Release of cell surface proteoglycans from differentiating colon cells proceeds by cleavage of lipophilic anchor peptides

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Heparan sulphate proteoglycans are rapidly released from VACO 10MS colon cancer cells that are triggered with phorbol esters to undergo terminal differentiation. This lag-free temperature-sensitive process is correlated with a conversion of the lipophilic proteoglycans of the cell surface into non-lipophilic proteoglycans that accumulate in the culture medium. The released proteoglycans are very similar to their lipophilic precursors in size, buoyant density and glycosaminoglycan characteristics; however, they exhibit slightly smaller core proteins after chemical and enzymic deglycosylation. The lipophilicity of the larger-sized core proteins of the cell-associated proteoglycans is also correlated with the presence of an easily iodinated domain; this domain is missing in the released proteoglycans. Exogenous proteases (i.e. chymotrypsin, V8, trypsin and proteinase K) readily cleave this segment from the larger protease-resistant region of the proteoglycan structure. It is also released intact by treatment of the isolated proteoglycans with methanolic HCl. This component appears to be peptide in character, in that proteases readily degrade it and release iodotyrosines when the precursor has been iodinated. No evidence for the presence of covalently attached fatty acids in the cell-associated proteoglycans was found. These results are consistent with the hypothesis that the altered proteoglycan metabolism that is associated with the phorbol-ester-induced terminal differentiation of certain human colon cancer cells ensues upon the activation of a membrane-localized protease that cleaves a lipophilic anchor segment from the cell surface proteoglycans.

INTRODUCTION

Heparan proteoglycans are abundant in the plasma membranes of actively growing cells [1,2]. However, the level of this strongly acidic class of glycoproteins decreases rapidly as cells are triggered to differentiate terminally. For example, erythroleukaemia cells that are forced to differentiate with polar solvents exhibit a striking decrease in surface charge, a response which is partially attributable to the loss of heparan sulphate proteoglycans [3,4]. Similarly, PC12 pheochromocytoma cells lose about 70 % of their cell surface heparan sulphate to the culture medium as they differentiate in response to nerve growth factor [5]. Finally, our laboratory has shown previously that the differentiation of certain human colon cancer cells under the influence of phorbol esters is correlated with a rapid release of cell surface heparan sulphate proteoglycans to the culture medium [6]. This loss of proteoglycans in experimentally differentiated cell systems, along with immunological evidence showing a relative deficiency of these entities in the more differentiated members of epithelial cell lineages *in vivo* [7–9], prompts the hypothesis that heparan sulphate proteoglycan metabolism may play a key role in regulating the balance between cell replication and cell differentiation.

To explore this concept, our laboratory has studied the mechanism underlying the phorbol-ester-induced release of cell surface heparan sulphate proteoglycans from cells of the VOM human colon cancer line [6,10]. This research has revealed that cell-associated heparan sulphate proteoglycans contain a lipophilic peptide domain that is missing from the proteoglycans that have been released to the medium. Accordingly, it is postulated that proteoglycan release under the influences of the phorbol ester proceeds via protein kinase C activation of a membrane-associated protease that cleaves the lipophilic membrane-

anchoring domain from the cell-associated proteoglycan, releasing the heparan sulphate-bearing domain to the culture medium. The potential importance of this process in the control of malignant cell growth is discussed.

MATERIALS AND METHODS

Materials

Pronase, Zwittergent 3-14 and CHAPS detergent were from Calbiochem. Triton X-100 (membrane grade), phosphatidyl-inositol phospholipase C (*Bacillus cereus*) and proteinase K were from Boehringer Mannheim. PMA was from LC Services, Woburn, MA, U.S.A. CsTFA, octyl-Sepharose CL4B, DEAE-Sepharose and Sepharose CL4B were from Pharmacia. TPCK-treated trypsin was from Cooper Biomedical. V8 protease, Ba(NO₃)₂ and heparitinase were from ICN Biochemicals. Guanidine hydrochloride dextran sulphate 8000, heparinase and TLCK-treated chymotrypsin were from Sigma. Phenol and sodium thiocyanate were from Mallinckrodt. Trifluoroacetic acid, CPC, CNBr and acetyl chloride were from Aldrich. Iodobeads were from Pierce. Aquasure scintillation fluid, Na₂³⁵SO₄ (400–700 mCi/mmol), [¹⁴C]glucosamine HCl (54 mCi/mmol) and Na¹²⁵I (17 Ci/mg) were from New England Nuclear.

Cell culture

VACO 10MS (VOM) colon cancer cells [10] were grown as described previously [6], in Dulbecco's modified Eagle's medium (4.5 g of glucose/litre) with 2 % (v/v) fetal-bovine serum (HyClone, Logan, UT, U.S.A.) (DMEM/2 % FBS). The cells were passaged at 5–7-day intervals in cultures typically maintained and used at densities of (2–20) × 10⁴ cells/cm² of growth surface area.

Abbreviations used: CPC, cetylpyridinium chloride; CsTFA, caesium trifluoroacetate; DMEM, Dulbecco's Modified Eagle's Medium; S-MEM, Ca²⁺-free minimal essential medium; FBS, fetal-bovine serum; NEM, *N*-ethylmaleimide; PMSF, phenylmethanesulphonyl fluoride; PMA, phorbol 12-myristate 13-acetate; TLCK, tosyl-l-lysylchloromethane; TPCK, tosylphenylalanylchloromethane; VOM, VACO 10MS colon cancer cell line.

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Protocol for proteoglycan prelabelling

VOM cells were grown in DMEM/2% FBS containing 5 μ Ci of [35 S]sulphate/ml for 16–18 h. Specific radioactivities were 100 mCi/mmol (50 μ M-sulphate; for release assays) or 6 mCi/mmol (800 μ M-sulphate; for isolation and lipophilic fractionation of proteoglycans). Alternatively, cells were cultured in DMEM/2% FBS with [14 C]glucosamine (10 μ Ci/ml) and 0.2 g of glucose/l. Each labelling regimen included a further 90 min incubation in label-free medium.

Proteoglycan release assay

Prelabelled cells in 10 cm² wells were fed on medium containing PMA (100 nM) or vehicle [0.025% (v/v) ethanol]. At the indicated incubation times the medium was removed and clarified by centrifuging at 1000 g for 10 min and then mixed with 100 μ g of dextran sulphate (M_r 8000; Sigma) as a co-precipitate for the released proteoglycans. The cells were removed from dishes with 0.05% (w/v) trypsin, 0.01% (w/v) EGTA and 0.01% (w/v) dextran sulphate in S-MEM (15–30 min at room temperature). An equal volume of a solution containing 0.2% (w/v) CPC, 10 mM-EDTA, 10 mM-sulphate and 10 mM-acetate (pH 6) was added to each of these samples in silicone-treated tubes. Final salt concentrations were less than 100 mM-Na⁺. After 60 min at 36 °C, CPC–proteoglycan precipitates were collected on glass fibre filters (Whatman GF-C) and counted for radioactivity by liquid scintillation in Aquasure fluid.

Proteoglycan isolation [11]

Prelabelled cells were lysed with 1% (w/v) Zwittergent 3-14, 100 mM-NaCl, 50 mM-Tris/HCl, 10 mM-EDTA, 2 mM-*N*-ethylmaleimide (NEM), 0.5 mM-phenylmethanesulphonyl fluoride (PMSF) and 100 μ g of pepstatin A/ml. After 15 min at 4 °C, the soluble fraction was collected by centrifugation at 1000 g for 15 min. Detergent-insoluble materials were rinsed once with the same detergent solution, and the combined extracts were used for proteoglycan isolation by density centrifugation.

Solutions were adjusted to contain 5% (w/v) guanidinium chloride, 50 mM-Tris/HCl and 0.8% Zwittergent 3-14, then extracted with phenol three times (heated to 60 °C during the first extraction). This procedure removes non-glycosylated proteins and lipid. The aqueous plus interfacial phases were then extracted with ether to remove phenol and then concentrated to approx. $\frac{1}{8}$ vol. by vacuum centrifugation (SpeedVac, Savant Instruments, Hicksville, NY, U.S.A.). Samples were then rediluted to give 4 M-guanidinium chloride, adjusted to pH 7.5, reduced with 10 mM-dithiothreitol for 2 h at 48 °C, and alkylated with 25 mM-*N*-ethylmaleimide.

This solution was adjusted to contain 1% (v/v) Triton X-100, mixed with an equal volume of CsTFA (density 1.7 g/ml), and the sample was layered over 2 vol. of CsTFA (1.7 g/ml). After centrifugation for 44 h at 45000 rev./min in an SW50.1 rotor operated at 20 °C, fractions were collected from the bottom and assayed for density (by weighing 100 μ l samples) and radioactivity. Fractions with proteoglycan were concentrated by Centricon 30 centrifugal ultrafiltration (Amicon Corp., Lexington, MA, U.S.A.).

For the density isolation of the cell-released proteoglycans, the culture medium was collected by centrifugation to remove non-adherent cells and debris. It was then precipitated with 0.1% CPC as described above (except without the dextran sulphate carrier). The CPC–proteoglycan complexes were sedimented at 10000 g for 60 min at 20 °C, dissolved in 4 M-guanidinium chloride and then used for density sedimentation as described above for the cell-associated proteoglycans.

Chromatographic separations of proteoglycans

Octyl-Sepharose CL4B in Pasteur pipettes (0.4 cm diameter $\times$ 5 cm bed volume) was pre-equilibrated with 4 M-guanidinium chloride in 50 mM-Na-EDTA/50 mM-sodium acetate (pH 6) before receiving the above sample in the same solution. After adsorption had occurred for 1 h, non-adsorbed materials were collected by washing each column with 5 column vol. of the same solution. A linear gradient of 0–1% Triton X-100 in this solution was then flowed through at 1.5 ml/h for 5 h; 0.5 ml fractions were collected throughout this elution period. Lipophilic proteoglycans are defined as proteoglycans that adsorb to octyl-Sepharose in 4 M-guanidinium chloride and can be subsequently eluted with Triton X-100 at approx. 0.2% Triton X-100. For this study, non-lipophilic proteoglycans are those which fail to adsorb to this matrix in 4 M-guanidinium chloride.

Lipophilic proteoglycans for structural studies described herein were obtained from the high-density (1.5–1.75 g/ml) regions of the gradient and were further purified by adsorption to octyl-Sepharose and subsequent elution with a Triton X-100 gradient (0–1%) in 4 M-guanidinium chloride. Proteoglycan-containing fractions were then concentrated by ultrafiltration (Centricon 30), washed twice on the filters with 4M-guanidinium chloride (to remove Triton X-100), and then recovered in CHAPS buffer (20 mM-CHAPS detergent, 100 mM-NaCl, 50 mM-Tris/HCl, pH 7.5 at 20 °C). These proteoglycan preparations were stored at this step at –20 °C in CHAPS buffer. These purification methods are detailed elsewhere [11].

Gel filtration of such products on 0.8 cm $\times$ 30 cm columns of Sepharose CL-4B was performed where indicated in 4 M-guanidinium chloride / 50 mM-EDTA / 50 mM-acetate (pH 6) / 0.2% Triton X-100 at flow rates of 1.5 ml/h. Blue Dextran and Phenol Red were used as void- and total-volume markers respectively.

DEAE-Sepacel anion-exchange chromatography for analytical purposes was done by adsorbing the same on to a 2 cm³ gel bed column in 0.1 M-NaCl/20 mM-Tris/HCl/0.1% Triton X-100 in 8 M-urea. Elution employed a linear gradient of 0.1–1.0 M-NaCl in the same buffer. Conductivity measurements were used to determine NaCl concentration.

¹²⁵I labelling

CsTFA-purified proteoglycan preparations in CHAPS buffer were adjusted to contain 8 M-urea, two Iodobeads and 1 mCi of Na¹²⁵I in a final volume of 0.2 ml and incubated for 15 min at 20 °C. Proteoglycans were adsorbed from this solution to 3 cm³ columns of DEAE-Sepacel, freed of unincorporated iodide by washing with 0.1 M-NaCl in 20 mM-Tris/HCl (pH 7.5) containing 0.1% Triton X-100, and eluted with a gradient of 0.1–0.8 M-NaCl in the same buffer. This step also allowed removal of labelled non-proteoglycan macromolecules (~20% of total DEAE-Sepacel-bound label) from cell-derived proteoglycans, and of bovine serum-derived chondroitin sulphate proteoglycans from culture-medium-derived proteoglycans. Iodinated cell-associated proteoglycans were fractionated on octyl-Sepharose for segregation of lipophilic and non-lipophilic species.

Liposome incorporation

Appropriately labelled proteoglycans in CHAPS buffer were added to a 1 mg thin film of dipalmitoylphosphatidylcholine; 0.5 ml of 150 mM-CHAPS detergent in 4 M-guanidinium chloride/50 mM-Tris/HCl, pH 7.5, was added and the tube was heated to 48 °C for 1 min. After dialysis against two changes of 200 vol. each of detergent-free 4 M-guanidinium chloride for 16 h at room temperature, each preparation was further dialysed extensively against 0.1 M-NaCl/50 mM-Tris/HCl, pH 7.5 (Tris-buffered saline).

Enzyme digestions

Proteases were added as 10 μ l of a 1 mg/ml solution either in 2% SDS (V8 or proteinase K) or in 0.1 M-HCl with 1 mM- CaCl_2 (TLCK-treated chymotrypsin or TPCK-treated trypsin) to 30 μ l of labelled proteoglycan in CHAPS buffer. Pronase digestion (10 mg/ml, predigested) of the methanolic-HCl-cleaved peptide was done in 0.25% SDS/5 mM-EDTA/50 mM-Tris pH 7.5. Incubations were for 18–24 h at 36 °C under 10 μ l of toluene.

Heparinase (EC 4.2.2.7) and heparitinase (EC 4.2.2.8) (0.5 unit of each) were mixed with the iodinated proteoglycan in CHAPS buffer, additionally containing 10 mM-NEM, 1 mM-PMSF, 10 mM-EDTA, 8 mM- CaCl_2 and 340 μ g of pepstatin A/ml [12]. Incubation was for 18 h at 20 °C.

Chemical degradations

HNO_2 was prepared by mixing equal volumes of cold 1 M- $\text{Ba}(\text{NO}_3)_2$ and 1 M- H_2SO_4 and pelleting the resultant BaSO_4 at 15000 g for 5 min [13]. Then 20 μ l of 1 M- HNO_2 was mixed with 5 μ l of proteoglycan in CHAPS buffer with 20 μ g of BSA. After 5 min at 20 °C, the HNO_2 was removed by evaporation using a high-vacuum centrifuge, and the non-volatile materials were dissolved in 50 μ l of SDS/PAGE buffer.

Alkaline elimination of glycosaminoglycans from core proteins was effected by adding 0.2 ml of a mixture of 0.1 M-NaOH and 0.5 M- NaBH_4 and incubating at 48 °C for 18 h. After neutralization with 50% (v/v) acetic acid, H_2 was allowed to evolve for 2 h before analysis of the sample by gel filtration.

Methanolic HCl (0.6 M) was prepared by adding 0.25 ml of acetyl chloride to 5 ml of anhydrous methanol and allowing it to cool for 1 h under N_2 . Then 50 μ l of this solution was added to freeze-dried proteoglycan. After 18 h of incubation at 20 °C under N_2 , methanol and HCl were removed by vacuum centrifugation.

Hydroxylamine hydrochloride at 1 M in 33 mM-Tris/HCl buffer, pH 8, was added as 0.1 ml to 10 μ l of proteoglycan in CHAPS buffer and incubated at 20 °C for 5 h.

Gel electrophoresis

Samples were run on 2–20% (w/v) polyacrylamide gradient gels [0.1–1% (w/v) bisacrylamide cross-linker] with 2.5% (w/v) cross-linked polyacrylamide stacking gels and Laemmli discontinuous buffer containing 1% (w/v) SDS [14]. Samples were heated to 95 °C for 5 min in 1% SDS/2% (v/v) 2-mercaptoethanol before electrophoresis. For direct autoradiography of ^{125}I , electrophoresed gels were wrapped in Saran film and placed on X-ray film over an intensifying screen at –80 °C. Fixation and staining were for 60 min in 0.1% (w/v) Coomassie Blue in 12.5% (w/v) trichloroacetic acid with 3% acetic acid, followed by extensive washing in 7% acetic acid. For ^{35}S autoradiography, fixed gels were impregnated with 5% (v/v) glycerol in 5% acetic acid and dried between Cellophane sheets. M_r standards included lactalbumin (M_r 14000), carbonic anhydrase (M_r 31000), rabbit actin (M_r 43000), BSA (M_r 68000), *Escherichia coli* β -galactosidase (M_r 116000), and bovine myosin (M_r 200000). The position of a M_r -400000 protein species from the myosin preparation was also routinely noted.

Iodotyrosine identification

^{125}I -labelled proteoglycan or derived fragments in CHAPS buffer were digested sequentially with chymotrypsin (500 μ g/ml, 3 h), carboxypeptidase A (400 μ g/ml, 18 h) and Pronase (5 mg/ml, 24 h). After each protease addition, 5 μ l of toluene was added. Samples with or without authentic iodotyrosines as an internal control (Sigma; 50 μ g of each) were spotted on silica-

gel t.l.c. plates and chromatographed in benzyl alcohol/acetone/1 M- NH_3 (1:4:1, by vol.) [15]. ^{125}I -labelled spots were detected by autoradiography, and standards were located as the yellow photochemical reaction product generated during u.v. irradiation of the t.l.c. plate (1 h at 0.8 m from a 30 W G.E. germicidal light).

RESULTS

Characteristics of the PMA-labile pool of VOM-cell-associated proteoglycans

VOM cells rapidly release cell surface proteoglycans in response to a phorbol ester treatment that leads to terminal differentiation [6]. Most of these [^{35}S]sulphate-prelabelled macromolecules can be recovered from the culture medium by precipitation with the cationic amphiphile CPC. This release process is independent of new protein synthesis and is rapidly initiated in the cultured cells without an appreciable lag period (Fig. 1a). The release process thus appears to be effected through activation of pre-existing enzymes. Temperatures below 20 °C dramatically

Fig. 1. Kinetics of proteoglycan release: effect of different temperatures

VOM cultures, prelabelled with [^{35}S]sulphate as described in the Materials and methods section, were temperature-equilibrated, drained, and refed with fresh medium at the appropriate temperature, with or without 100 nM-PMA. After incubation at the indicated temperature, the media were harvested for CPC-precipitable radioactivity as described in the Materials and methods section. Total cell-associated CPC-precipitable label at zero time was (a) 103000 c.p.m. and (b) 14000 c.p.m. Data represent CPC-precipitable label in culture medium (means $\pm$ s.d.; $n = 3$). (a) Constant temperature; (b) effect of transferring 36 °C-incubated PMA-treated cells to 4 °C at the times indicated by arrows.

Fig. 2. Subcellular origin of released proteoglycans

VOM cells were prelabelled with [^{35}S]sulphate in low-sulphate medium as described in the Materials and methods section. PMA or control medium was added to triplicate dishes for each time point shown. Cultures were incubated at 36 °C for the indicated time periods, after which the medium was centrifuged to sediment non-adherent cells and debris, and the combined cells plus dish-associated materials were treated for 20 min at 4 °C with 0.5 ml of either (a) 100 μg of Pronase/ml in 50 mM-Tris-buffered saline, or (b) 4 M-guanidinium chloride/50 mM-Tris, pH 8.2. The resultant cell suspensions were then rapidly diluted 4-fold with Tris-buffered saline (mixed by inversion) and centrifuged at 15000 g for 30 min at 4 °C in an Eppendorf micro-centrifuge. Separated media, solubilized cells and residual insoluble fractions (the last sonicated for 4 s in 4 M-guanidinium chloride/1 % Triton X-100) were each diluted and adjusted to contain 100 mM-guanidinium chloride, 0.025 % Triton X-100, 50 mM-acetate, pH 6, 50 mM-EDTA, 50 mM-sulphate, 10 μg of dextran sulphate 8000/ml, and 0.1 % CPC. After incubation at 36 °C in silicone-treated glass tubes, the resulting CPC-[^{35}S]polyanion complexes were filtered on to Whatman GF-C glass-fibre discs and counted for radioactivity by liquid scintillation. S.D. bars are shown at the bottom of each data set for cell-associated fractions.

decrease initial rates of release, in accord with the operation of membrane-based enzymic processes [16]. In addition, the kinetics of proteoglycan release at different sub-physiological temperatures suggests that the proteoglycans may exist in more than one susceptible pool. The finding that the release of proteoglycans can be decreased to the control level by chilling the PMA-activated cells to 4 °C (Fig. 1b) shows that it is the PMA-induced event that is both temperature-sensitive and rate-limiting. In addition, proteoglycan release was only minimally affected by treating VOM cells with 1 mM-KCN (51 % of total [^{35}S]sulphate-prelabelled polyanion released by treatment with PMA alone, compared with 53 % released after 90 min of combined treatment; release by KCN alone was similar to the control, 14 versus 9 %).

The proteoglycan which was recovered from the culture medium appears to be soluble, rather than associated with cell fragments or membrane vesicles. Centrifugation (100000 g at 4 °C for 2 h) of the culture medium of PMA-treated [^{35}S]sulphate-prelabelled cells sediments less than 5 % of the polyanionic molecules. Thus the phenomenon is distinct from the release of proteoglycan-containing 'adherons' as previously described for cultured cells of mesenchymal and neural-crest origin [17]. [^{35}S]Sulphate-prelabelled proteoglycan isolated from the media of PMA-treated VOM cells (by adsorption to DEAE-Sephacel and elution with a denaturant-free NaCl gradient) failed to reassociate with growing VOM cells (< 1 % bound at either 4 °C or 36 °C).

The released proteoglycans appear to be derived from cell-surface-exposed integral membrane proteins. The increase in soluble proteoglycan is correlated with a decrease in that fraction of proteoglycans which are releasable on treatment of the cells with trypsin [6] or pronase at 4 °C (Fig. 2a). In spite of this extracellular localization, heparin (100–500 $\mu\text{g}/\text{ml}$, 4 °C or 36 °C) caused less than a 10 % increase in release of [^{35}S]sulphate-prelabelled polyanions from control cells, compared with saline alone. This finding suggests that the vast majority of these cell-

surface polyanions are anchored by a mechanism that is distinct from the electrostatic associations that bind proteoglycans to some cell types [2]. Furthermore, 40–60 % of [^{35}S]sulphate-prelabelled polyanions remained associated with the cell residue when prelabelled control cells were treated with 4 M-guanidinium chloride, a strongly ionic protein denaturant which dissociates most intermolecular associations other than covalent bonding or lipophilic interactions. Irrespective of whether the cells had been treated with PMA or not, 80–95 % of cell-associated polyanionic [^{35}S]sulphate from prelabelled VOM cells can be solubilized by 1 % solutions (in saline) of non-denaturing detergents, Triton X-100, deoxycholate or Zwittergent 3-14. Accordingly, the fraction of proteoglycans which decreases most precipitously upon PMA treatment appears to exist in some hydrophobic association with the VOM cells.

When [^{35}S]sulphate-prelabelled cells are treated with PMA, the guanidine-insoluble fraction of polyanions declines by half of its control cell value within 30 min (Fig. 2b). The corresponding 4 M-guanidine-removable fraction, representing molecules held by interactions dependent on native protein conformations or ionic interactions, was stable during this initial time period, or even increased. Consistent with the temperature-sensitivity of proteoglycan release, sub-physiological temperatures inhibited the PMA-induced decline in guanidine-insoluble ^{35}S -labelled polyanions; although this fraction was decreased to 48 % of the starting level after 20 min of PMA treatment at 36 °C, it was still 74 % after 20 min at 25 °C, 97 % at 15 °C, and 104 % at 4 °C.

Physical properties of cell-associated and phorbol-ester-released proteoglycans

The unique effectiveness of detergents in solubilizing the PMA-releasable fraction of proteoglycans of VOM cells suggested that these molecules carry a strongly lipophilic domain which is the target of PMA action. To test this premise, proteoglycans from lysates of [^{35}S]sulphate-labelled cells or their culture medium were isolated and examined for their ability to adsorb to octyl-

Fig. 3. Effect of PMA on pre-labelled lipophilic proteoglycan of VOM cells

VOM cells were prelabelled with [³⁵S]sulphate as described in the Materials and methods section and refed with DMEM/2% FBS containing either 100 nM-PMA or vehicle (0.025 % ethanol; control). At this time (0 h) and after 5 h of incubation, cells were lysed with 1 % (w/v) Zwittergent 3-14, and the detergent-soluble and -insoluble fractions of cells and the culture media were separately harvested by centrifugation. Proteoglycans were isolated from each fraction by phenol extraction and isopycnic CsTFA centrifugation: phenol-insoluble fractions with densities of 1.35–1.75 g of CsTFA/ml from each preparation were pooled and concentrated. The detergent-soluble cell-associated pool was further fractionated into lipophilic and non-lipophilic components by adsorption to octyl-Sepharose CL-4B (non-lipophilic components flow through, while lipophilic components are desorbed at approx. 0.2 % Triton X-100 during a gradient of 0–1 % detergent in 4 M-guanidinium chloride; see the Materials and methods section for details). Data are the radioactivity present in samples representing an equivalent proportion of cell or media fractions from each culture, either at the start of treatment (0 h) or after 5 h of incubation with or without 100 nM-PMA.

Sepharose [11]. When the proteoglycan preparations from control VOM cells were applied to this matrix in 4 M-guanidinium chloride, 30–50 % of total label adsorbed to the resin and was eluted with 0.2 % Triton X-100, the critical micelle concentration of this detergent in 4 M-guanidinium chloride. PMA treatment

of cells was associated with a progressive depletion of this pool. There was an associated increase, however, in the exclusively (> 95 %) non-lipophilic species recoverable from the culture medium (Fig. 3). This conversion from lipophilic to non-lipophilic state was very rapid; in one experiment, 30 min of PMA treatment decreased the lipophilic proteoglycan fraction to 40 % of its starting level. In contrast, the non-lipophilic cell-associated pools {consisting of detergent-soluble and -insoluble glycosaminoglycans, proteoglycans, and a minor (< 5 % of total label) unidentified sulphated macromolecule [11]} remain at 80–90 % of their starting levels during 5 h of treatment with PMA or control medium. Thus PMA appears to cause a preferential proteoglycan release from a pre-existing lipophilic pool. Consistent with this interpretation, the guanidinium-insoluble fraction discussed above was found to be highly enriched in octyl-Sepharose-binding proteoglycans.

As a test of the potential for membrane intercalation of the lipophilic proteoglycans, preparations of cell-associated proteoglycan which had been adsorbed to octyl-Sepharose and then eluted with Triton X-100 were mixed with detergent-dispersed dipalmitoyl phosphatidylcholine in the presence of 4 M-guanidinium chloride and dialysed to obtain liposomes. When these preparations were centrifuged, 70 % of lipophilic proteoglycan, but less than 3 % of culture-medium-derived proteoglycans, co-sedimented with the vesicles (Table 1). Treatment of these preparations with either trypsin or detergent before centrifugation solubilized the liposome-associated proteoglycans, whereas a high-salt solution had no effect. This finding is taken as evidence that these molecules are externally displayed and lipid-anchored. This distinctly different lipophilicity of the PMA-released proteoglycan and the cell-associated precursor suggests that the latter is structurally altered en route to its release.

As revealed in the high-resolution density profile of these isolated fractions (Fig. 4), the two proteoglycan classes have very similar densities. Accordingly, one must conclude that the lipophilic determinants of the cell-associated proteoglycans either have low mass relative to the glycosaminoglycan-bearing segments or have a similar density to the glycosaminoglycans. These densities (1.65 ± 0.10 g/ml) also suggest that the ratio of protein (density 1.2–1.4 g/ml) to glycosaminoglycan (density as high as 2.2 g/ml in CsTFA) in the two products is quite similar, or is simply dominated by the high density of sulphated polysaccharides.

Both classes of proteoglycans migrated through Sepharose CL4B gel-filtration media as very large and polydisperse molecules with similar size distributions (90 % of label at *K*_{av}. 0.25 ± 0.20; Fig. 5). Upon reductive alkaline-elimination

Table 1. Incorporation of proteoglycans into phosphatidylcholine liposomes

Proteoglycans were mixed with dipalmitoyl phosphatidylcholine in 4 M-guanidinium chloride containing CHAPS, and dialysed to produced liposomes as detailed in the Materials and methods section. A portion of each liposome suspension was added to 10 vol. of Tris-buffered saline containing (a) no addition, (b) 100 µg of TPCK-treated trypsin/ml, (c) 0.1 % Triton X-100 or (d) 1 M-NaCl. After incubation for 5 h at 36 °C, each preparation was centrifuged at 15000 g for 10 min at 4 °C to separate soluble from sedimentable materials. Data represent c.p.m./fraction; ND, not done.

Proteoglycan		Treatment							
		None		Trypsin		Detergent		NaCl	
		Pellet	Soluble	Pellet	Soluble	Pellet	Soluble	Pellet	Soluble
Released	³⁵ S	30	1050	40	1040	20	1060	40	1050
Lipophilic	³⁵ S	810	320	40	1190	20	1160	850	370
Released	¹²⁵ I	50	870	70	850	10	960	ND	ND
Lipophilic	¹²⁵ I	1080	280	600	620	40	1480	ND	ND

Fig. 4. Isopycnic centrifugation of high-density lipophilic cell-associated proteoglycan compared with released proteoglycan

[^{35}S]sulphate-prelabelled proteoglycans from control VOM cells or culture medium of PMA-treated cells were isolated by phenol extraction and CsTFA centrifugation. Fractions corresponding to density regions 1.5–1.75 g/ml were pooled; the preparation derived from the cells was further fractionated by octyl-Sepharose adsorption and elution into 4 M-guanidinium chloride containing Triton X-100. This latter preparation and the proteoglycan from the culture medium were placed in 50 mM-Tris/HCl/2 M guanidinium chloride/2 M-CsTFA/0.5 % Triton X-100 (density 1.4 g/ml) and layered over an equal volume of CsTFA (1.7 g/ml). After 44 h at 35000 rev./min in an SW50.1 rotor, the gradients were fractionated from the bottom and analysed for radioactivity and density.

treatment, both yielded large heparan sulphate glycosaminoglycans which were indistinguishable by size (90 % with K_{av} 0.40 ± 0.20 ; Fig. 5) and exhibited the same chemical nature (> 98 % degraded by HNO_3 or *Flavobacterium heparitinas*; see below). In addition, the behaviour of the proteoglycans on DEAE-Sepharose was indistinguishable, in that 0.45 ± 0.05 M-NaCl was required for elution in each case. Thus one is led to conclude that the glycosaminoglycans are of similar sizes and have similar levels of sulphate substitution (i.e. charge density) on the carbohydrate chains. In view of these data, there appeared to be little loss of molecular mass during the release process.

Studies of the proteoglycan core proteins

In attempts to characterize the source of lipophilicity in cell-associated proteoglycans, the proteoglycan preparations from cells and medium respectively were iodinated by using immobilized chloramine-T (Iodobeads) and Na^{125}I . Under standard labelling conditions, the lipophilic cell-associated proteoglycans were found to incorporate 5–10 times as much ^{125}I as did an equivalent amount of [^{35}S]sulphate-labelled non-lipophilic proteoglycans from either cells or culture medium. SDS/polyacrylamide-gradient-gel-electrophoretic analysis of ^{125}I -labelled proteoglycan followed by counting of gel slices for γ -emissions revealed that over 95 % of the released material and

Fig. 5. Gel-filtration behaviour of high-density lipophilic and released proteoglycans

[^{35}S]sulphate-prelabelled proteoglycans were isolated from density regions 1.5–1.75 g/ml of CsTFA gradients (as in Fig. 4). Released lipophilic proteoglycans were either analysed unmodified (left panels) or first subjected to alkaline β -elimination (right panels) as described in the Materials and methods section. Samples were run on 0.8 cm $\times$ 30 cm columns of Sepharose CL-4B in 4 M-guanidinium chloride with 0.2 % Triton X-100 together with Blue Dextran and Phenol Red to indicate the void- and total-volume fractions respectively.

about 80 % of lipophilic material isolated from cells co-migrated with ^{35}S -labelled proteoglycans. In both cases the products fractionated as extremely large molecules (M_r 200000–800000), but the lipophilic proteoglycans, when labelled with ^{125}I (or ^{35}S ; see below) exhibited slightly lower average electrophoretic mobilities than did released proteoglycans (Fig. 6, lanes 1 and 2). In addition, a labelled component of M_r 18000 was routinely present on gels of the ^{125}I -labelled lipophilic proteoglycan. This pronase-sensitive component comprised about 5 % of ^{125}I /radioactivity in these preparations.

In accord with the behaviour of the [^{35}S]sulphate-labelled lipophilic proteoglycan, the ^{125}I -labelled lipophilic proteoglycans were also efficiently incorporated into phosphatidylcholine micelles (Table 1). In this case, however, trypsin treatment released only 50 % of iodine label from the lipid vesicles as compared with the release of > 95 % of label when [^{35}S]sulphate-labelled proteoglycans were analysed. These data suggested that portions of the lipophilic proteoglycan core proteins which were devoid of glycosaminoglycans had been ^{125}I -labelled and were likely to be intercalated into the lipid vesicles, in view of this insensitivity to the proteolytic action of trypsin. Again, the ^{125}I -

Fig. 6. Products of glycosaminoglycan cleavage from ^{125}I -labelled proteoglycans

Proteoglycans isolated and labelled with ^{125}I as described in the Materials and methods section were treated for 18 h with protease-inhibitor-containing CHAPS buffer (lanes 1 and 2), with heparinase and heparanase in the same buffer (lanes 3 and 4, HTNase), or for 10 min with 0.8 M- HNO_2 , pH 1.5 (lanes 5 and 6, HNO_2). Products were electrophoresed on 3–20% (w/v) polyacrylamide gels and then fixed with trichloroacetic acid and dried before autoradiography. Lanes 1, 3, and 5, lipophilic proteoglycan (L); lanes 2, 4, and 6, released proteoglycan (R). Arrow indicates the stacking-gel/running-gel interface. M_r markers are: lysozyme, 14000; carbonic anhydrase, 31000; rabbit actin, 43000; BSA, 68000; β -galactosidase, 116000; myosin, 200000; unidentified M_r -400000 protein from the myosin preparation.

labelled proteoglycan from the culture medium, lacking the lipophilic domain, failed to be incorporated into liposomes (Table 1) or bind to octyl-Sepharose (results not shown).

Core proteins reflecting the two classes of proteoglycans were generated by specific degradation of glycosaminoglycan with either heparanase or HNO_2 (Fig. 6). In each case the deglycosylated ^{125}I -labelled proteoglycan products were decreased in size to M_r 30000–40000 (70–80%) and M_r 110000–150000 (10–30%). The similarity of the sizes of products resulting from both chemical and enzymic degradation of the two proteoglycan preparations suggests that the two major bands in each are representative of two distinct core proteins rather than artifacts of either deglycosylation treatment (e.g. cross-linking adventitious proteases or acid cleavage). The small electrophoretic-mobility difference between deglycosylated core proteins of the major species of lipophilic and released proteoglycan (M_r ~37000 and ~3200 respectively) suggests again that the lipophilic determinant of the cell-associated proteoglycans is rather small. Both HNO_2 and heparanase spared approx. 20% of the high- M_r ^{125}I -labelled material from the lipophilic preparation. In separate analyses (results not shown), unidentified

Fig. 7. Methanolic-HCl cleavage of a peptide from lipophilic proteoglycan

^{125}I -labelled proteoglycans were freeze-dried and treated with 0.5 M-HCl in anhydrous methanol as described in the Materials and methods section. After removal of the methanol and HCl by vacuum centrifugation, the residue was dissolved in 1% SDS plus 2% β -mercaptoethanol and electrophoresed on 3–20% (w/v) gradient polyacrylamide running gel in Laemmli SDS buffer. Arrow indicates the stacking-gel/running-gel interface. Lanes 1 and 3, lipophilic proteoglycan (L); lanes 2 and 4, released proteoglycan (R). Lanes 1 and 2, untreated; lanes 3 and 4, methanolic HCl (MeOH/HCl), 18 h, 20 °C. An autoradiograph is shown of a trichloroacetic acid-fixed and dried gel. M_r markers were those of Fig. 6.

heparitinase- and chondroitinase-resistant glycoproteins of this size were recovered in great abundance from lower-density (1.4–1.5 g/ml) regions of the CsTFA gradient during proteoglycan isolation from VOM cells.

Several analyses were used to determine whether or not the lipophilic determinants of the major cell-associated proteoglycans were covalently bound lipid. To test for the presence of glycosylphosphatidylinositol anchorage, lipophilic proteoglycans in phosphatidylcholine liposomes were treated with phosphatidylinositol-specific phospholipase C. In this experiment, 83% of the proteoglycan co-sedimented with liposomes whether or not they were treated with the phospholipase (250 m-units/ml in Tris-buffered saline for 5 h at 36 °C). This concentration of phospholipase was sufficient to release >95% of carcinoembryonic antigen (a phosphatidylinositol-anchored glycoprotein) from MKN-45 cells within 1 h. To determine further whether thiol- or oxy-ester-linked fatty acid could be responsible for lipophilicity, proteoglycan (labelled on glycosaminoglycan with either [^{35}S]-sulphate or [^{14}C]-glucosamine) was treated with 1 M-hydroxylamine, pH 8, or 0.5 M-HCl in anhydrous methanol and then tested for a change in octyl-Sepharose absorbability. Hydroxyl-

Fig. 8. Protease-digestion products of isolated [^{35}S]sulphate-labelled proteoglycans

Isolated proteoglycans labelled on glycosaminoglycan with [^{35}S]sulphate were treated as described in the Materials and methods section with the following proteases: lane 1, no protease; lane 2, TLCK-treated chymotrypsin (Cht); lane 3, V8 protease (V8); lane 4, TPCK-treated trypsin (Try); lane 5, proteinase K (PrK). (a) Lipophilic proteoglycan; (b) released proteoglycan. After treatment, samples were run on a 3–20% (w/v) gradient polyacrylamide/SDS gels; autoradiographs of fixed and dried gels are shown. Arrow indicates the top of the running gel. M_r markers are the same as in Fig. 6.

Fig. 9. Protease-digestion products of isolated ^{125}I -labelled proteoglycans

Isolated proteoglycans, labelled on the core protein with ^{125}I , were treated as described in the Materials and methods section with the following proteases: lane 1, no protease; lane 2, TLCK-treated chymotrypsin (Cht); lane 3, V8 protease (V8); lane 4, TPCK-treated trypsin (Try); lane 5, proteinase K (PrK). (a) Lipophilic proteoglycan; (b) released proteoglycan. After treatment, samples were run on 3–20% (w/v) gradient polyacrylamide/SDS gels. Autoradiographs of fresh frozen gels are shown. Arrow indicates the top of the running gel. M_r markers are the same as in Fig. 6.

amine treatment caused less than a 5% loss of lipophilicity (regardless of label), suggesting the absence of such bonding. Treatment with methanolic HCl (a desulphating agent, obviating the use of [^{35}S]sulphate as a radiolabel) caused a > 90% loss of lipophilicity of the [^{14}C]glucosamine-labelled lipophilic proteoglycan. This change was accompanied by slight increases in gel-filtration mobility attributable to complete desulphation and partial glycosidic cleavage of the protein-linked glycosaminoglycans.

Most strikingly, methanolic HCl treatment of the ^{125}I -labelled lipophilic proteoglycans (but not the released proteoglycans) resulted in the production of a M_r -18000 ^{125}I -labelled species (Fig. 7). This latter material appeared to be peptide in that it was chloroform/methanol-insoluble, could be fixed in polyacrylamide gels with 25% (w/v) trichloroacetic acid, has been efficiently electroblotted on to polyvinylidene difluoride membranes, and was digestible with Pronase. The ^{125}I -labelled products of Pronase digestion co-migrated on silica-gel t.l.c. with authentic iodotyrosines. In contrast with methanolic HCl treatment, only limited amounts of this peptide were generated during treatment of the iodinated lipophilic proteoglycan for 18 h with aqueous solutions of 5% (v/v) HCl or 75% (v/v) trifluoroacetic acid, thereby suggesting a requirement for anhydrous conditions for efficient cleavage.

The unique presence of iodinated peptide structures on the lipophilic proteoglycans is also revealed by protease treatment of the two preparations. As previously shown [11], the glycosaminoglycan-bearing segment of these molecules is little decreased in size by any of four commonly used proteases, as revealed by the electrophoretic migration of [^{35}S]sulphate-labelled products in polyacrylamide gels (Figs. 8a and 8b). If ^{125}I -labelled lipophilic proteoglycans are subjected to the same analyses, a series of protease-generated peptides can be shown to account

for > 80% of the ^{125}I label (Fig. 9a). These easily iodinated peptide segments show high affinity for octyl-Sepharose after cleavage from the parent molecules (J. A. McBain, unpublished work). The proteoglycans of the medium (i.e. PMA-released products) retain label as polydispersed high- M_r species after protease treatment (with the notable exception of chymotrypsin) (Fig. 9b).

DISCUSSION

Work from this laboratory has documented that the initiation of terminal differentiation of the human colon cancer cell VACO-10MS by phorbol esters and other agents which activate protein kinase C [18] is associated with a rapid release of cell surface proteoglycans of the heparan sulphate type [6]. This PMA response, which is followed by an acceleration of proteoglycan resynthesis, was also observed in other growth-arrest-susceptible cells, including human adenocarcinoma cell lines derived from colon (VACO 1, SW1222 and butyrate-treated VACO 5), breast (MCF-7 and GRM) and kidney (ACHN) (J. A. McBain & G. C. Mueller, unpublished work). Thus cell surface proteoglycan abundance appears generally to be under the direct control of protein kinase C and is altered in conjunction with changes in growth properties. The present paper indicates that cleavage of a lipophilic peptide domain from this surface-membrane-associated molecule leads directly to its release from the cell.

The proteoglycans of VOM cells which are affected most strikingly by phorbol ester treatment appear to be anchored in the plasma membrane by lipophilic peptide domains. They may thus be homologous to previously isolated species with integral membrane core proteins [19–23], such as the syndecan family

[20–23], as opposed to the glycosylphosphatidylinositol-anchored types [24,25]. At the present level of resolution, it is clear that at least one of the two VOM-cell proteoglycan core proteins undergoes proteolytic cleavage en route to its release from the cell. A similar cleavage has been postulated [26,27] to occur during release of syndecan, which is, like the VOM-cell proteoglycans, absent from terminally differentiated cells [7–9]. The high degree of interspecies amino acid sequence conservation involving the syndecan intra- and juxta-cellular domains suggests that these molecules may be similarly recognized by cytoplasmic, cytoskeletal and plasma-membrane-associated proteins which are involved in proteoglycan turnover. In VOM cells, neither spent medium of PMA-treated cells nor any of a variety of commercially available protease inhibitors was able to alter the rates of spontaneous or PMA-induced proteoglycan release (J. A. McBain & G. C. Mueller, unpublished work). Accordingly, this release may be initiated by a novel membrane-localized protease [28] that is activated by a protein kinase C-mediated phosphorylation. However, it is also possible that proteoglycan release follows core protein phosphorylation by protein kinase C at specific sites on the cytoplasmic domains. This might facilitate proteoglycan translocation along the plane of the membrane and complexation with existing active proteases. The extraordinary susceptibility of the lipophilic anchor peptides to mild methanolic-HCl-catalysed cleavage may be an additional clue to the release mechanism. This chemical cleavage may be an N-to-O acyl shift, involving transfer of a peptide chain from the amino to the hydroxyl group of a susceptible serine or threonine by way of a hydroxyoxazolidine intermediate [29], followed by hydrolysis of the resulting ester. We are, however, unaware of enzymic reaction mechanisms which combine the extraordinary dehydration and proton donation necessary for such a cleavage.

The relationship of an accelerated proteoglycan release to terminal differentiation is unclear at present, but, since heparan sulphate proteoglycans can bind to certain growth factors [30–33], cross-link specific sites on extracellular-matrix components, facilitating their assembly [34,35], span the membrane to contact the intracellular cytoskeleton [36], and act as precursors for nuclear targeting sulphated glycosaminoglycans [37], a variety of effects or roles can be visualized [6]. In particular, the PMA-induced alterations in the cell surface density of proteoglycan might derange the growth-factor responses which determine cell survival. For example, heparan sulphate glycosaminoglycans are essential components of the high-affinity class of fibroblast-growth-factor receptors [38–40] and withdrawal of specific heparin-binding growth factors from growing cells for defined periods of time are sufficient to trigger either terminal differentiation [41,42] or apoptotic cell death [43,44]. An additional possible effect of cell surface proteoglycan release on viability is the hyperpolarization of transmembrane potential (owing to loss of polyanion from one face of the membrane), with attendant effects on cation (e.g. Ca^{2+})-transport rates.

Thus it appears that altered cell surface proteoglycan metabolism may be generally coupled to processes that regulate the balance between cell replication and terminal growth states. Whatever the mechanism, this cellular response to PMA is of considerable interest in that it is associated with the triggering of terminal differentiation in adenocarcinoma cells. We hope that knowledge of the underlying process will explain how two unique activators of protein kinase C, bryostatin 1 and PMA, exhibit diametrically opposite effects on both cell growth and proteoglycan metabolism in VACO-10MS [45] and the other differentiation-responsive cells. Most importantly, it can be hoped that this knowledge will provide new routes to the therapy of certain human malignancies, a therapy that selectively forces cancer cells to undergo terminal differentiation despite the

blockade of normal differentiation programs by an otherwise dominating oncogenic event.

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