Evidence of a small hydrophobic domain in the core protein of the heparan sulfate proteoglycan from human colon carcinoma cells

Renato V. Iozzo, Cynthia L. Ketterer and Diana J. Slaymaker

Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, 3400 Spruce Street/G1, Philadelphia, PA 19104, USA

Received 25 July 1986

We demonstrate that the cell surface heparan sulfate proteoglycan of human colon carcinoma cells has an affinity for a hydrophobic matrix. This property is mediated by sequences in the core protein, since papain- or alkaline borohydride-released heparan sulfate chains do not bind to the matrix. Trypsin releases a [^{3}H]leucine-rich, unsulfated, hydrophobic peptide, with $M_r \sim 5000$. This domain is present in neither the proteoglycan released into the medium nor in the intracellular degradation products. It is proposed that this peptide may represent the portion of the core protein intercalated into the plasma membrane.

Heparan sulfate proteoglycan *Hydrophobic peptide* *Core protein* *(Human colon carcinoma cell)* *Octyl-Sepharose*

1. INTRODUCTION

We have previously shown [1] that human colon carcinoma cells synthesize a high-M_r heparan sulfate proteoglycan which is localized at the cell surface. Several lines of evidence indicate that this molecule is an integral membrane component. First, it cannot be displaced by the addition of heparin, a much more anionic compound, under conditions that would displace heparan sulfate proteoglycans from hepatocytes [2]. Second, it requires detergent for solubilization and good recovery; and, finally, it can be released from the cell surface by mild proteolysis. In analogy with other systems [3,4], the proteolytic cleavage of the core protein occurs at a labile site near the plasma membrane [5]. This results in the splitting of the molecule in two segments: (i) a hydrophilic domain (ectodomain) which contains most of the core protein and heparan sulfate chains and which is released into the medium, and (ii) a cell-associated domain which presumably contains a portion of the core protein intercalated into the lipid bilayer. The results of the present investigation indicate that the cell-associated proteoglycan contains a small, hydrophobic, [^{3}H]leucine-rich region, with $M_r \sim 5000$, that is cleaved from the intact molecule by trypsin. This fragment is not present in the proteoglycan released into the medium or in the intracellular degradation intermediates.

2. MATERIALS AND METHODS

All the materials were of the best analytical grade and have been previously described [1,6]. Confluent cultures of human colon carcinoma cells, $\sim 10 \times 10^6$ cells/75 cm^2 flask, were incubated for 24–48 h in the presence of either [^{35}S]sulfate alone (40 μCi/ml) or in association with [3,4,5-^{3}H(n)]leucine (50 μCi/ml) with specific activities of ~900 and 140 mCi/mM, respectively.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50 © 1986 Federation of European Biochemical Societies

Experimental details for the isolation and purification of proteoglycans have been described in [1,6]. Briefly, the medium and cell layer samples were purified separately by sequential chromatography on Sephadex G-50 in 8 M urea buffer, 0.1 M NaCl [7] and DEAE-Sephacel with an NaCl gradient between 0.1 and 0.7 M. The proteoglycan peaks from the DEAE-Sephacel were concentrated by step-eluting them on 5 ml DEAE columns with 4 M guanidine HCl buffer [8], and further separated by analytical Sepharose CL-4B chromatography [6]. Hydrophobic chromatography was performed using the methods of Kjellen et al. [9] or Yanagishita et al. [8] with minor modifications. In the latter procedure, detergent-free samples were resuspended in ~2.5 ml of 4 M guanidine HCl buffer and applied to 10 ml octyl-Sepharose CL-4B columns. The columns were washed with ~20 ml buffer and then with a ~60 ml linear gradient (0–2%) of Triton X-100. The concentration of the detergent was estimated by UV absorbance at 280 nm and related to standard curves. Purified proteoglycans were treated with trypsin, papain, alkaline, borohydride or polyhydrogen fluoride as in [1,6,10].

3. RESULTS AND DISCUSSION

Human colon carcinoma cells synthesize a high-M_r species of heparan sulfate proteoglycan which is recovered from the medium as a single polydisperse peak (fig.1A), whereas the purified cell extract contains two major populations, A and B (fig.1B). As shown in [1], peak A is the precursor form of the medium proteoglycan and is slightly larger in hydrodynamic size, whereas peak B represents intracellular degradation products of peak A and is composed primarily of free glycosaminoglycan chains, approximately one-third their original size. These peaks were isolated and analyzed on octyl-Sepharose CL-4B (fig.2). As discussed previously [8], the high concentration of guanidine maintains the core protein in an unfolded state, thereby exposing the polypeptide chains to the hydrophobic gel matrix and thus favoring interactions which may not occur in the native conformation. Under these conditions, the majority of the medium form (fig.2A, filled circles, and table 1) did not bind to the column; the bound proteoglycan, which represented <25% of the total,

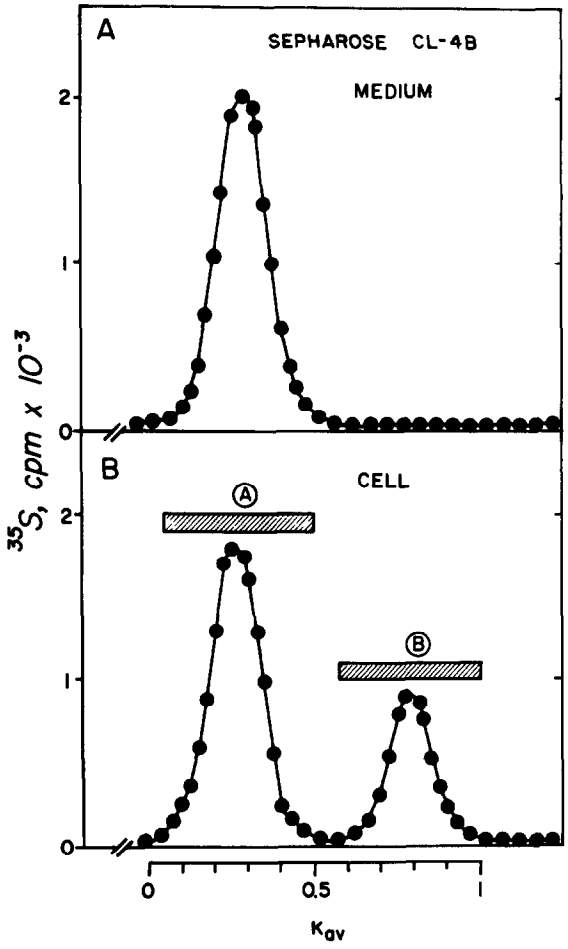

Fig.1. Elution profiles from an analytical Sepharose CL-4B column (0.7 × 90 cm) of ^{35}S-labeled heparan sulfate proteoglycans from the medium (A) and cell layer (B). The proteoglycans were twice purified by DEAE-Sephacel chromatography before applying to the column [1]. The cell layer peaks were pooled separately as indicated by the horizontal bars.

eluted as a single peak between 0.05 and 0.2% Triton X-100. In contrast, only ~19% of peak A (fig.2B, filled circles) did not bind to the hydrophobic matrix, whereas the bound proteoglycan (~81%) eluted at the same detergent concentrations. Nearly all of peak B (fig.2C) showed no affinity for the gel. Overall similar hydrophobic interactions were obtained with the methodology of Kjellen et al. [9] (not shown). Trypsin digestion prevented the binding of the ma-

jority of the proteoglycan particularly in the peak A species (fig.2B, unfilled circles, and table 1). In

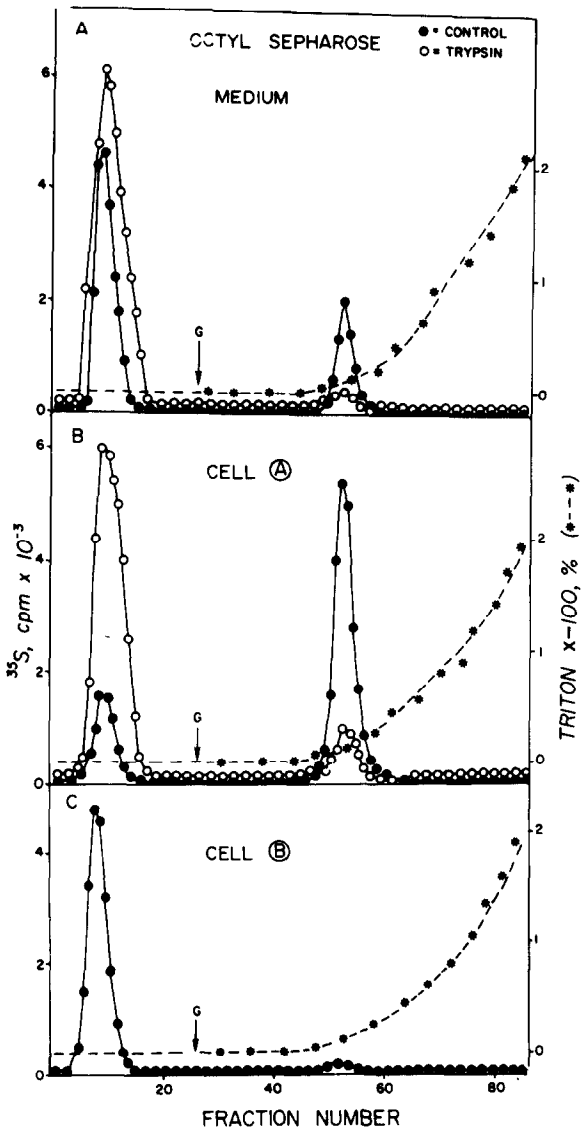

Fig.2. Elution profiles from octyl-Sepharose CL-4B of ^{35}S-labeled heparan sulfate proteoglycans from the medium (A) and cell layer (B,C) before (●) or after (○) trypsin treatment. Purified proteoglycans (see fig.1) were applied to 10 ml columns in 4 M guanidine HCl buffer and eluted with a Triton X-100 gradient (0–2%) at a constant flow rate of 3 ml/h. The detergent concentration (*---*) was measured by UV detection at 280 nm. The vertical arrow labeled 'G' designates the start of the gradient. Quantitation of the various components is provided in table 1.

agreement with these results is the report [11] that most of the medium and the trypsin-released heparan sulfate proteoglycan of hepatoma cells do not bind to octyl-Sepharose, whereas detergent is required to displace the majority of the cell-associated proteoglycan.

To determine whether the heparan sulfate chains contributed significantly to the hydrophobic behavior of the intact proteoglycans, purified samples of the medium proteoglycan and peak A were subjected to papain digestion or alkaline borohydride treatment and the released glycosaminoglycans were analyzed on octyl-Sepharose. The results (table 1) clearly show that the heparan sulfate chains released by either papain or alkali, did not have any significant affinity for the hydrophobic gel matrix. This is consistent with the report that ^{3}H-labeled heparin does not bind to this hydrophobic matrix under comparable experimental conditions [12]. In a separate experiment, the bound medium and peak A species were isolated and treated with polyhydrogen fluoride to remove most of the *O*-linked glycosaminoglycans and oligosaccharides leaving an intact core protein [1]. The deglycosylated core protein bound to the hydrophobic gel as the control samples (not shown). All of the above results strongly indicate that the hydrophobic interaction of the heparan sulfate proteoglycan is mediated by sequences in the core protein.

The difference in hydrophobic behavior between the medium and peak A species was further investigated in samples double-labeled with [^{3}H]leucine and [^{35}S]sulfate. Following trypsin treatment, both the medium proteoglycan (fig.3A) and the peak A species (fig.3B) had similar hydrodynamic size, with K_{av} ~0.3; however, in the peak A (fig.3B, unfilled circles) a significant proportion of [^{3}H]leucine-labeled material was cleaved from the proteoglycan and eluted near the total column volume. This unsulfated peptide was an integral component of the core protein since it could not be released by either heating at 100°C for 5 min or at 40°C for 30 min in the presence of 20 mM dithiothreitol and 1% SDS. This material bound to the octyl column in its entirety (not shown) and eluted from a Sephadex G-50 column with a K_{av} ~0.55, corresponding to M_r ~5000 (fig.3C). The results also indicate that this trypsin-labile region was highly enriched in leucine, since

it comprised ~20% of the total [^{3}H]leucine incorporated into peak A (fig.3B) though it accounted for only 2–3% of the total core protein mass [1].

In previous studies [2–4,9,11,12] the affinity of proteoglycans to hydrophobic matrices has been thought to represent evidence for their being integral membrane constituents. However, under appropriate solute conditions, several proteoglycans retrieved from the medium can exhibit affinity for the hydrophobic gels [8]. Indeed, ~25% of the medium proteoglycan bound to the column even though it lacked the trypsin-labile domain. It is likely, therefore, that other unglycosylated, hydrophobic sequences may be interspersed in the unfolded core protein between glycosaminoglycan chains and these regions may interact with the matrix. It is important to note, however, that a much greater proportion of the peak A proteoglycan (~81%) bound to the column. Since the only major difference between these two species was the presence in peak A of the trypsin-labile domain, we propose that this region may comprise the intercalated portion of the core protein. This relatively leucine-rich peptide would be responsible for the greater degree of hydrophobicity expressed by the peak A proteoglycan. Furthermore, this region retained its hydrophobic nature even after proteolytic cleavage and was not labeled with [^{35}S]sulfate, indicating that it was free of glycosaminoglycan chains. This is also consistent with a terminal location (i.e. at either end of the core protein) since its removal resulted in an intact but slightly smaller proteoglycan (fig.3B) without generation of glycosaminoglycan-peptide fragments. The estimated size of this hydrophobic region is similar to the transmembrane portion of other integral membrane proteins, such as the receptor for transferrin [13] or low-density lipoprotein [14]. It is possible that the heparan sulfate proteoglycan may be translocated intracellularly in a way similar to other transmembrane glycoproteins, in which an N-terminal region of 15–30 predominantly hydrophobic amino acids is required for their translocation across the membrane of the endoplasmic reticulum [15]. However,

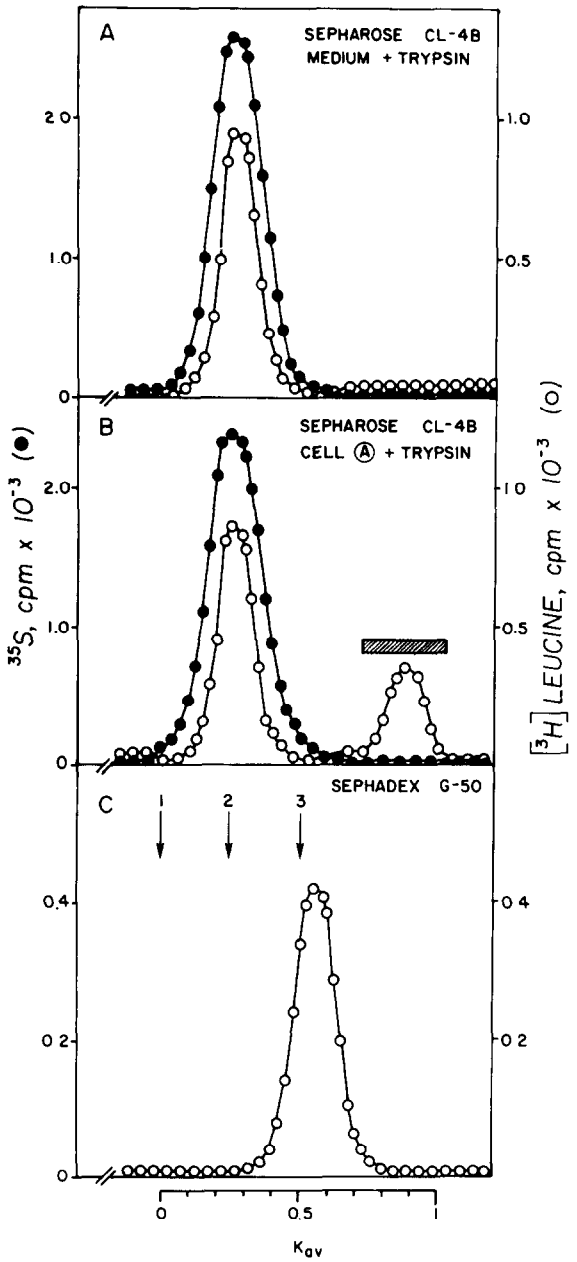

Fig.3. Elution profiles from an analytical Sepharose CL-4B column of trypsin-treated proteoglycan samples double-labeled with [^{35}S]sulfate (●) and [^{3}H]leucine (○). The [^{3}H]leucine-labeled peak (B) was pooled as indicated by the horizontal bar and further analyzed by analytical Sephadex G-50 chromatography (C). Both columns (0.7 × 90 cm) were eluted with 4 M guanidine HCl, 0.2% Triton X-100. The numbers in panel C designate the elution position of ^{14}C-labeled carbonic anhydrase, M_r ~30000 (1), ^{14}C-labeled lactoalbumin, M_r ~18000 (2) and ^{125}I-labeled insulin, M_r ~5800 (3), respectively.

Table 1

Affinity of the heparan sulfate proteoglycan for octyl-Sepharose gel

Treatment	Percentage ^{35}S-labeled proteoglycan					
	Medium		Cell, peak A		Cell, peak B	
	Unbound	Bound	Unbound	Bound	Unbound	Bound
Control (n = 3)	75 ± 2	25 ± 5	19 ± 6	81 ± 6	96 ± 3	4 ± 2
Trypsin (n = 5)	83 ± 6	17 ± 6	90 ± 7	10 ± 4		
Papain (n = 3)	97 ± 4	3 ± 1	98 ± 5	2 ± 1		
OH/BH_4 (n = 3)	97 ± 4	3 ± 2	94 ± 8	6 ± 4		

The purified proteoglycans, free of detergent, were subjected to each designated treatment and quantitated on octyl-Sepharose columns as described in the legend to fig.2 and in the text. The values represent the means ± SD

knowledge of the amino acid sequence would be necessary to confirm the existence and to determine the specific location of this hydrophobic domain.

ACKNOWLEDGEMENTS

This research was supported in part by a grant (CA-39481) from the National Institutes of Health and by a Junior Faculty Research Award (JFRA-95) from the American Cancer Society.

REFERENCES

[1] Iozzo, R.V. (1984) J. Cell Biol. 99, 403–417.
[2] Kjellen, L., Oldberg, Å. and Höök, M. (1980) J. Biol. Chem. 255, 10407–10413.
[3] Rapraeger, A. and Bernfield, M. (1983) J. Biol. Chem. 258, 3632–3636.
[4] Rapraeger, A. and Bernfield, M. (1985) J. Biol. Chem. 260, 4103–4109.
[5] Iozzo, R.V. (1985) Lab. Invest. 53, 373–396.
[6] Iozzo, R.V. (1985) J. Biol. Chem. 260, 7464–7473.
[7] Yanagishita, M. and Hascall, V.C. (1983) J. Biol. Chem. 258, 12847–12856.
[8] Yanagishita, M., Midura, R.J. and Hascall, V.C. (1986) Methods Enzymol., in press.
[9] Kjellen, L., Petterson, I. and Höök, M. (1981) Proc. Natl. Acad. Sci. USA 78, 5371–5375.
[10] Iozzo, R.V. and Wight, T.N. (1982) J. Biol. Chem. 257, 11135–11144.
[11] Robinson, J., Viti, M. and Höök, M. (1984) J. Cell. Biol. 98, 946–953.
[12] Lark, M.W. and Culp, L.A. (1984) J. Biol. Chem. 259, 6773–6782.
[13] McClelland, A., Kuhn, L.C. and Ruddle, F.H. (1984) Cell 39, 267–274.
[14] Russell, D.W., Schneider, W.J., Yamamoto, T., Luskey, K.L., Brown, M.S. and Goldstein, J.L. (1984) Cell 37, 577–585.
[15] Sabatini, D.D., Kreibich, G., Morimoto, T. and Adesnik, M. (1982) J. Cell Biol. 92, 1–22.