Journal of Cellular Biochemistry 65:574–590 (1997)

Heparan Sulfate-Binding Peptide Promotes the Deposition of Proteoglycans in the Extracellular Matrix

P. Colburn, E. Kobayashi, and V. Buonassisi*

Departamento de Bioquímica, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, Brazil

Abstract A synthetic peptide, which was shown to bind extracellular matrix heparan sulfate chains with a high degree of affinity and specificity [Colburn et al. (1996): Arch Biochem Biophys 325:129–138], has now been found to promote the transfer and the deposition of endothelial cell surface proteoglycans in the extracellular matrix. The peptide also induces preferential binding of extracellular matrix heparan sulfate proteoglycans, which have been added to the supernatant growth medium, and the requirement for its presence is stringent in that only a negligible amount of proteoglycans are bound to the cell layer in the absence of the peptide. In addition, antibodies directed against the peptide detect the accumulation of the peptide in the matrix compartment where the peptide is found associated with the proteoglycans transferred from the cell surface.

The sequence of events induced by the peptide appears to be an extension of a naturally occurring process since proteoglycans with properties similar to those of the species ordinarily present in the extracellular matrix have been observed to transfer from the cell surface to the matrix during a pulse-chase experiment. We suggest that formation of the complex peptide-proteoglycan with consequent displacement of the proteoglycan from its anchorage on the cell, initiates the process of transfer of the heparan sulfate-bound peptide from the cell surface to the extracellular matrix. J. Cell. Biochem. 65:574–590. © 1997 Wiley-Liss, Inc.

Key words: endothelial cells; tissue factor pathway inhibitor (TFPI); heparan sulfate proteoglycans

The purpose in initiating this series of experiments was to obtain some insights into the process that results in the deposition of proteoglycans in the extracellular matrix. A difficulty we previously encountered in performing experiments aimed at elucidating the requirements for the insertion of proteoglycans in the matrix (such as, for instance, the relevance of the structural properties of the chains), is that the addition to the culture medium of proteoglycans or glycosaminoglycan chains obtained from different cellular compartments, including those derived from the extracellular matrix, constantly results in the fixation of very little amount of proteoglycan or glycosaminoglycans to the matrix of cultured endothelial cells. Of interest, in the light of these observations, was the report that a proteoglycan species that is normally present in basement membranes is also found in the conditioned medium of cultured aortic endothelial cells [Saku and Furthmayr, 1989]. We assumed that the accumulation of this proteoglycan in the culture medium originates mostly from a species released from the endothelial cell surface and hypothesized that this phenomenon may be the consequence of an alteration of the normal transfer process of proteoglycans to the extracellular matrix.

Heparan sulfate moieties extracted from the extracellular matrix of cultured aortic endothelial cells bind, with a high level of specificity [Colburn et al., 1996], a synthetic peptide patterned upon the amino acid sequence of the carboxyl terminal region of a N-glycansulfated, endothelial cell protein, which acts as an inhibitor of the coagulation pathway activated by tissue factor. Two forms of the inhibitor are found in the culture medium: a more abundant 45 kDa species [Colburn and Buonassisi, 1988] missing a sequence of about 25 amino acids at the carboxyl terminus (truncated form) and the intact 47 kDa form, present in low concentration, that binds more tightly to the heparin-

Contract grant sponsors: CNPq, FAPESP, FINEP.

*Correspondence to V. Buonassisi, Dept. de Bioquímica, Escola Paulista de Medicina, Universidade Federal de Saõ Paulo, Rua Três de maio, 100, CEP 04044-020 Saõ Paulo, Brazil. E-Mail: Buonassisi.Bioq@EPM.BR

Received 2 August 1996; accepted 20 February 1997

© 1997 Wiley-Liss, Inc.

Sepharose column; the 45 kDa form is derived from the 47 kDa molecule by proteolytic cleavage [Colburn et al., 1991]. We hypothesized that, because of its high affinity for the glycosaminoglycan chains, the peptide could be used to probe the endothelial cell surface for the presence of proteoglycans bound to the cell through their polysaccharide chains. The administration of the peptide would, then, cause the displacement of these proteoglycans from their binding on the cell and given its high specificity for the glycosaminoglycan chains of the matrix [Colburn et al., 1996], the peptide could be of use in revealing the presence on the endothelial cell surface of proteoglycans, which are in transit to the extracellular matrix.

In the course of this work, we have detected the presence on the endothelial cell surface of heparan sulfate proteoglycans whose rate of transfer to the extracellular matrix sharply increases in the presence of the synthetic peptide. In addition, in the presence of the peptide, the accumulation of proteoglycans, which is usually observed in the culture medium of control cells, is drastically reduced. Because the deposition of these proteoglycans in the matrix is an event that is observed also in the absence of the peptide (e.g., the peptide simply augments the rate of transfer of these species) we hypothesize that the role of the peptide is to overcome a rate-limiting step represented by the availability of a protein that, by forming a complex with the proteoglycans through their glycosaminoglycan chains, promotes the release of these molecules from their anchorage to the cell surface and their deposition in the extracellular matrix.

METHODS

Cell Culture

Culture conditions for the endothelial cell line derived from rabbit aorta [Buonassisi and Venter, 1976] were as originally described: the cells were grown as a monolayer in 35-mm tissue culture dishes (Falcon, Lincoln Park, NJ) in F-12 basal medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Cultilab, Campinas, Brazil) in a humidified, 2.5% CO_2 atmosphere at 37°C. Three- to six-week postconfluent cultures were used.

Synthetic Peptide

The amino acid sequence (KTKRKKKKQPV-KITYVETFVKK-COOH) of the synthetic peptide [Colburn et al., 1996] is based on the carboxyl terminal region (amino acids 278 through 299) of a rabbit lipoprotein-associated coagulation inhibitor (LACI) [Wesselschmidt et al., 1990]. This inhibitor and the coagulation inhibitor synthesized by cultured rabbit endothelial cell [Colburn and Buonassisi, 1988; Colburn et al., 1991] are now considered to be the same molecule [Warn-Cramer et al., 1991] under the name of tissue factor pathway inhibitor (TFPI). The peptide was synthesized in the laboratory of Dr. John Crabb, W. Alton Jones Cell Science Center (Lake Placid, NY).

Cell Labeling and Differential Extraction of the Cell Layer

To study the flow of proteoglycans between cellular compartments (in the absence or in the presence of the peptide) without the excessive background given by labeling conditions approximating the steady state, short labeling times were employed and therefore the data obtained cannot be taken as an indication of the relative proportions of different proteoglycans observed at the steady state. The cells were labeled for 1 to 2 h with 200 μCi of [^{35}S]sulfate (carrier-free, Instituto de Energia Nuclear, São Paulo, Brazil) per ml of serum-free, F-12 basal medium in the absence or presence of 30 μM peptide. At the end of the incubation, the plates were transferred to ice, the medium was removed, and the cells were washed 3 times with 2 ml of ice-cold phosphate buffered saline (PBS). Differential extraction of the cell layer with 0.5% Triton X-100 (cell surface proteoglycans), and with 2% sodium dodecyl sulfate (SDS) (extracellular matrix proteoglycans) was performed as reported [Colburn et al., 1994]. This sequential extraction gives results that are reproducible and also consistent with our knowledge of the organization of the cell layer: the [^{35}S]sulfate-labeled fibronectin [Colburn et al., 1987] synthesized by these cells, a cell product that is considered to be mainly an extracellular matrix protein in normal cells, is found largely in the SDS (matrix) extract; on the other hand, most of the N-glycansulfated proteins, which are characteristic of this cell type [Colburn et al., 1987; Heifetz and Allen, 1982] and are thought to be principally located on the cell surface [Raub et al., 1994], are extracted with Triton X-100. The volume used for the extraction of the material present in each cellular compartment was 1 ml/tissue culture dish except where

noted in the figure legends. Fifty microliters of the samples were analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The extracts from different cell compartments were also fractionated by ion exchange chromatography; for these experiments, urea was used to solubilize the matrix (urea, 8.0 M; Triton X-100, 0.25%; NaCl, 0.125 M; Tris-HCl, 0.05 M, pH 7.5) instead of SDS [Colburn et al., 1994]. In some experiments, poly-L-lysine (Sigma, MW 3120, St. Louis, MO) or protamine (Sigma) were used, instead of the peptide, at the concentration of 50 μM and 50 μg/ml, respectively.

Pulse-Chase Labeling

Pulse-chase experiments were performed to follow the transfer of proteoglycans from the cell surface to extracellular matrix. Cells were pulse-labeled for 1 h and the incorporated radioactivity chased for 4 h, in the presence or absence of 30 μM peptide, under conditions that effectively inhibited further incorporation of [^{35}S]sulfate [Colburn et al., 1994]. At various time points, the culture medium was removed, the cells washed, and the cell surface and extracellular matrix extracts were obtained. The amount of radioactivity incorporated into protein-bound carbohydrates was determined by adding a 50 μl aliquot to 10 ml of 7% trichloroacetic acid (TCA) in ice. After a 10-min period, the solution was passed through a wetted cellulose filter disk (Millipore, Bedford, MA, GSWP, 25 mm, 0.22 μm pore size). Vacuum was applied and the filters washed twice with 10 ml of 7% TCA. The amount of radioactivity bound to the filter was determined by liquid scintillation spectrometry. Free glycosaminoglycan chains are neither precipitated by TCA nor do they bind to the filter disks.

Gel Filtration and Ion Exchange Chromatography

Ion exchange chromatography was used to separate proteoglycans present in different cell compartments at the end of 1 h pulse with radioactive sulfate. Seven 35-mm plates of post-confluent culture were used per set. To remove unincorporated [^{35}S]sulfate and to exchange buffer prior to application to the ion exchange column, the medium and Triton X-100 extract were passed through a Sephadex G-50 column, 1.5 × 18 cm, equilibrated with urea buffer. The fractions eluted with the void volume were pooled and applied to a DEAE-Sepharose (Pharmacia, Piscataway, NJ) column (3 ml of packed gel) equilibrated with urea buffer. The extracellular matrix extract was centrifuged and the supernatant applied to the ion exchange column; the precipitate, which contained a negligible amount of radioactivity, was discarded. The column was washed with 20 ml urea buffer. Different proteoglycans were separated with a convex gradient of NaCl (0.125 to 0.5 M, 40 ml total volume) in urea buffer followed by stepwise concentrations of NaCl of 0.5 and 1.0 M (10 ml each). The flow rate was 15 ml/h and the fraction volume 1 ml. Under these conditions, the radioactive material is separated into three peaks: the first (peak I) is composed of sulfated glycoproteins, the other peaks (II and III) contain the proteoglycans. The radioactivity recovered from the various DEAE column runs was calculated by adding the amount of radioactivity found in the by-pass and wash fractions to the total amount eluted from the column and dividing this sum by the amount of radioactivity applied to the column. These values were obtained by determining the amount of radioactivity present in 50-μl aliquots of the samples by liquid scintillation spectrometry and adjusting for total sample volume. The recovery from the various DEAE columns ranged from 98–109%.

Identification of Glycosaminoglycan Moieties With Enzymatic Treatment

Two hundred microliters of [^{35}S]sulfate-labeled species contained in the DEAE peak fractions II and III were dialyzed against PBS at 4°C for 1.5 h in a microdialysis chamber (Pierce, Rockford, IL) using an 8,000 molecular weight cut-off dialysis membrane, which had been previously exposed for 2 h to 10% fetal bovine serum in PBS. After dialysis, 40 μl of sample were added to 20 μl enzyme solution (heparitinase I [Nader et al., 1990] and chondroitinase ABC [Yamagata et al., 1968]: 0.1 U in 0.05 M ethylenediamine acetate buffer [EDA], pH 7.0; Proteinase K [Sigma]: 1 mg/ml in 0.02 M Tris-HCl buffer, pH 7.5). Twenty microliters of 0.05 M EDA buffer was added to the control samples. The samples were incubated overnight at 30°C. Thirty microliters of 3 × sample buffer (Tris-HCl, 0.19 M, pH 6.8; beta-mercaptoethanol, 2.14 M; SDS, 8%; glycerol, 30%) were added to each sample and the samples were placed in a boiling-water bath for 5 min prior to addition of bromophenol blue and application to the poly-

acrylamide gel. After electrophoresis, the gel was fixed and stained with Coomassie blue R250 in 50% methanol and 10% acetic acid, destained, and dried to visualize the position of the molecular weight markers (Sigma). Radioactive compounds were visualized by autoradiography using SB-5 X-ray film (Kodak, Rochester, NY).

Heparin-Sepharose Column Chromatography

Heparin-Sepharose chromatography [Colburn and Buonassisi, 1988] was used for the separation of the truncated (45 kDa) form from the intact (47 kDa) form of the inhibitor present in the endothelial cell culture medium. The two forms of the inhibitor were identified by determining their inhibitory activity on trypsin using SDS-polyacrylamide gel electrophoresis or, in the case of the intact form, also with antibodies raised against the synthetic peptide (see below). In addition, the column fractions were assayed for their inhibitory activity on activated factor X and in a thromboplastin assay as previously described [Colburn et al., 1991].

Electrophoresis on Polyacrylamide and Agarose Gel

Submerged agarose gel (for the separation of heparan sulfate and chondroitin sulfate chains) was performed at pH 8.6 [Colburn et al., 1994]; fixation, staining, and quantitation of the radioactive band were as described [Nader et al., 1984]. Reduced, SDS polyacrylamide gel electrophoresis for the separation of proteoglycans was as published [Castillo et al., 1987].

For the detection of protease inhibitory activity, electrophoresis was performed under nonreducing conditions on SDS-polyacrylamide gel slabs containing porcine gelatin as the substrate [Hanspal et al., 1983] at a concentration of 1.5 mg/ml acrylamide solution. Aliquots of fractions from the heparin-Sepharose column were applied to the gel and after the resolution of the sample proteins, the species endowed with inhibitory activity were detected using bovine trypsin at a concentration of 2 mg/100 ml 10 mM Tris-HCl, pH 7.5, for 2 h at 37°C. The bands of inhibition were revealed by staining the undegraded gelatin with Coomassie blue. Prior to the incubation with trypsin, the gel was washed for 1 h with 2% Triton X-100 in 10 mM Tris-HCl, pH 7.5. Prestained molecular weight marker were used to estimate molecular mass.

Binding of Exogenous Glycosaminoglycans and Proteoglycans to Cell Cultures

[^{35}S]Sulfate-labeled proteoglycans or heparan sulfate glycosaminoglycan chains (isolated from either the cell surface or the extracellular matrix of labeled endothelial cell cultures) in F-12 basal medium were added to unlabeled post-confluent cell cultures in the presence and the absence of 30 μM peptide. At the end of the incubation (4 h), the radioactivity remaining in the medium was determined by liquid scintillation spectrometry. Purified [^{35}S]sulfate-labeled heparan sulfate glycosaminoglycan chains were isolated from 48 h labeled endothelial cell cultures as previously described [Colburn et al., 1996]. The amount of radioactivity added/plate of [^{35}S]sulfate-labeled heparan sulfate chains derived from the cell surface and extracellular matrix was 24,000 and 48,000 cpm, respectively. [^{35}S]Sulfate-labeled proteoglycans were obtained from cell cultures that had been labeled for 72 h with 100 μCi of [^{35}S]sulfate/ml of F-12 basal medium supplemented with 10% fetal bovine serum. The preparations were enriched for heparan sulfate proteoglycans by DEAE column chromatography followed by microdialysis prior to application to the cells. More than 90% of the radioactivity was incorporated into heparan sulfate proteoglycans, the remainder into chondroitin sulfate proteoglycans. The amount of radioactivity added per plate of labeled proteoglycan extracted from the cell surface and from the matrix was 74,000 and 24,500 cpm, respectively. Percentage of radioactivity removed from the medium was calculated: ([cpm at zero time-cpm at 4 h]/cpm at zero time) × 100%.

Release of Cell Surface Proteoglycans by Different Agents at Low Temperature

Four-week post-confluent endothelial cultures in 35-mm culture dishes were pulse-labeled for 2 h with 300 μCi [^{35}S]sulfate per ml of F-12 nutrient medium. The dishes were transferred to ice, the medium removed, and the cells washed three times with ice-cold PBS. One milliliter ice-cold F-12 basal medium (supplemented with 0.8 mM $MgSO_4$ and additions as indicated) was added per plate for 15 min with gentle agitation. The incubation fluid (supernatant) was removed and the cell layer was differentially extracted in 1 ml extraction buffers for each cellular compartment. Three volumes of ethanol were added to 300 μl aliquots of the

various samples after addition of carrier compounds (25 μg chondroitin 6-sulfate, 50 μg bovine pancreas heparan sulfate, 1 μl fetal bovine serum). After precipitation at −20°C overnight, the samples were centrifuged at 12,500*g* for 15 min. The pellets were dissolved in 100 μl Tris-HCl (0.02 M, pH 7.5) containing Proteinase K (1 mg/ml). The proteins were digested by incubation overnight at 37°C. Twenty-five microliter aliquots were applied to an agarose gel slab and the glycosaminoglycans were separated by electrophoresis using Tris-borate-EDTA buffer [Colburn et al., 1994]. The gels were then fixed, dried, standard compounds visualized by staining and the radioactive species by autoradiography. Radioactive species were removed from the gel by cutting and the amount of radioactivity was quantitated by liquid scintillation spectrometry.

Peptide Binding by Endothelial Cell Cultures

For the experiments aimed at determining the cellular location of the synthetic peptide accumulation, the cells were exposed to a peptide concentration of 30 μM for 1 h at 37°C. The cell layer was then differentially extracted, a 40 μl aliquot of the samples resolved by SDS-PAGE under reducing conditions, electroblotted onto nitrocellulose, and the membrane treated with antibodies. The antibodies were raised in chickens against the synthetic peptide immobilized on agarose beads; the antiserum was freeze-dried and redissolved in PBS at a concentration of 25 mg/ml w/v. Following electrotransfer to a nitrocellulose membrane, the membranes were blocked by incubation for 2 h at room temperature in 0.5% non-fat dry milk, 0.2% gelatin in PBS; exposed to antibody 1:1,000 dilution in buffer (0.25% non-fat dry milk; 0.1% gelatin; 0.05% Tween 20, in PBS) for 2 h; washed in PBS for 5 min three times; exposed to the 2nd antibody (horseradish peroxidase conjugated rabbit anti-chicken IgG, 1:500 dilution in the above antibody buffer) for 1 h washed, and finally the peroxidase activity was developed using 2 mg 3,3′ diaminobenzidine in 50 ml PBS containing 0.06% hydrogen peroxide and 0.03% cobalt chloride. This methodology is not quantitative since a portion of the peptide passes through the membrane during electrotransfer due to its small size. This was evidenced by detection of the peptide by the antibody on both sides of the nitrocellulose membrane.

To show that the antibodies directed against the synthetic peptide recognize the intact, 47 kDa form of the inhibitor, fractions from the heparin-Sepharose column containing the intact or the truncated form of the inhibitor were used. The procedures used for electrophoresis, electrotransfer, and visualization with antibody were the same as those for the detection of the peptide.

Affinity Cross-Linking

The peptide was biotinylated by adding 100 μl peptide (10 mM) to 50 μl DMSO containing 1 mg biotinamide caproate N-hydroxysuccinimide (Sigma). After vortexing, the sample was placed on ice for 45 min followed by addition of 350 μl 20 mM Tris-HCl, pH 7.4, and 500 μl 1 M lysine.

For the affinity cross-linking experiments, cell cultures were incubated in the presence or absence of 1 mM peptide conjugated with biotin in F-12 medium for 15 min at 37°C. At the end of the incubation, the following procedure was performed (on ice, except where indicated): the culture medium was removed, the cells washed 3 times with PBS followed by exposure to freshly prepared disuccinimidyl suberate (Sigma, 0.1 M in DMSO, 10 μl per ml of PBS per culture dish) for 15 min with rocking. The cross-linker was removed, the cells washed with PBS followed by a 2-min incubation with 20 mM Tris-HCl, pH 7.4. The cultures were washed with PBS and sequentially extracted with 0.5% Triton X-100 and 20 mM ammonium hydroxide as indicated earlier. The matrix preparations left in the culture dish after the Triton X-100 and ammonium hydroxide extractions were washed 3 times with PBS followed by addition of 1 ml 0.1 M ethylenediamine acetate buffer, pH 7.0 ± heparitinase I. After 1.5 h incubation at 37°C, the solution was removed, the matrices washed with PBS as above, followed by solubilization with 1 ml 2% SDS. The matrix samples were subjected to PAGE under reducing conditions. The position of the cross-linked peptide was detected, after electroblotting the resolved proteins onto PVDF membrane, by incubation with avidin-conjugated horseradish peroxidase and development of peroxidase activity as indicated above.

RESULTS AND DISCUSSION

Addition of the Synthetic Peptide to the Culture Medium Causes a Redistribution of Heparan Sulfate Proteoglycans Between the Various Cellular Compartments

The exposure of endothelial cell cultures to the peptide brings about a reduction in the

amount of sulfated proteoglycans on the cell surface (Fig. 1A, compare lanes 3 and 4). The decrease, seen as a "clearing" of the proteoglycan shadows in the autoradiogram, affects particularly the uppermost part of the gel where the high molecular weight species migrate. Careful scrutiny of the autoradiography also suggests that the amount of proteoglycans in the medium of peptide-treated cells is lower than that found in the medium of control cells and that the extracellular matrix is the cellular compartment where an increase has taken place. Proteoglycans are recognized in the autoradiography as diffuse shadows partially covered and obscured by numerous, well-defined bands that represent the N-glycansulfated proteins characteristic of cultured vascular endothelial cells whether primary cultures derived from human umbilical vein [Heifetz and Allen, 1982] or an established line derived from rabbit aorta [Colburn et al., 1987]. The distribution of these glycoproteins appears unaffected by the exposure to the peptide. Densitometric scanning of the autoradiogram (Fig. 1B) shows that the area under the broader peaks (proteoglycans) is in fact decreased in the medium and surface compartments and increased in the matrix of peptide-treated cells. The sharper peaks (N-glycansulfated proteins) do not seem affected; this is more easily assessable in the lower part of the autoradiogram where the interference due to the high background absorbancy of the proteoglycan is absent and the peaks become superimposable. The N-glycansulfated species are predominantly cell membrane components with the exception of fibronectin (also an N-glycansulfated protein in this cell type [Colburn et al., 1987), which is predominantly an extracellular matrix component scarcely represented on the cell membrane (compare lane 3 with lane 5 of Fig. 1A) but present in the conditioned medium (Fig. 1, lane 1). These observations indicate that among the sulfated species synthesized by cultured endothelial cells only proteoglycans are affected by the exposure to the peptide and provide an internal control of the validity of the method for the differential extraction of the cell layer, which we have used to separate cell surface proteoglycans from those of the matrix. Thus, this initial experiment provides a clear suggestion that the exposure to the synthetic peptide may cause a reduction in the amount of proteoglycans on the cell surface (and in the amount of those released from the cell surface into the culture medium), which is paralleled by an increase in the extracellular matrix.

We analyzed the kinetics of transfer of proteoglycans from other cellular compartments to the extracellular matrix by quantitating the amount of [^{35}S]sulfate incorporated into macromolecular form at different times during the period of "chase" following 1 h pulse (Fig. 2). Since, as shown in Figure 1, the N-glycansulfated proteins are not affected, this procedure will only measure the variations, in different cellular compartments, of the amount of the [^{35}S]sulfate-labeled glycosaminoglycan moieties of the proteoglycans. Note that the reduced (by about 75% at the end of the chase) accumulation of proteoglycans in the culture medium (Fig. 2A) and the depletion of the cell surface compartment (Fig. 2B), which are observed in cell cultures exposed to the peptide, are accounted for by a corresponding increase in the extracellular matrix (Fig. 2C). In fact, analysis of the data obtained during the period of chase supports the conclusion that the peptide only promotes a redistribution of the proteoglycans between cellular compartments without influencing appreciably the rate of degradation of these molecules since the sum of the amounts of [^{35}S]sulfate-labeled proteoglycans found in different cell compartments is equal to that of control cells (Fig. 2D). Also, after 1 h lag, the fall of the incorporated radioactivity is logarithmic (Fig. 2D). This is due in large part to the fast turnover rate of the cell surface species. The modest decrease that is observed during the chase period in the extracellular matrix of the peptide-treated cells is likely to be due to the fast turnover rate of the fastest moving proteoglycan (Fig. 1A, lane 6) as will be discussed below. Also, note (Table I) that neither polylysine nor protamine (a protein that owes its basicity to the presence of clusters of four to five arginine residues along the polypeptide chain) shows an activity comparable to that of the peptide (whose structure contains a cluster of basic amino acids) in promoting proteoglycan deposition in the extracellular matrix: only the peptide acts in a consistent manner by lowering the level of [^{35}S]sulfate-labeled species in the medium and on the cell surface while increasing the amount of these molecules in the extracellular matrix. These results make it less likely

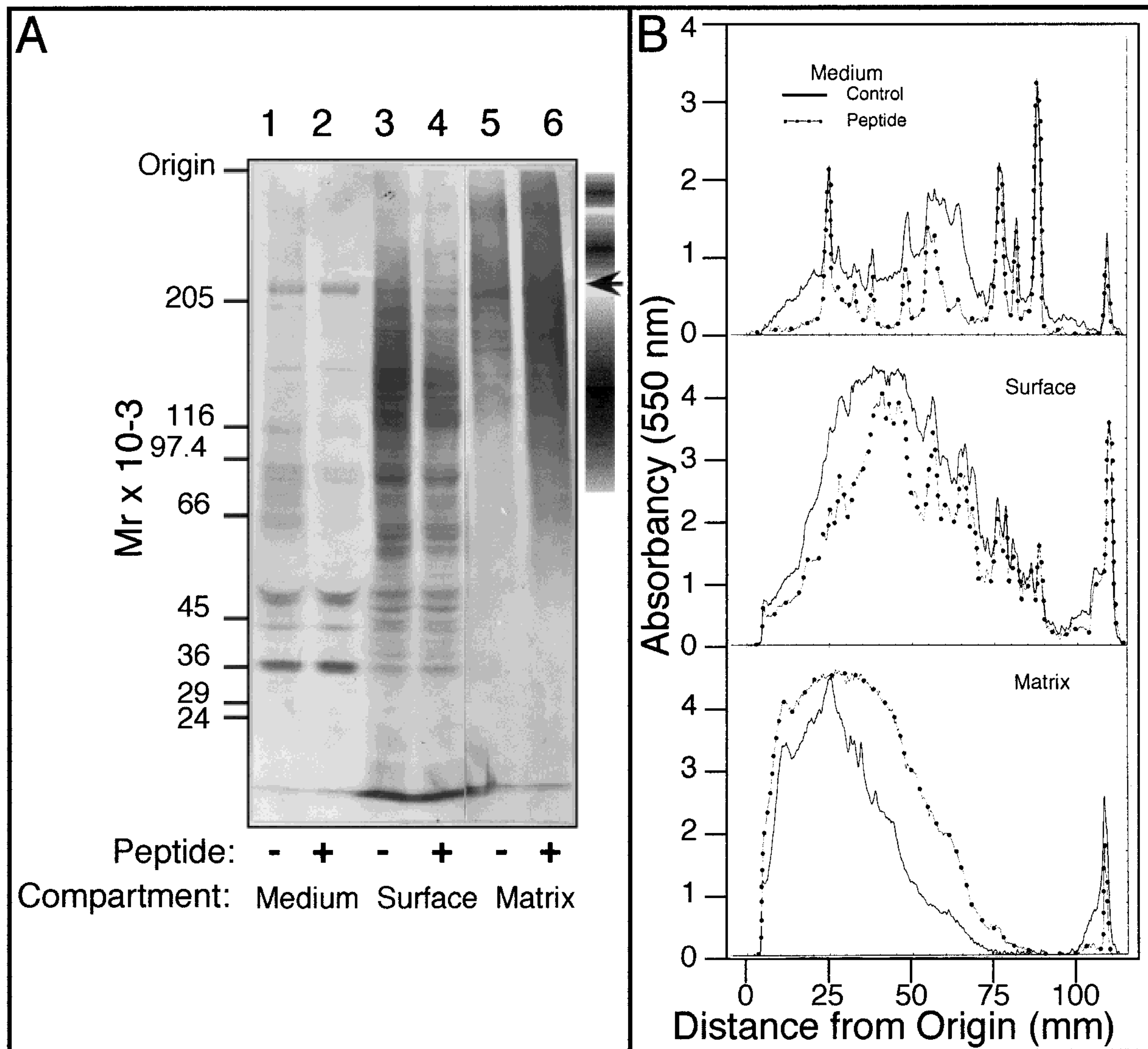

Fig. 1. Electrophoretic analysis of the [^{35}S]sulfate-labeled species obtained from different compartments of cells exposed to the peptide. **A:** Endothelial cell cultures were labeled for 2 h in the absence or the presence of 30 μM peptide. The cell layer was then differentially extracted to separate the [^{35}S]sulfate-labeled proteoglycans of the cell surface from those of the extracellular matrix. Aliquots of the extracts were analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions. After electrophoresis, the gel was stained, dried, and the radioactive compounds were visualized by autoradiography. Shaded bars on the right side of the figure indicate the position of different proteoglycans. Arrow indicates [^{35}S]sulfate-labeled fibronectin. **B:** Densitometric scanning of the autoradiogram shown in A.

that the activity of the peptide is purely the result of a charge effect.

Peptide Treatment Seems to Affect Only the Distribution of Proteoglycans

To follow the transfer of different proteoglycans from the cell surface to the extracellular matrix, the cells were labeled with [^{35}S]sulfate for 1 h and the [^{35}S]sulfate-labeled proteoglycan material subjected to ion exchange chromatography. Ion exchange chromatography also separates the numerous N-glycansulfated proteins, which would interfere with the visualization of the proteoglycans in the autoradiogram of a polyacrylamide gel slab. The results obtained by this chromatographic procedure confirm the redistribution in the amounts of proteoglycans between different cellular compartments that occurs in the presence of the peptide, namely, the reduction of these molecules on the

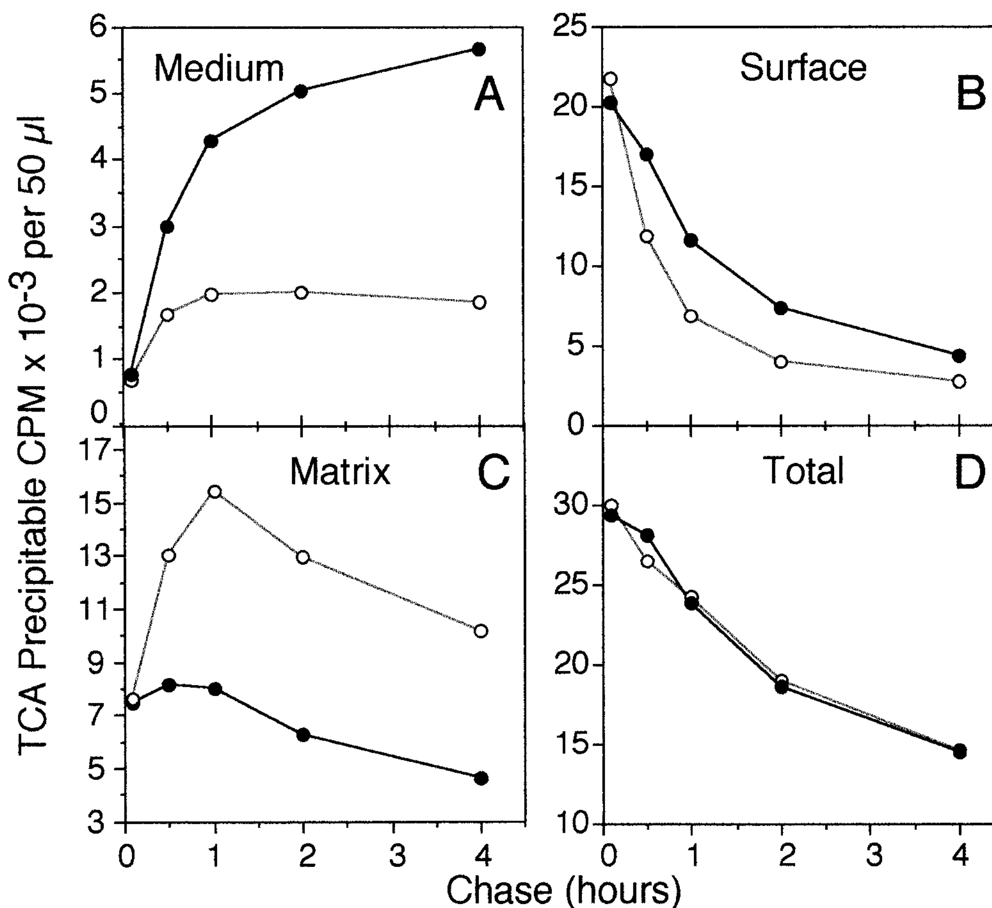

Fig. 2. Kinetics of transfer of proteoglycans to the extracellular matrix in the presence of the peptide. Cultured endothelial cells were pulse-labeled for 1 h and then the incorporated radioactivity chased for 4 h in the presence or absence of the peptide. Conditions for the chase were as indicated in Methods. At the times indicated, differential extraction of the cell layer was performed as in the experiment shown in Figure 1. The incorporated radioactivity present in various cellular compartments was determined by precipitation with 7% TCA. The data in D were calculated by summing the amount of radioactivity found in the three compartments. Control cells, ●; peptide-treated cells, ○.

TABLE I. Activity of Different Compounds on the Distribution of [^{35}S]Sulfate-Labeled Molecules Synthesized by Endothelial Cell Cultures*

	Percent TCA precipitable radioactivity per compartment		
Addition	Medium	Surface	Matrix
Control	15.5 (8,433 ± 458)	56.5 (30,812 ± 474)	28.0 (15,287 ± 721)
Peptide (50 μM)	10.9 (5,534 ± 255)	48.2 (24,432 ± 342)	40.8 (20,695 ± 661)
Polylysine (50 μM)	13.6 (6,826 ± 218)	64.4 (32,214 ± 344)	22.0 (11,014 ± 697)
Protamine (50 μg/ml)	14.5 (6,764 ± 62)	64.7 (29,987 ± 398)	20.7 (9,585 ± 324)

*The experiment was performed as in Figure 1 with the additions stated in the table being present during the entire incubation period (2 h). At the end of the incubation, cell fractions were obtained and the amount of trichloroacetic acid (TCA) precipitable radioactivity was determined on 50-μl aliquots as stated in Methods. The total amount of radioactivity recovered was calculated by adding the amount present in the three compartments. The percent TCA precipitable radioactivity per compartment was calculated by dividing the amount present in each compartment by the total amount recovered. The number was then multiplied by 100. The data in parenthesis represents the mean ± standard deviation (n = 3) of the actual cpm present in 50-μl aliquots as determined by liquid scintillation spectrophotometry.

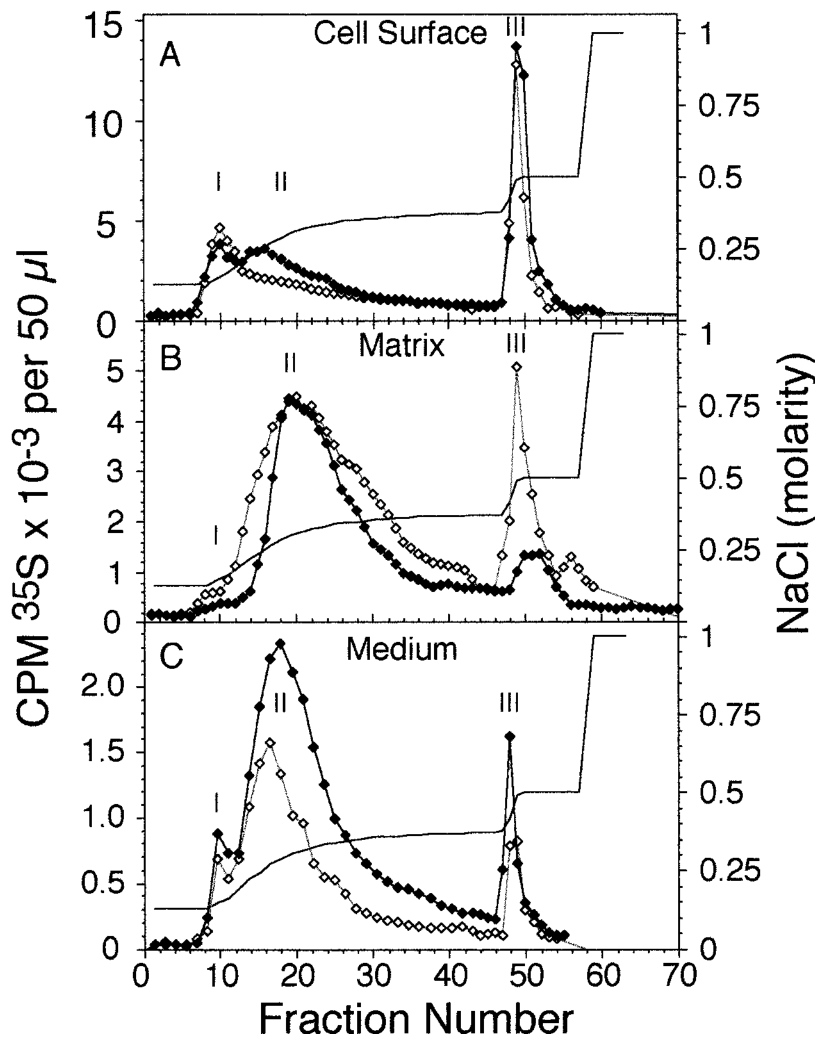

Fig. 3. DEAE chromatography of material obtained from cells pulse-labeled for 1 h with [^{35}S]sulfate. At the end of 1-h pulse with [^{35}S]sulfate, the cells were differentially extracted and the labeled proteoglycans present in various cellular compartments separated by ion-exchange chromatography. Samples from control cultures, –◆–; samples from peptide-treated culture, –◇–; NaCl molarity, ____. Roman numerals indicate the positions of the three main peaks. The amount of radioactivity eluted from the ion exchange columns of control samples were: 2,313,240 cpm, cell surface extract; 1,510,520 cpm, matrix extract; and 580,660 cpm, medium; from the DEAE columns of samples from peptide-treated cultures the amounts were: 1,847,180 cpm, cell surface; 2,209,260 cpm, matrix extract; and 335,620 cpm, medium.

cell surface and their increase in the extracellular matrix. With respect to the chromatographic profile of the material extracted from the cell surface (Fig. 3A), note that the decrease affects mostly the second and to a minor extent the third peak (both containing proteoglycans, see heading below) while the amount of [^{35}S]sulfate-labeled material eluted with the first peak (N-glycansulfated proteins) is not affected. The analysis of the matrix extract clearly reveals the increase in proteoglycan content of the second (main) peak and in an even greater proportion, compared to the control, the increase of the third (minor) peak, the one eluted at higher salt molarity (Fig. 3B). The experiment depicted in Fig. 3C confirms the reduction of the amount of proteoglycans (peaks II and III) in the supernatant medium of cells exposed to the peptide. Together with the results of the kinetic experiments shown in Figure 2, this experiment provides assurance that the increased level of proteoglycans in the extracellular matrix of peptide-treated cells also derives from deposition of species that, in control cells, accumulate in the culture medium for the duration of the experiment after their release from the

endothelial cell surface. Further, analysis of the data obtained at the end of the 1-h pulse (the experiment shown in Fig. 3) supports the conclusion that the peptide treatment does not affect the rate of synthesis of the proteoglycans but only their distribution since there is no difference between control and experimental cells with respect to the total amount of [^{35}S]sulfate-labeled proteoglycans that can be extracted from the various cellular compartments. The increase of [^{35}S]sulfate-labeled proteoglycans in the matrix of peptide-treated cells is about 50% over control cells. Half of this amount is contributed by the cell surface compartment but this amount represents only 20–25% of the heparan sulfate proteoglycans normally present on the cell.

Analysis of the Proteoglycans Transferred to the Extracellular Matrix in the Presence of the Peptide

After ion exchange chromatography, the proteoglycan species that transfer from the cell surface to the extracellular matrix and those already present in the matrix were analyzed by polyacrylamide gel electrophoresis and characterized on the basis of the sensitivity of their glycosaminoglycan chains to treatment with different degradative enzymes (Fig. 4). This analysis reveals that at the end of the 1-h labeling period, three proteoglycans bearing heparan sulfate chains can be detected in the cell surface compartment (compare lanes 1 and 3 of Fig. 4A); these species are eluted with DEAE peak II, a chromatographic fraction that seems to contain only heparan sulfate proteoglycans. The fastest moving (partially obscured by two overlapping, sharper bands, presumably N-glycansulfated proteins), which extends below 205 kDa molecular weight marker and possibly includes more than one proteoglycan, becomes more visible after longer labeling periods: compare lane 1 of Figure 4A (1-h labeling) with lane 3 of Figure 1A (2-h labeling period); and at the steady state or when the cells are subjected to particular stimuli such as endotoxin [Colburn et al., 1994] it becomes by far the predominant heparan sulfate proteoglycan species of the cell membrane. This heparan sulfate proteoglycan is likely to be the cellular counterpart of the one previously isolated in our laboratory from the conditioned medium of this endothelial cell line and characterized as a relatively small proteoglycan (M_r = 100 kDa), bearing two to three heparan sulfate chains, no oligosaccharides, and a protein core with a molecular mass of 22 kDa; this conditioned medium proteoglycan exhibited an N-terminus amino acid sequence of 25 residues [Castillo et al., 1987] identical to that of amphiglycan [David et al., 1992], a member of the syndecan family: syndecan-4 [Elenius and Jalkanen, 1994].

With respect to the relative proportions of the various species of heparan sulfate proteoglycans, at the end of 1-h pulse one sees in the extracellular matrix the reverse of what is observed in the cell surface compartment: in the matrix compartment, the two slower moving proteoglycan species represent more than 80% of the total radioactivity incorporated. The core protein of the slowest-moving proteoglycan (designated as HS1 in Fig. 4C) exhibits a molecular mass of ~450 kDa after enzymatic deglycosylation with heparitinase I; after treatment with this enzyme, the core protein runs as a sharp band, above the electrophoretic position of the other two intact heparan sulfate proteoglycans, HS2 and HS3 (not shown), suggesting a limited number of heparan sulfate moieties. This proteoglycan has properties similar to those of the species that was reported to be present in the conditioned medium of cultured aortic endothelial cells as well as in various basement membranes, namely, a proteoglycan composed of a large core protein bearing 2 to 3 heparan sulfate chains [Saku and Furthmayr, 1989], now known as perlecan [Noonan et al., 1991]. No attempt to characterize the other high molecular weight heparan sulfate proteoglycan designated as HS2 (Fig. 4A and C) has been made. The remainder of the incorporated radioactivity extracted from the extracellular matrix is found in a faster moving heparan sulfate proteoglycan species (HS3) migrating between the 205 and the 116 kDa markers (Fig. 4C).

A relatively minor fraction of both high and low molecular weight heparan sulfate proteoglycans extracted either from the cell surface or the extracellular matrix elute from the ion exchange column with peak III at higher salt molarity than the bulk of the heparan sulfate proteoglycans, which are eluted with peak II (Fig. 4B and D). The significance of this chromatographic behavior is not known. Also, treatment with proteolytic enzyme modifies the electrophoretic migration of the [^{35}S]sulfate-labeled compounds derived from both the cell surface and the extracellular matrix (Fig. 4, lanes 7 and 8). This result demonstrates that the gly-

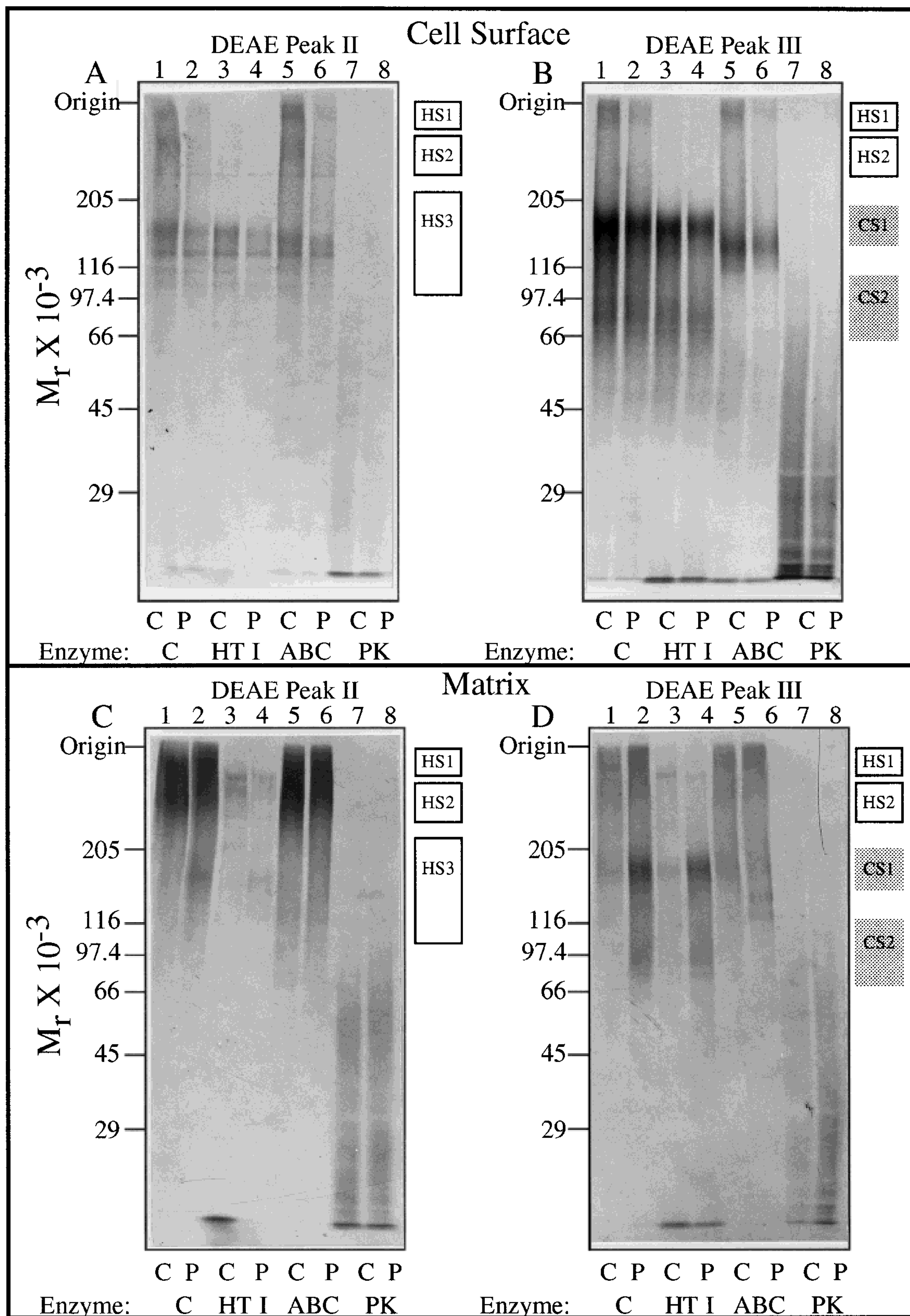

Fig. 4. Polyacrylamide gel electrophoresis analysis of the [^{35}S]sulfate-labeled material present in the various peaks eluted from the DEAE column. [^{35}S]Sulfate-labeled species present in the various peaks eluted from the DEAE-Sepharose column were analyzed by polyacrylamide gel electrophoresis before and after treatment with enzymes. C, samples obtained from untreated cultures; P, samples obtained from peptide-treated cultures. Enzyme: C, no enzyme added; HT-I, heparitinase I; ABC, chondroitinase ABC; PK, Proteinase K. Bars on the right side of the figure indicate the position of different proteoglycans: HS, heparan sulfate; CS, chondroitin sulfate.

cosaminoglycan chains are bound to a protein core.

In summary, the process of redistribution of the heparan sulfate proteoglycans between cellular compartments, induced by the peptide, results in the reduction of the level of these molecules in the cell surface compartment that affects the high molecular weight heparan sulfate species (HS1 and HS2, Fig. 4) which migrate above the 205 kDa marker and a faster moving species (HS3) migrating between 205 and 116 kDa. The level of these species correspondingly increases in the matrix; however, relative to the control cells, the highest increase in the matrix is that involving the proteoglycan designated as HS3 in Figure 4. It should be noted that this proteoglycan can be easily visualized in the matrix compartment at the end of the 1-h pulse, but in a long-term labeling period, it amounts only to 10% of the total heparan sulfate proteoglycans present in the matrix; together, these data suggest a short half-life in the matrix for this proteoglycan. The experimental results shown so far are also consistent with the notion that the peptide binds to the cell surface proteoglycans and promotes their deposition in the extracellular matrix; in the absence of the peptide, part of these proteoglycans would accumulate in the culture medium. Another suggestion compatible with the results of the experiments shown in the preceding sections is that the administration of the synthetic peptide only increases the rate of a normally occurring transfer process. The validity of this interpretation, namely that the transfer of proteoglycans from the cell surface to the matrix is an extension of a physiological process, and not an artifactual event induced by the administration of the peptide, is strengthened by the demonstration (Fig. 4C, lanes 1 and 2) that species with the characteristics of the heparan sulfate proteoglycans whose transfer is enhanced by the peptide are ordinarily present in the extracellular matrix.

The cell surface compartment also contains two proteoglycans, eluted with DEAE peak III and designated as CS1 and CS2, whose glycosaminoglycan chains are sensitive to chondroitinase ABC (Fig. 4B, compare lane 1 with lane 5). This result is in agreement with earlier reports on the presence of chondroitin sulfate chains in cultured aortic endothelial cells of rabbit [Buonassisi, 1973; Buonassisi and Colburn, 1980] or bovine derivation [Humphries et al., 1986]. Unlike the cell surface, the matrix does not seem to contain an appreciable amount of chondroitin sulfate (Fig. 4D, compare lane 1 with lane 5) and it is only in the presence of the peptide that both of these proteoglycan species are clearly visible in the matrix (Fig. 4D, compare lane 2 with lane 6). These are likely to be proteoglycans released into the medium since, unlike the proteoglycans bearing heparan sulfate chains, the level of these proteoglycans in the cell surface compartment is not affected by exposure of the cells to the peptide (compare lane 1 with lane 2 in Fig. 4B). Thus, the 20 to 30% decrease in the amount of radioactivity present in peak III (Fig. 3A, cell surface extract prepared from cells exposed to the peptide) is likely to be caused by the removal by the peptide of the cell surface heparan sulfate proteoglycans present in this peak; the chondroitin sulfate is left essentially unchanged. This is because in peptide-treated cells the Triton X-100 extract reflects essentially what is left on the cell surface (e.g., the proteoglycans, which are not removed by the peptide) while what is seen in the matrix of peptide-exposed cells is the result of two concomitant processes: the proteoglycans removed from the surface and those that instead of accumulating in the medium as a result of the continuous release, are deposited in the extracellular matrix. Note that in test tube assays [Colburn et al., 1996] it can be shown that the peptide has a higher affinity for heparan sulfate than for chondroitin sulfate. This selectivity is not as apparent under the conditions of the experiments presented here since chondroitin sulfate proteoglycans, present in the medium, seem to be interacting to a certain extent with the peptide and as a consequence are found in the extracellular matrix of peptide-exposed cells (Fig. 4D). The fact that no appreciable amounts of chondroitin sulfate proteoglycans are removed from the cell surface by the peptide may mean that the link between these proteoglycans and the endothelial cell surface is different from that of the proteoglycans bearing heparan sulfate moieties, namely, it is not an association that can be displaced by the peptide.

Proteoglycans Added to the Culture Medium Bind to the Cell Layer Only in the Presence of the Peptide

The experiment shown in Figure 5 provides evidence that the availability of the peptide is a

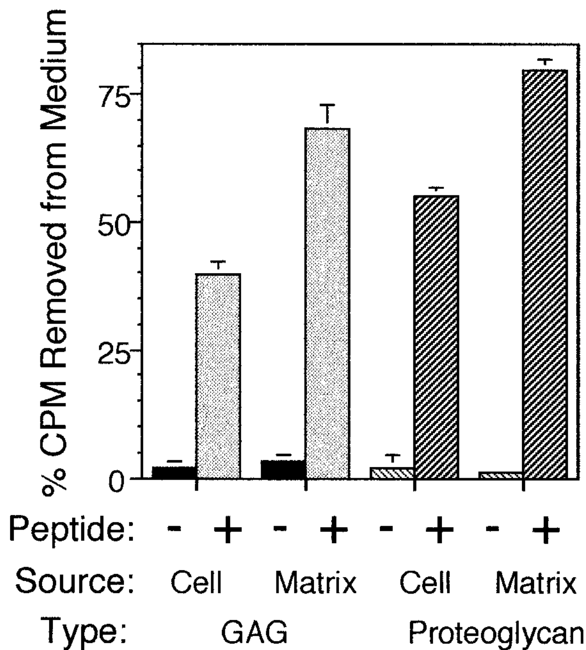

Fig. 5. Binding to the cell layer of free glycosaminoglycan chains and proteoglycans added to the culture medium. [^{35}S]Sulfate-labeled proteoglycans or heparan sulfate glycosaminoglycan chains (isolated from either the cell surface or matrix of labeled endothelial cell cultures) in F-12 basal medium were added to unlabeled post-confluent cell cultures in the presence and the absence of peptide. At the end of the incubation, the amount of radioactivity remaining in the medium was determined. The bars represent the average of duplicate determinations; error bars represent the standard deviation. Source: Cell, material isolated from the Triton X-100 extract of labeled cultures; Matrix, material isolated from the SDS extract of labeled cultures. Type: GAG, [^{35}S]sulfate-labeled heparan sulfate glycosaminoglycan chains; Proteoglycan, [^{35}S]sulfate-labeled heparan sulfate proteoglycans.

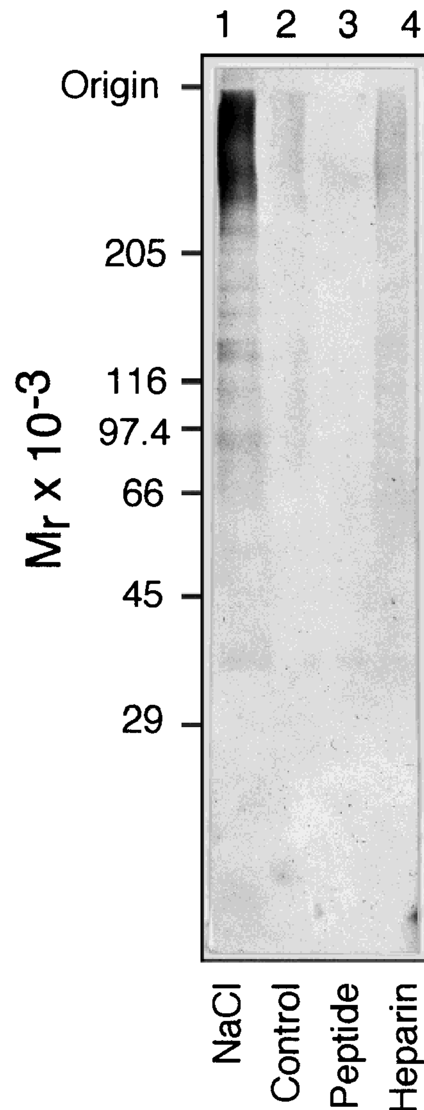

Fig. 6. Polyacrylamide gel electrophoresis of cell surface proteoglycans removed at low temperature by different agents. Endothelial cell cultures were labeled for 2 h with [^{35}S]sulfate and then transferred to ice. The radioactive medium was removed and the cells were treated with different agents as indicated in Table I and in Methods. Aliquots of the [^{35}S]sulfate-labeled material contained in the supernatant medium after 15 min of treatment were analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions. NaCl, supernatant from cells treated with 1.5 M sodium chloride; Control, supernatant from control cells treated with F-12 basal medium only; Peptide, supernatant from peptide-treated cells (50 μM); Heparin, supernatant from cells treated with heparin (100 μg/ml).

stringent requirement for the deposition of the proteoglycans since in its absence no significant amount of [^{35}S]sulfate-labeled heparan sulfate, added to the culture medium, binds to the cell layer. This is observed for both free chains and intact proteoglycans, implying that the interaction of the peptide with the proteoglycan takes place through the glycosaminoglycan moieties of the latter. Also note that the matrix glycosaminoglycans bind in higher amount to the cell layer than the glycosaminoglycan chains from the cell surface, confirming the binding specificity previously reported [Colburn et al., 1996]. The experiment represented in Figure 5 also shows that the binding specificity of the intact proteoglycan is the same as that of the isolated carbohydrate chains, namely, the matrix proteoglycans bind preferentially. This confirms that the proteoglycans bind through the carbohydrate moieties and implies that, in this case, the binding specificities of the proteoglycan may be governed by the structural peculiarities of the chains. The essential role of the glycosaminoglycan moieties in the binding of the peptide to the proteoglycan suggests the

TABLE II. Peptide-Promoted Displacement and Transfer of Proteoglycans at Low Temperature*

	Counts per minute radioactivity/area					
	Supernatant		Cell surface		Matrix	
	Heparan sulfate	Chondroitin sulfate	Heparan sulfate	Chondroitin sulfate	Heparan sulfate	Chondroitin sulfate
Control	121 ± 21	14 ± 3	9,499 ± 125	5,638 ± 322	8,564 ± 33	851 ± 61
NaCl (1.5 M)	1,165 ± 48	208 ± 4	8,944 ± 66	5,677 ± 65	8,685 ± 103	959 ± 16
Peptide (50 μM)	14 ± 5	0	8,369 ± 158	5,657 ± 76	10,097 ± 48	1,112 ± 9

*Pulse-labeled (2 h) endothelial cell cultures were transferred to ice and subjected to treatment with different agents for 15 min. Glycosaminoglycans obtained from different cell compartments and the supernatant were separated by agarose gel electrophoresis. The gels were processed for autoradiography, the area occupied by the [^{35}S]sulfate-labeled glycosaminoglycans excised, and the radioactivity quantitated by liquid scintillation counting. Data are expressed as the mean ± the standard error from triplicate electrophoretic analyses.

speculation that any impairment to the formation of the complex proteoglycan-peptide caused, for instance, by alterations of the structural integrity of the chains, could result in a decreased deposition of the proteoglycan in the extracellular matrix. Although the experiment shown in Figure 5 demonstrates that in the presence of the peptide a large fraction of the exogenous glycosaminoglycans or proteoglycans added to the culture medium are fixed by the cell layer, this result does not necessarily imply that the route normally followed by the proteoglycans, which are in transit to the extracellular matrix, is through the medium.

Peptide-Promoted Transfer of Proteoglycans From the Cell Surface and Their Insertion in the Extracellular Matrix Are Not Prevented by Low Temperature

Previous ligand-binding experiments had shown that the peptide interacts with the heparan sulfate chains obtained from the extracellular matrix of cultured endothelial cells with high affinity and specificity [Colburn et al., 1996]. This suggested that some of the proteoglycan species involved in the process of transfer from the cell compartment to the extracellular matrix may be bound to the cell surface through their glycosaminoglycan moieties and that the formation of the complex glycosaminoglycan/peptide results in the displacement of the proteoglycan from its anchorage to the cell. This type of association should be disrupted by high salt molarities. Treatment with high NaCl concentrations of the cell layer at low temperature does in fact remove the high molecular weight heparan sulfate proteoglycans (Fig. 6, lane 1) and the species removed are the same as those displaced by the peptide (Fig. 1, lane 4; these species are designated as HS1 and HS2 in Fig. 4A). An important observation gathered from these experiments is that, whereas the proteoglycans removed by high salt are found in the wash fluid, those removed by the peptide cannot be shown in the supernatant (Fig. 6, lane 3) because they have been deposited in the extracellular matrix in an amount close to that removed from the cell surface by high salt (Table II). Under the conditions used in this series of experiments, we do not detect, in the 1.5 M NaCl supernatant, any appreciable amount of HS3, the electrophoretically fast moving heparan sulfate proteoglycan found in the cell surface extracts (Fig. 4A), which, as noted above, may be considered a member of the syndecan family. Also, heparin causes the release of only a modest amount of proteoglycans (Fig. 6, lane 4). This is not too surprising in the light of the results of previous peptide-binding experiments that demonstrate that heparin is not as effective as a competitor against endothelial cell heparan sulfate as heparan sulfate from other organs [Colburn et al., 1996]. These results and the fact that the transfer of the proteoglycans to the extracellular matrix induced by the peptide can take place in the cold (Table II), contribute additional evidence that, in the case of the high molecular weight proteoglycans, the peptide may act by displacing these species from their glycosaminoglycan-mediated binding to the cell surface.

Synthetic Peptide Added to the Culture Medium Is Found Associated With the Proteoglycans Transferred to and Deposited in the Extracellular Matrix

Using antibodies directed against the synthetic peptide we were able to demonstrate that

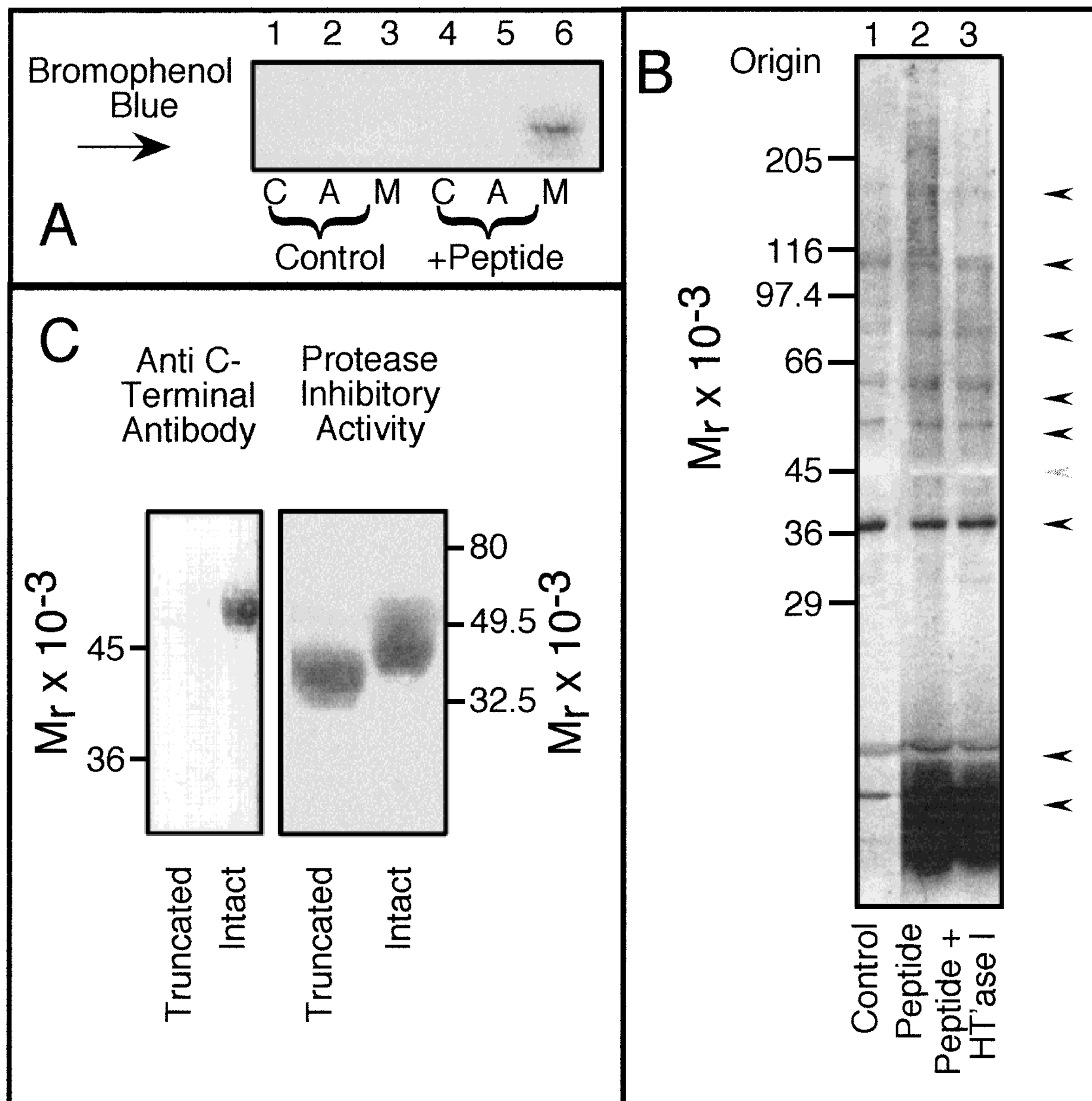

Fig. 7. Binding of the synthetic peptide to the matrix proteoglycans. **A:** Peptide accumulation in the matrix. Cultured endothelial cells incubated in the absence or presence of peptide were differentially extracted, aliquots of the extracts applied to a SDS-polyacrylamide gel, and the proteins resolved by electrophoresis under reducing conditions. After transfer to a nitrocellulose membrane, the membrane was treated with antibodies directed against the synthetic peptide. Only the lower portion of the blot is shown and the electrophoretic position of bromophenol blue (dye front) is indicated by an arrow. M, extracellular matrix extract; A, ammonium hydroxide extract; C, cell surface extract. The ammonium hydroxide wash is ordinarily performed prior to the extraction of the extracellular matrix [Colburn et al., 1994]. **B:** Removal of the bound peptide from the matrix by digestion with heparitinase I. Endothelial cell cultures were incubated with biotinylated peptide for 15 min. The cells were then washed and exposed to cross-linker for 15 min. After the incubation with cross-linker, extracellular matrices were obtained and exposed to heparitinase I. Following enzyme digestion, the matrix preparations were extracted with SDS and resolved by SDS-polyacrylamide electrophoresis. The biotinylated proteins were transferred to a PVDF membrane and detected using avidin-conjugated horseradish peroxidase. Arrows indicate the position of protein bands that reacted with avidin in the absence of the biotinylated peptide. **C:** Aliquots of fractions from the heparin-Sepharose column chromatography of endothelial cell culture medium were resolved by SDS-PAGE under reducing conditions and electroblotted to a nitrocellulose membrane. The membrane was exposed to antibodies to the synthetic peptide. In a separate experiment, aliquots of the heparin-Sepharose column fractions were resolved by SDS-PAGE under non-reducing conditions and tested for protease inhibitory activity as indicated in Methods.

the increased deposition of the proteoglycans in the extracellular matrix is paralleled by the accumulation of the peptide (Fig. 7A) and that the peptide is closely associated with the matrix proteoglycans (Fig. 7B). As expected from the experiments shown in the two previous sections, this association is maintained in the matrix by the glycosaminoglycan chains since degradation with heparitinase I results in the decrease of the amount of peptide interacting with the proteoglycans in the matrix: the action of the enzyme is evidenced by the "clearing" observed in the upper portion of lane 3 in Figure 7B. Note that the antibodies directed against the synthetic peptide recognize the intact (47 kDa) form of the parental protein but do not visualize the truncated (45 kDa) form (Fig. 7C). Both forms, however, are detected in the preparation when tested for protease inhibitory activity (Fig. 7C).

With respect to the possible physiological implications of the experimental results obtained with the peptide, we speculate that the accumulation in the extracellular matrix of the synthetic peptide and its association with the matrix proteoglycans may suggest that the extracellular matrix is the place of storage [as it has been proposed for other proteins: Vlodavsky et al., 1987; Raines and Ross, 1992] for the endothelial cell coagulation inhibitor. Alternatively, the peptide sequence may be sufficiently similar to that of the protein that normally interacts with the proteoglycans and participates in the transfer and deposition of these species in the extracellular matrix. In either case (e.g., whether the synthetic peptide mimics the action of the endothelial cell inhibitor from which it was derived or that of an entirely new protein), a possible interpretation of the experiments performed with the peptide is that the first step in the process that leads to the deposition of the proteoglycan in the extracellular matrix is the binding of the protein, participating in the transfer process, to the proteoglycan through the heparan sulfate chains of the latter. The first consequence of the formation of the complex proteoglycan/protein would then be the displacement of the proteoglycan from its anchorage to the cell surface; this would be followed by the deposition of the complex in the extracellular matrix, the presence of the bound protein being a stringent requirement for initiating the insertion of the proteoglycan in the extracellular matrix. Although in the interpretation of the data emphasis has been placed on the proteoglycan as the molecule being transferred, on the basis of the reports cited above it may be argued that the formation of the complex protein-proteoglycan could be considered as the first step in the mechanism of transfer of biological effectors from the cell surface to their place of storage in the extracellular matrix.

The action of the synthetic peptide on the proteoglycans released in the culture medium (an action that results in the fixation of the proteoglycans to the cell layer) requires further comment. The release of proteoglycans into the supernatant medium of cultured cells (shedding) has been interpreted as being part of the mechanism of turnover of integral membrane heparan sulfate proteoglycans [Yanagishita, 1992] and has been attributed to proteolytic cleavage of the protein core of the syndecans at the junction of the transmembrane and extracellular domains [Bernfield et al., 1992]. It may be hypothesized that, in endothelial cell cultures, shedding is the result of two processes: the release of syndecan-4 by enzymatic cleavage of the core protein near the membrane junction and the release of the peripheral proteoglycan (presumably perlecan), which is bound to the cell surface through its glycosaminoglycan moieties. Under the culture conditions ordinarily used, these molecules accumulate steadily into the conditioned medium of the endothelial cell line where both the heavy molecular weight (predominantly matrix) and the lower molecular weight (predominantly cell surface) proteoglycans are found. Because in the presence of the peptide the level of proteoglycans in the supernatant medium is greatly reduced and is paralleled by an increased deposition of the peptide and the proteoglycan in the extracellular matrix, the accumulation of proteoglycans in the medium of cultured endothelial cells (a phenomenon that does not seem to be taking place in vivo since only trace amounts of heparan sulfate are found in the blood stream) may be hypothesized to be an alteration, caused by the decreased availability of a protein ligand, of a process that ordinarily would result in the deposition of the complex in the extracellular matrix.

ACKNOWLEDGMENTS

This work was funded by grants from CNPq (Conselho Nacional de Desenvolvimento Cien-

tïfico e Tecnológico), FAPESP (Fundação de Amparo a Pesquisa do Estado de São Paulo), and FINEP (Financiadora de Estudos e Projetos), Brazil.

REFERENCES

Bernfield M, Kokenyesi R, Kato M, Hinkes MT, Spring J, Gallo RL, Lose EJ (1992): Biology of the syndecans: A family of transmembrane heparan sulfate proteoglycans. Annu Rev Cell Biol 8:365–393.

Buonassisi V (1973): Sulfated mucopolysaccharide synthesis and secretion in endothelial cell cultures. Exp Cell Res 76:363–368.

Buonassisi V, Colburn P (1980): Hormone and surface receptors in vascular endothelium. In Altura BM (ed): "Advances in Microcirculation." Vol. 9 Basel. S Karger, pp. 76–94.

Buonassisi V, Venter JC (1976): Hormone and neurotransmitter receptors in an established vascular endothelial cell line. Proc Natl Acad Sci USA 73:1612–1616.

Castillo CJ, Colburn P, Buonassisi V (1987): Characterization and N-terminal sequence of a heparan sulfate proteoglycan synthesized by endothelial cells in culture. Biochem J 247:687–693.

Colburn P, Buonassisi V (1988): Identification of an endothelial cell product as an inhibitor of tissue factor activity. In Vitro Cell Dev Biol 24:1133–1136.

Colburn P, Buonassisi V, Dietrich CP, Nader HB (1987): N-glycansulfated fibronectin: one of the several sulfated glycoproteins synthesized by endothelial cells in culture. Biochem Biophys Res Commun 147:920–926.

Colburn P, Crabb JW, Buonassisi V (1991): Enhanced inhibition of tissue factor by the extended form of an endothelial cell glycoprotein (an extrinsic pathway inhibitor). J Cell Physiol 148:320–326.

Colburn P, Kobayashi E, Buonassisi V (1994): Depleted level of heparan sulfate proteoglycan in the extracellular matrix of endothelial cell cultures exposed to endotoxin. J Cell Physiol 159:121–130.

Colburn P, Dietrich CP, Buonassisi V (1996): Alterations of heparan sulfate moieties in cultured endothelial cells exposed to endotoxin. Arch Biochem Biophys 325:129–138.

David G, van der Schueren B, Marynen P, Cassiman JJ, van den Berghe H (1992): Molecular cloning of amphiglycan, a novel integral membrane heparan sulfate proteoglycan expressed by epithelial and fibroblastic cells. J Cell Biol 118:961–969.

Elenius K, Jalkanen M (1994): Function of the syndecans: A family of cell surface proteoglycans. J Cell Sci 107:2975–2982.

Hanspal JS, Bushell GR, Ghosh P (1983): Detection of protease inhibitors using substrate-containing sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Anal Biochem 132:288–293.

Heifetz A, Allen D (1982): Biosynthesis of cell surface sulfated glycoproteins by cultured vascular endothelial cells. Biochemistry 21:171–177.

Humphries DE, Silbert CK, Silbert JE (1986): Glycosaminoglycan production by bovine aortic endothelial cells cultured in sulfate-depleted medium. J Biol Chem 261:9122–9127.

Nader HB, Ferreira TMPC, Paiva PJF, Medeiros MGL, Jeronimo SMB, Paiva VMP, Dietrich CP (1984): Isolation and structural studies of heparan sulfates and chondroitin sulfates from three species of mollusks. J Biol Chem 259:1431–1435.

Nader HB, Porcionatto MA, Tersariol ILS, Pinhal MAS, Oliveira OFW, Moraes MCT, Dietrich CP (1990): Purification and substrate specificity of heparitinase I and heparitinase II from Flavobacterium heparinum. J Biol Chem 265:16807–16813.

Noonan DM, Fulle A, Valente P, Cai S, Horigan E, Sasaki M, Yamada Y, Hassel JR (1991): The complete sequence of perlecan, a basement membrane heparan sulfate proteoglycan, reveals extensive similarity with laminin A chain, low density lipoprotein receptor, and the neural cell adhesion molecule. J Biol Chem 266:22939–22947.

Raines EW, Ross R (1992): Compartmentalization of PDGF on extracellular binding sites dependent on exon-6-encoded sequences. J Cell Biol 116:533–543.

Raub TJ, Sawada GA, Kuentzel SL (1994): Expression of a widely distributed, novel sulfated membrane glycoprotein by bovine endothelia and certain transporting epithelia. J Histochem Cytochem 42:1237–1259.

Saku T, Furthmayr H (1989): Characterization of the major heparan sulfate proteoglycan secreted by bovine aortic endothelial cells in culture. Homology to the large molecular weight molecule of basement membranes. J Biol Chem 264:3514–3523.

Vlodavsky I, Folkman J, Sullivan R, Fridman R, Ishai-Michaeli R, Sasse J, Klagsburn M (1987): Endothelial cell-derived basic fibroblast growth factor: Synthesis and deposition into subendothelial extracellular matrix. Proc Natl Acad Sci USA 84:2292–2296.

Warn-Cramer BJ, Maki SL, Rapaport SI (1991): A sulfated rabbit endothelial cell glycoprotein that inhibits factor VIIa/tissue factor is functionally and immunologically identical to rabbit extrinsic pathway inhibitor (EPI). Thromb Res 61:515–527.

Wesselschmidt RL, Girard TJ, Broze GJ (1990): cDNA sequence of rabbit lipoprotein-associated coagulation inhibitor. Nucleic Acids Res 18:6440.

Yamagata T, Saito H, Habuchi O, Suzuki S (1968): Purification and properties of bacterial chondroitinases and chondrosulfatases. J Biol Chem 243:1523–1535.

Yanagishita M (1992): Glycosylphosphatidylinositol-anchored and core protein-intercalated heparan sulfate proteoglycans in rat ovarian granulosa cells have distinct secretory, endocytotic, and intracellular degradative pathways. J Biol Chem 267:9505–9511.