunoprecipitation.

Immunoprecipitation of macromolecules secreted into the medium

Antibodies to the pepsinized form of type VI collagen [19, 20], raised in guinea pigs, were concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation (45% saturation) and purified by chromatography on carboxymethyl-Affi-Gel Blue and on a series of affinity columns with fibronectin, collagen types I–V and finally type VI collagen, bound to Sepharose. Culture medium, or 0–30%-satn.- and 30–80%-satn.- $(\text{NH}_4)_2\text{SO}_4$ precipitates, redissolved as above, were incubated with the purified antibodies for 2 h at room temperature and overnight at 4 °C. The IgG fraction of an anti-(guinea-pig IgG) serum raised in rabbits was added and the mixture incubated as above. The proportions of medium and antiserum were optimized by preliminary titration experiments. Immunoprecipitates were washed twice with 62 mM-Tris/HCl buffer, pH 6.8, containing phenylmethanesulphonyl fluoride (1 mM) before analysis.

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Characterization of macromolecules secreted into the medium

Labelled macromolecules were analysed by SDS/polyacrylamide-gel electrophoresis in the absence and in the presence of β -mercaptoethanol (5%, w/v) [21] followed by fluorography [22]. Fluorograms were scanned by a laser densitometer (LKB 2202 Ultrosan). M_r values were determined by reference to non-collagenous standards of known M_r . Samples were digested with purified bacterial collagenase with and without prior reduction and alkylation, as previously described [11].

Glycoproteins of interest were recovered by careful excision from gels after SDS/polyacrylamide-gel electrophoresis and fluorography. [^3H]Proline-labelled components were hydrolysed in 6 M-HCl for 24 h, and the relative amounts of hydroxyproline and proline estimated by scintillation spectrometry following their separation on an automatic amino acid analyser [5]. Values quoted are the means $\pm$ S.D. for three determinations. Peptide 'mapping' was carried out on [^3H]proline- and [^3H]fucose-labelled components after digestion with *Staphylococcus aureus* V8 proteinase or CNBr [12].

RESULTS AND DISCUSSION

The macromolecules present in the 0–30%-satn.- and 30–80%-satn.-(NH_4) $_2\text{SO}_4$ precipitates from medium of cells labelled with [^3H]fucose in the presence of ascorbate are shown in Fig. 1. The major fucosylated glycoproteins

in the 0–30%-satn.-(NH_4) $_2\text{SO}_4$ precipitate had M_r values of 140 000 and 240 000 (track 7) and those in the 30–80%-satn.-(NH_4) $_2\text{SO}_4$ precipitate had M_r values of 150 000 and 250 000 (track 10) when analysed in the reduced state. In the absence of a reducing agent only the glycoprotein of M_r 150 000 was observed, the other three glycoproteins being present as disulphide-bonded multimers of high M_r (tracks 2 and 5). However, the intensity of the [^3H]fucose-labelled glycoprotein of M_r 150 000 was often observed to increase after reduction, suggesting that it was partially disulphide-bonded.

The possible relationship between the secreted glycoprotein of M_r 140 000 and 150 000 and the 140K(VI) component of type VI collagen [12] was investigated by using a specific anti-(type VI collagen) antibody. Comparable experiments with anti-(microfibrillar protein) serum could not be carried out as this antiserum [3] is no longer available, and attempts in this and in other laboratories to produce an antiserum with similar specificity have been unsuccessful. The glycoprotein of M_r 140 000 present in the 0–30%-satn.-(NH_4) $_2\text{SO}_4$ fraction was immunoprecipitated by the anti-(type VI collagen) antibody (Fig. 1, tracks 3 and 8), but the glycoprotein of M_r 150 000 present in the 30–80%-satn.-(NH_4) $_2\text{SO}_4$ fraction was not precipitated (Fig. 1, tracks 4 and 9). The two glycoproteins therefore appeared to be immunologically distinct. This result was confirmed by the immunoprecipitation of whole medium, followed by (NH_4) $_2\text{SO}_4$ fractionation of the immunosupernatant,

Fig. 1. Differential solubility and immunoprecipitation of [^3H]fucose-labelled glycoproteins synthesized by cultured fibroblasts

Cells were labelled with [^3H]fucose in the presence of ascorbate, and the medium was fractionated and immunoprecipitated with an anti-(type VI collagen) antibody, as described in the text. Samples were analysed by SDS/7%-(w/v)-polyacrylamide-gel electrophoresis in the absence (tracks 1–6) and in the presence (tracks 7–14) of β -mercaptoethanol. Tracks 1 and 6, standard of [^3H]acetylated types I and V collagens respectively; tracks 2 and 7, 0–30%-satn.-(NH_4) $_2\text{SO}_4$ precipitate; tracks 3 and 8, immunoprecipitate from 0–30%-satn.-(NH_4) $_2\text{SO}_4$ precipitate; tracks 4 and 9, immunoprecipitate from 30–80%-satn.-(NH_4) $_2\text{SO}_4$ precipitate; tracks 5 and 10, 30–80%-satn.-(NH_4) $_2\text{SO}_4$ precipitate; tracks 11 and 12, reduced and alkylated immunoprecipitate before and after digestion with purified bacterial collagenase; tracks 13 and 14, as tracks 11 and 12 but without prior reduction and alkylation.

when no fucosylated glycoprotein of M_r 140 000 was recovered in the 0–30%-satn.-(NH₄)₂SO₄ precipitate but the glycoprotein of M_r 150 000 was present in the 30–80%-satn.-(NH₄)₂SO₄ precipitate (result not shown). The immunoprecipitated glycoprotein of M_r 140 000 co-migrated on SDS/polyacrylamide-gel electrophoresis with the 140K(VI) component extracted from tissues [12] and was susceptible to bacterial collagenase digestion only after prior reduction and alkylation (Fig. 1, tracks 11–14), this property being characteristic of type VI collagen [11]. It is therefore concluded that the secreted glycoprotein of M_r 140 000 and the 140K(VI) component of type VI collagen are identical. The disulphide-bonded component of M_r 240 000, which was also immunoprecipitated by this antibody (Fig. 1, tracks 3, 8 and 13), was apparently non-collagenous, as judged by its resistance to bacterial collagenase even after reduction and alkylation (Fig. 1, tracks 11–14), and is therefore unlikely to be a precursor or cross-linked form of the 140 K(VI) component. Indeed, incubation of cells in the presence of β -aminopropionitrile, an inhibitor of collagen cross-linking, did not decrease the amount of the 240 000- M_r component (results not shown). It was also distinct from fibronectin, since the anti-(type VI collagen) antibody was purified through a fibronectin-linked affinity column. It is possible that the component of M_r 240 000 could form a complex with the 140K(VI) component, and therefore be immunoprecipitated non-specifically, as was proposed for the undoubtedly analogous species GP140 and GP250 secreted by lung fibroblasts [23].

Analysis of culture medium from cells labelled with [³H]proline in the presence of ascorbate indicated that the glycoproteins of M_r 150 000 and 250 000 were the major proline-labelled components in the 30–80%-satn.-(NH₄)₂SO₄ precipitate. In contrast, the 140K(VI) component in the 0–30%-satn.-(NH₄)₂SO₄ precipitate could not be identified without immunoprecipitation since it represented only a very small proportion of the total proline-labelled polypeptides in this fraction (results not shown). Consequently, for further characterization, the 140K(VI) component was excised from gels after SDS/polyacrylamide-gel electrophoresis of immunoprecipitates. The glycoprotein of M_r 150 000 was excised from gels after SDS/polyacrylamide-gel electrophoresis of the 30–80%-satn.-(NH₄)₂SO₄ precipitates that had first been immunoprecipitated with the anti-(type VI collagen) antibody to avoid any possible contamination with the 140K(VI) component.

When fibroblasts were cultured and labelled with [³H]fucose in the absence of ascorbate, the 140K(VI) component immunoprecipitated by the anti-(type VI collagen) antibody was substantially decreased compared with that observed in ascorbate-supplemented cultures, but its mobility was unaffected (Fig. 2, tracks 1 and 2). In contrast, the glycoprotein of M_r 150 000 in the 30–80%-satn.-(NH₄)₂SO₄ precipitate was not significantly affected by the absence of ascorbate, but its mobility was increased (Fig. 2, tracks 3 and 4). Similar results were obtained with medium labelled with [³H]proline in the absence of ascorbate. These observations provide further evidence that the glycoprotein of M_r 150 000 is distinct from the 140K(VI) component of type VI collagen.

The physicochemical properties of the glycoprotein of M_r 150 000, i.e. solubility in 30%-saturated (NH₄)₂SO₄,

Fig. 2. Effect of ascorbate on the synthesis of [³H]fucose-labelled glycoproteins by cultured fibroblasts

Cells were labelled with [³H]fucose in the presence (+) and in the absence (–) of ascorbate, and the medium was fractionated or immunoprecipitated with an anti-(type VI collagen) antibody as described in the text. Samples were analysed by SDS/6.5%-(w/v)-polyacrylamide-gel electrophoresis in the presence of β -mercaptoethanol. Tracks 1 and 2, immunoprecipitate; tracks 3 and 4, 30–80%-satn.-(NH₄)₂SO₄ precipitate.

molecular mass and synthesis in the presence or absence of ascorbate, indicated that it was identical with the collagenous glycoprotein MFPI [4, 5]. This suggestion was confirmed by estimating the percentage proline hydroxylation of the excised glycoprotein of M_r 150 000, the value of $32.5 \pm 1.5\%$ observed agreeing closely with that found for glycoprotein MFPI [5]. The percentage proline hydroxylation of the excised 140K(VI) component was virtually identical ($33.2 \pm 3.8\%$) and also agreed with values found for the tissue-extracted 140K(VI) component [10–13]. Thus the possibility that the glycoprotein of M_r 150 000 (MFPI) represented a different molecular form of type VI collagen, with altered physicochemical and immunological properties, could not be excluded. However, when the 140K(VI) component and glycoprotein MFPI (labelled with either [³H]proline or [³H]fucose) were digested with *S. aureus* V8 proteinase, the resulting peptide ‘maps’ were quite different (Fig. 3). In particular, fucose was restricted to only two of the peptides derived from the 140K(VI) component, but was present on many of the MFPI-derived peptides. Distinct peptide profiles were also observed after the digestion of the two proteins with CNBr (results not shown). It is therefore concluded that type VI collagen and glycoprotein MFPI are distinct components of the extracellular matrix but exhibit similar degrees of proline hydroxylation. The glycoprotein of M_r 240 000 that was immunoprecipitated by the anti-(type VI collagen) antibody also showed

Fig. 3. Peptide 'mapping' of [^3H]proline-labelled and [^3H]fucose-labelled glycoproteins synthesized by cultured fibroblasts

Components labelled with either [^3H]proline [scans (a)–(c)] or [^3H]fucose [scans (d)–(f)] were excised from SDS/polyacrylamide gels after electrophoresis and digested with *S. aureus* V8 proteinase. The resulting peptides were separated by electrophoresis on SDS/15%-(w/v)-polyacrylamide gels, processed for fluorography and the fluorograms scanned as described in the text. Scans (a) and (d), 140K(VI) component immunoprecipitated by the anti-(type VI collagen) antibody; scans (b) and (e), glycoprotein MFPI from 30–80%-(NH $_4$) $_2$ SO $_4$ precipitate; scans (c) and (f), glycoprotein of M_r 240000 immunoprecipitated as in (a) and (d).

distinct peptide profiles after digestion with *S. aureus* V8 proteinase (Fig. 3). This result confirms the earlier suggestion that the glycoprotein of M_r 240000 is not directly related to the 140K(VI) component. Type VI collagen and MFPI have been tentatively assigned to microfibrillar structures that are non-elastin- and elastin-associated respectively [5, 14, 15], but further studies will be required to confirm these observations and to establish their functions.

It may be noted that a glycoprotein complex consisting of several disulphide-bonded subunits (M_r 150000–240000) designated 'myotendinous antigen' has been observed in the medium of cultured chick-embryo skin fibroblasts and skeletal-muscle cells [24, 25]. It was suggested that this complex, which appears early in chick limb morphogenesis, could be a component of the

microfibrils that connect developing muscle fibres, tendons and bone. Whether this glycoprotein complex is related to the glycoproteins reported in the present paper is not known. It is possible that there are different 'types' or 'families' of collagenous and non-collagenous glycoproteins which form the specific structural elements of the many microfibrillar structures that provide a link between the major extracellular matrix macromolecules (collagens, proteoglycans, elastin), basal lamina and cells. Further characterization of these glycoproteins may result in their 'classification' and provide further insight into their function.

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REFERENCES

- Anderson, J. C. (1976) *Int. Rev. Connect. Tissue Res.* **7**, 251–322
- Aplin, J. D. & Hughes, R. C. (1982) *Biochim. Biophys. Acta* **694**, 375–418
- Kewley, M. A., Steven, F. S. & Williams, G. (1977) *Immunology* **32**, 483–489
- Sear, C. H. J., Kewley, M. A., Jones, C. J. P., Grant, M. E. & Jackson, D. S. (1978) *Biochem. J.* **170**, 715–718
- Sear, C. H. J., Grant, M. E. & Jackson, D. S. (1981) *Biochem. J.* **194**, 587–598
- Sear, C. H. J., Grant, M. E., Anderson, J. C. A. & Jackson, D. S. (1975) *Biochem. Soc. Trans.* **3**, 138
- Taylor, C. M., Oelbaum, R. S. & Grant, M. E. (1982) *Connect. Tissue Res.* **10**, 77–89
- Shuttleworth, C. A., Berry, L. & Wilson, N. H. F. (1982) *Arch. Oral Biol.* **27**, 645–650
- Shuttleworth, C. A. & Smalley, J. W. (1983) *Int. Rev. Connect. Tissue Res.* **10**, 211–247
- Gibson, M. A. & Cleary, E. G. (1982) *Biochem. Biophys. Res. Commun.* **105**, 1288–1295
- Knight, K. R., Ayad, S., Shuttleworth, C. A. & Grant, M. E. (1984) *Biochem. J.* **220**, 395–403
- Ayad, S., Chambers, C. A., Shuttleworth, C. A. & Grant, M. E. (1985) *Biochem. J.* **230**, 465–474
- Jander, R., Troyer, D. & Rauterberg, J. (1984) *Biochemistry* **23**, 3675–3681
- Gibson, M. A. & Cleary, E. G. (1983) *Collagen Relat. Res.* **3**, 469–488
- von der Mark, H., Aumailley, M., Wick, G., Fleischmajer, R. & Timpl, R. (1984) *Eur. J. Biochem.* **142**, 493–502
- Aumailley, M., von der Mark, H. & Timpl, R. (1985) *FEBS Lett.* **182**, 499–502
- Crawford, S. W., Featherstone, J. A., Holbrook, K., Yong, S. L., Bornstein, P. & Sage, H. (1985) *Biochem. J.* **227**, 491–502
- Gibson, M. A. & Cleary, E. G. (1985) *J. Biol. Chem.* **260**, 11149–11159
- Abedin, M. Z., Ayad, S. & Weiss, J. B. (1982) *Biosci. Rep.* **2**, 493–502
- Jander, R., Rauterberg, J. & Glanville, R. W. (1983) *Eur. J. Biochem.* **133**, 39–46
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Laskey, R. A. & Mills, A. D. (1975) *Eur. J. Biochem.* **56**, 335–341
- Carter, W. G. (1984) *J. Cell Biol.* **99**, 105–114
- Chiquet, M. & Fambrough, D. M. (1984a) *J. Cell Biol.* **98**, 1926–1936
- Chiquet, M. & Fambrough, D. M. (1984b) *J. Cell Biol.* **98**, 1937–1946