Identification of a heparin-binding protein using monoclonal antibodies that block heparin binding to porcine aortic endothelial cells

Walter A. PATTON, II,*† Catherine A. GRANZOW,†‡ Lori A. GETTS,*† Sandra C. THOMAS,*† Lucy M. ZOTTER,*†‡
Kristin A. GUNZEL* and Linda J. LOWE-KRENTZ*†§

*Department of Chemistry, ‡Department of Molecular Biology and †Center for Molecular Bioscience and Biotechnology, Lehigh University, Mountaintop Campus, Iacocca Building Bethlehem, PA 18015, U.S.A.

The binding of heparin or heparan sulphate to a variety of cell types results in specific changes in cell function. Endothelial cells treated with heparin alter their synthesis of heparan sulphate proteoglycans and extracellular matrix proteins. In order to identify a putative endothelial cell heparin receptor that could be involved in heparin signalling, anti-(endothelial cell) monoclonal antibodies that significantly inhibit heparin binding to endothelial cells were prepared. Four of these antibodies were employed in affinity-chromatographic isolation of a heparin-binding protein

from detergent-solubilized endothelial cells. The heparin-binding protein isolated from porcine aortic endothelial cells using four different monoclonal antibodies has an M_r of 45000 assessed by SDS/PAGE. The 45000- M_r heparin-binding polypeptide is isolated as a multimer. The antibody-isolated protein binds to heparin-affinity columns as does the pure 45000- M_r polypeptide, consistent with its identification as a putative endothelial heparin receptor.

INTRODUCTION

Heparan sulphate proteoglycans (HSPGs) and heparin are known to have a number of important functions (reviewed in ref. [1]), such as providing the non-thrombogenic nature of the endothelial glycocalyx [2–5] and serving as the receptor for tissue-synthesized lipoprotein lipase on endothelial cell layers where it is active in plasma lipoprotein triacylglycerol hydrolysis [6–10]. More recently it has become clear that the HSPGs and heparin also play roles in signalling pathways. For example, they serve as reservoirs of growth factors by protecting them from proteolytic degradation [11–13], and cell-surface HSPGs (or mixed chondroitin sulphate/heparan sulphate proteoglycans) act as growth factor receptors [14–20].

Some specific signalling involves heparin/heparan sulphate-binding sites. Heparin and endothelial HSPGs inhibit the growth and migration of vascular smooth-muscle cells [21,22], presumably by binding to the approx. 10^5 specific heparin/heparan sulphate-binding sites/cell [23]. Heparin binding to smooth-muscle results in synthetic changes [24–28]. Heparin also binds to endothelial cells [29–32], where its binding leads to changes in synthesis of extracellular matrix protein [33] and causes an increase in secreted HSPGs, and possibly other HSPG changes as well [34–36]. Binding studies with human umbilical-vein endothelial cells indicate between 10^6 [31] and 10^7 [29] binding sites/cell. Direct evidence indicates internalization of cell-bound heparin in endothelial cells [30,32]. Although the intracellular location of the heparin has not been identified in endothelial cells, some of the internalized heparin is known to go to the nucleus in HeLa cells [25] and hepatocytes [37, 38]. Cultured porcine endothelial cells retain a heparin-releasable HSPG population on their surfaces which is presumably associated with the heparin/heparan sulphate-binding sites [39]. Heparin and heparan sulphate also modulate endothelial cell growth by a mechanism that might involve their interaction with growth factors [40].

Examples of other stimuli which cause changes in endothelial-

cell HSPG synthesis that may involve heparin/heparan sulphate receptors include the following. (1) Extracellular matrix removal or vessel injury induces endothelial cells to proliferate and synthesize a new matrix including sulphated proteoglycans [41]. (2) Wounding of cultured endothelial cells leads to an alteration in the synthesis of radiolabelled proteoglycans which localize in the cell layer [42]. (3) Cultured bovine endothelial cells increase the percentage of radiolabelled proteoglycan incorporated into their extracellular matrix after reaching confluence [43]. (4) Growth of endothelial cells on HSPG-depleted matrix results in higher levels of HSPG synthesis compared with growth on normal matrix [44].

In vitro changes in endothelial HSPG synthesis may serve as clues to similar changes that occur in disease situations. In atherosclerosis, the endothelial extracellular matrix may contain less HSPG than the original matrix [45–47]. Changes in the amount and type of endothelial-layer glycosaminoglycans that accumulate during the disease process are proposed to be involved in the build-up of lipid in the atherosclerotic plaque [45,47–48]. Vascular complications of diabetes also include altered patterns of endothelial HSPG synthesis in the affected blood vessels [49–53].

Despite significant progress in understanding heparin involvement in signalling, the involvement of a heparin receptor(s) and its mechanism(s) remain unclear. The identification of a heparin receptor is critical to understanding the receptor-mediated aspects of heparin signalling. The identity of a specific endothelial heparin-binding protein, which is therefore a putative receptor, is the subject of the present report.

EXPERIMENTAL

Materials

[35 S]Methionine and [3 H]heparin were obtained from DuPont deNemours (NEN, Wilmington, DE, U.S.A.). Heparin, PMSF, acrylamide, some immunochemicals, ExtrAvidin-Phosphatase

conjugate, 5-bromo-4-chloro-3-indolyl phosphate, cell-culture-grade penicillin-streptomycin, amphotericin B, gelatin and chemicals for media were obtained from Sigma Chemical Co (St. Louis, MO, U.S.A.). Nitro Blue Tetrazolium was obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Biotinylated and prestained M_r markers were from Bio-Rad (Richmond, CA, U.S.A.), and Rainbow M_r markers were from Amersham (Arlington Heights, IL, U.S.A.). The biotinylating reagent NHS-LC-Biotin [sulphosuccinimidyl-6-(biotinamido)hexanoate] was obtained from Pierce Chemical Co. (Rockford, IL, U.S.A.). Chromatography resins, including heparin-Sepharose, were obtained from Pharmacia Biotech Inc. (Piscataway, NJ, U.S.A.). The anti-mouse IgG agarose-affinity resin was obtained from Sigma. Antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA, U.S.A.) or Sigma. Anti-LH receptor monoclonal antibodies produced with the same myeloma cells as used in the present study were generously given by Dr. Eugene Nau of the Molecular Biology Department at Lehigh University. Budget Solve and Econo-Safe (Research Products International Corp., Mount Prospect, IL, U.S.A.) were employed as scintillation fluids. Kodak (Rochester, NY, U.S.A.) XOMAT AR film was used for autoradiography.

Cell culture

The isolation and maintenance of porcine aortic endothelial (PAE) cells have been described previously [34,39,54]. Briefly, cells were grown at 37 °C with 5% CO₂ on gelatin-coated dishes in Dulbecco's modified Eagle's medium with 10% heat-inactivated serum and antibiotics as reported. When the cells had become established, they were weaned into 10% heat-inactivated Serum Plus (JRH Biosciences, Hazelton Biologics, Lenexa, KS, U.S.A.) by using a 50:50 mixture of heat-inactivated serum and heat-inactivated Serum Plus for one passage. For preparation of radiolabelled putative heparin receptor, PAE cells were labelled with [³⁵S]methionine at 2 µCi/ml in normal growth medium. After 48 h, the cells were harvested by removal of media, washing with PBS, scraping and pelleting of the cells in PBS, and solubilization of the pellets in PBS with 0.2% CHAPS to solubilize the cell membranes and protease inhibitors. Removal of cell debris was accomplished with a low-speed spin (2000 g) before further analysis.

Hybridoma production and culture

Female Balb/c mice (10 weeks old) from Jackson Laboratories were immunized with 2.5×10^6 endothelial cells in Freund's complete adjuvant by subcutaneous injection into the abdomen. Booster injections of about 2.5×10^6 cells in PBS were given every 2.5–4.5 weeks. Antibodies to whole endothelial cells (determined by ELISA [55] with whole endothelial cells grown on multiwell plates) were observed after two or three booster injections. Fusions were accomplished essentially as described [55] using the murine myeloma cell line P3X63-Ag8653 obtained from a subculture used by Stamatoglou and Keller [56] originally from the Institute of Medical Research (Camden, NJ, U.S.A.). Poly(ethylene glycol) was used to stimulate cell fusion. The hybridoma cells were grown in 96-well plates with hypoxanthine/amethopterin/thymidine (HAT) medium as described [55] to select against myeloma cell survival. The medium was a 1:1 mixture of the above medium and Swiss mouse 3T3 cell-conditioned medium (containing 20% rather than 10% heat-inactivated fetal bovine serum). For long-term hybridoma cultivation, HAT was removed from the medium, and the serum reduced to 15% in non-conditioned medium.

Identification of heparin-blocking hybridomas

Initially, hybridoma lines were selected on the basis of their ability to bind endothelial cells in an ELISA. Selection continued using a function-based assay. Briefly, PAE cells were grown in multiwell (30 mm diameter) dishes until confluent. The spent growth medium was removed and replaced with 1 ml of conditioned hybridoma medium (or, in the case of some controls, unsupplemented fresh medium) and incubated at 37 °C. After 10 min, radiolabelled heparin (20 µg/ml final concentration except for experiment no. 1 where the final concentration was 100 µg/ml) was added and the incubation continued for 30 min. The medium containing unbound [³H]heparin was removed and the radioactivity determined by scintillation counting. After a brief wash in cold PBS, the amount of [³H]heparin associated with the cells was also determined. To ensure separation of cell-bound heparin from extracellular-matrix-bound heparin, the cells were removed from the matrix by gentle scraping before solubilization for scintillation counting. Triplicate samples were analysed for each hybridoma supernatant and compared with controls and other hybridoma supernatants tested in the same experiment. Cell-bound [³H]heparin as a percentage of the total was determined for each sample, thus eliminating error due to minute differences in the amount of [³H]heparin added. The average [³H]heparin bound for all of the hybridoma supernatants in an experiment (five to 15 hybridoma supernatants were tested in a single experiment) was used as the benchmark for that experiment. Assays were performed over a 4-month period of time with a variety of different endothelial-cell populations.

Isolation of antibody and putative heparin receptor

The antibodies produced by the hybridoma cells were separated from other protein components of the hybridoma supernatants using affinity chromatography on 1 ml columns of anti-mouse IgG-agarose at 4 °C, with monitoring of column flow at A_{280} . The hybridoma supernatants were passed twice over the columns and then washed extensively with PBS until baseline was maintained. The antibodies were eluted from the affinity resin with glycine (0.1 M, pH 2.5) and neutralized with Tris/HCl buffer, pH 9.0, or 0.1 M NaHCO₃. In some experiments, tubes used for antibody collection were coated with carrier protein (immunoassay-grade BSA).

The radiolabelled solubilized PAE cells, prepared as described above were mixed 1:1 (v/v) with purified antibodies [between 5 and 30 µg of antibody, 18H6, 18E9, 12B1, 18B6, 18C4, anti-(LH receptor) or non-immune IgG] and allowed to bind for 48 h at 4 °C. The antibody/cell extract was passed over a 2 ml affinity column of Protein G-Sepharose at 4 °C in the same buffer as was used for cell solubilization. Loading buffer was used to wash the column until there was no protein or radioactivity in the eluted fractions. Glycine (0.1 M, pH 2.5, with 0.2% CHAPS) was used to disrupt the antibody interaction with the affinity column. The eluted sample was dripped into Tris/HCl buffer, pH 9.0, (or 0.1 M NaHCO₃) with 0.2% CHAPS for neutralization. Protease inhibitors (50 µM *ε*-aminocaproic acid, 5 mM benzamidine, 2 mM *N*-ethylmaleimide and 1 mM PMSF) were included in the incubation mixtures which did not contain carrier albumin.

One alternative isolation using antibody supernatant and anti-mouse IgG columns involved mixing the cells 1:2 (v/v) with antibody 18H6 and binding to anti-mouse IgG-agarose in the same buffer as above. In this procedure, 4 M urea in PBS with 0.2% CHAPS was employed to elute the radiolabelled sample from the affinity column. The urea-elution protocol resulted in trapping of some radiolabelled protein in the column. Therefore

most isolations were routinely accomplished as described above using glycine to disrupt the antibody–resin interactions.

Biotinylation of cell-surface proteins

Biotinylation of cell-surface proteins was carried out with whole cells and the water-soluble biotinylation reagent NHS-LC-Biotin. The reaction was carried out essentially as recommended by the supplier. NHS-LC-Biotin (final concentration 1 mM in PBS) was added to 150 mm-diameter dishes of confluent cells, which had been carefully washed with PBS (pH 7.5) to remove excess amines from the culture medium. The cells were incubated at 25 °C for 30 min. The reaction was stopped by removing the biotin solution, and any remaining biotin quenched with PBS containing 50 mM Tris/HCl (final pH 7.8) and 0.01 % BSA. The cells were washed three times with the same solution before receptor preparation as above for radiolabelled samples. Typically one plate of cells was used for each experiment, and a second identical plate for a non-immune mouse IgG control. Protease inhibitors (50 μ M ϵ -aminocaproic acid, 5 mM benzamidine, 2 mM *N*-ethylmaleimide, and 1 mM PMSF) were included in the incubations of biotinylated proteins and antibodies.

Analytical technique

Chromatography columns (1 cm $\times$ 100 cm) were packed with Sepharose CL4B, CL6B and Sephacryl S-300. The columns were equilibrated in CHAPS buffer (0.1 M Tris/HCl, pH 6.5, 0.1 % CHAPS, 1 mM EDTA, 1 mM PMSF) or CHAPS buffer with 2 M urea at 4 °C as indicated. Some columns were also run in 0.2 % CHAPS or 0.2 % octyl β -D-glucopyranoside instead of 0.1 % CHAPS. Fractions of 1.8 ml each were collected, and aliquots of each fraction were analysed by scintillation counting. All sizing columns were calibrated using BSA and ovalbumin. β -Galactosidase and alcohol dehydrogenase were also employed in the calibration of CL4B columns. Protein elution was determined by monitoring A_{280} .

Heparin-affinity column chromatography employed 0.5 cm $\times$ 15 cm columns of heparin–Sepharose in the CHAPS column buffer noted above at 4 °C. Radiolabelled proteins were applied to the affinity column and the column was washed with CHAPS column buffer. Elution of radiolabelled proteins was by batch with 100 μ g/ml heparin in CHAPS buffer followed by 2 M urea in CHAPS buffer. Alternatively, bound material was eluted with a 100 ml linear gradient of 0–2 M NaCl in CHAPS buffer. In the gradient elution, 1.8 ml fractions were collected and monitored for A_{280} and radioactivity.

SDS/PAGE was accomplished using the Laemmli technique [57]. Samples with radioactive protein were prepared by mixing with SDS sample buffer, concentration in Amicon centricons, incubation at 55 °C for 20 min and dialysis against SDS sample buffer; they were then boiled for 5 min before loading on the gel. Gels were stained with Coomassie Brilliant Blue, and individual lanes cut into 2 mm slices. The slices were either oxidized using H_2O_2 or solubilized with TS-2 (Research Products International) and analysed by scintillation counting. Other radioactive gels were dried after equilibration in 1 % glycerol/10 % acetic acid and analysed by autoradiography using a Lanex Regular intensifying screen (Kodak) to improve detection. Some Coomassie Blue-stained gels and autoradiograms were scanned with an LKB UltroScan XL laser densitometer.

Isolated biotinylated protein samples were precipitated by adding trichloroacetic acid to 10 % (v/v). The precipitate was pelleted at high speed for 5 min in a Microfuge, the liquid carefully removed, and the pellet dissolved in SDS sample buffer. As above, an extended time (24 h at 4 °C) was required between

boiling the sample and running the gel. After electrophoresis as above, the proteins were transferred to nitrocellulose membranes using the Towbin buffer system [58]. After transfer, the membrane was blocked with 1 % fish gelatin/1 % polyvinylpyrrolidone/0.1 % NaN_3 in TBST overnight at 4 °C, washed 3 $\times$ 10 min in TBST and incubated with 1 : 20000 ExtrAvidin–Phosphatase in TBST for 2 h at 25 °C, (where TBST is 150 mM NaCl/50 mM Tris/HCl, pH 7.5, containing 0.1 % Tween-20). The membrane was washed as before and developed with Nitro Blue Tetrazolium/5-bromo-4-chloro-3-indolyl phosphate in 100 mM NaCl/100 mM Tris/HCl/5 mM $MgCl_2$ as recommended by the enzyme–conjugate supplier. Biotinylated protein M_r standards were run in adjacent gel lanes.

RESULTS

Identification of heparin-blocking antibodies

In order to obtain antibodies that bound to a putative heparin receptor on PAE cells, whole PAE cells were used to immunize mice for hybridoma production as described in the Experimental section. From the resulting hybridomas, those that produced antibodies to PAE cells were identified. Hybridoma supernatants from 170 of these lines were tested for their ability to inhibit binding of [3H]heparin to confluent PAE cells.

In each experiment, heparin binding in the presence of individual hybridoma supernatants was determined and compared with the average heparin binding for all hybridomas tested in the experiment and with controls containing no antibody. Nine candidate anti-(heparin receptor) antibodies were identified on the basis of their inhibition of heparin binding. The experiments in which the four antibodies used in the present studies were identified are illustrated in Figure 1. In these original assays, heparin binding to cells in the presence of hybridoma supernatants 18B6, 18E9, 18H6 and 12B1 was reduced by 49, 36, 54 and 64 % respectively when compared with average heparin binding in the presence of other hybridoma supernatants tested in the same experiment. These blocking antibodies also inhibited binding by 46, 41, 50 and 53 % respectively relative to heparin binding in control cells.

Antibody-based identification of a putative receptor polypeptide

Hybridoma antibodies were isolated from the supernatants using anti-mouse IgG–agarose affinity columns as described in the Experimental section. These antibodies were mixed with PAE cells grown with [^{35}S]methionine and solubilized in CHAPS buffer. The mixtures were subjected to affinity chromatography on Protein G–Sepharose. In order to analyse migration of the ^{35}S -radiolabelled antibody–putative heparin receptor samples by SDS/PAGE, SDS was exchanged for the CHAPS detergent and lipid as described in the Experimental Section. Under these conditions, more than 90 % of the radioactivity in the samples entered the gel. If CHAPS was not replaced with SDS, less than 50 % of the radioactivity entered the separating gel with the rest remaining in the stacking gel. Coomassie Blue staining of the gels revealed the expected antibody bands and carrier albumin, when present. A faint band at M_r 45000 was usually seen. Detection of the endothelial-derived proteins was accomplished by autoradiography or cutting and counting of the gel.

Antibodies 18B6, 18H6, 18E9, 12B1 and 18C4 (an IgM antibody) were each used for isolation of radiolabelled protein from endothelial cells. Autoradiography of the gel containing 18B6, 18H6 and 18E9 (2-week exposure) indicated a faint polypeptide band of M_r 45000 on each sample lane. A 4-week exposure yielded a stronger band at M_r 45000 (Figure 2a, lanes

Figure 1 Identification of antibodies that block heparin binding

Identification of blocking antibodies was accomplished as described in the Experimental section. The binding data for the experiments that resulted in identification of the antibodies used here are shown: (a) 18B6 (experiment no. 1); (b) 18E9 (experiment no. 7); (c) 18H6 (experiment no. 11); (d) 12B1 (experiment no. 15). Solid bars indicate the percentage of total heparin bound averaged for each antibody. The stacked open bars indicate standard deviations. B indicates the heparin binding for the blocking antibody identified, and numbered samples are non-blocking antibodies. C indicates controls with no antibody added, and A represents the average of the non-blocking antibodies. B' in (c) is a second blocking antibody identified in experiment no. 11 which was not used in the present study. Antibody 7 in (b) and antibody 6 in (c) were significantly lower than the average, but not enough to be chosen. Antibody 1 in (d) is an example of an antibody that significantly increased heparin binding.

1–3) and faint development of much of the remainder of the gel. An experiment with antibody 12B1 also produced one band at M_r 45000, whereas an identical sample with antibody 18C4 (IgM antibodies do not bind to Protein G) yielded no band at M_r 45000 (Figure 2a, lanes 4 and 5).

Figures 2(a) and 2(c) show a control isolation in which the autoradiograph was scanned with an LKB densitometer to show that using an unrelated monoclonal antibody [anti-(LH receptor)] did not result in purification of the 45000- M_r protein. The amounts of antibody were carefully controlled to ensure that at least as much anti-(LH receptor) antibody as 12B1 antibody was used with identical numbers of radioactive cells from the same endothelial-cell preparation. Densitometric scans of the Coomassie Blue-stained gel indicated approx. 2.5 times more anti-(LH receptor) heavy chain than 12B1 heavy chain. Scintillation counting of 2 mm gel strips from a number of isolations also yielded one band at M_r 45000. Occasionally, a second radiolabelled polypeptide of M_r about 30000 was also observed.

Characterization of biotinylated protein

Whole endothelial-cell-surface proteins were biotinylated with the water-soluble long-chain reagent NHS-LC-Biotin as

suggested by Pierce Chemical Co. and described in the Experimental section. Putative receptors were isolated from the biotinylated cells as described above for the radiolabelled cells, separated by SDS/PAGE, and blotted to nitrocellulose. When the putative receptor was isolated from biotinylated confluent endothelial cells, a biotinylated protein of M_r 45000 was routinely obtained. Repeated experiments with the antibodies that blocked heparin binding consistently showed biotinylated protein at M_r 45000 whereas control non-immune mouse IgG did not (for example see Figure 3 in which antibody 18H6 was used). However, the non-specific bands that showed up in both samples varied from experiment to experiment. When blots from experiments with identical quantities of control and specific blocking antibodies were analysed by densitometric scanning, the blocking antibody samples had at least ten-fold more absorbance at M_r 45000 than samples isolated with an identical amount of non-immune mouse IgG. In most experiments there was no absorbance in the 45000- M_r region of the non-immune samples.

Analysis of intact putative receptor by gel filtration

In order to obtain evidence for the intact putative heparin receptor structure, antibody-radiolabelled putative heparin

Figure 2 SDS/PAGE of antibody-isolated proteins

(a) Antibodies from hybridomas 18E9 (lane 1), 18H6 (lane 2), 18B6 (lane 3), 12B1 (lane 4) and the IgM antibody, 18C4 (lane 5) were used to isolate radiolabelled proteins from solubilized endothelial cells as described in the Experimental section. The antibody-putative heparin receptor mixtures [containing carrier BSA (lanes 1–3) or not (lanes 4 and 5)] were exchanged into sample buffer as described in the Experimental section and subjected to SDS/PAGE on 10% gels. Autoradiographic analysis of the gels (4-week exposure to film) is shown in (a). Markers are identified by numbers beside the gels and the tops of the separating gels are marked by arrowheads. TD, Tracking dye. (b) and (c) show additional isolations with antibody 12B1 (b) and a non-heparin-blocking control [anti-(LH receptor) antibody] (c) where the autoradiograms were scanned with an LKB densitometer and the data transferred into a graphics program for plotting. The peak at about 50 mm in (b) is at M_r 45 000 compared with protein standards.

receptor mixture was analysed by gel-filtration chromatography under non-denaturing conditions (Figure 4a). The radioactivity eluted in the void volume of Sephacryl S-300, and the addition of 2 M urea did not alter the elution pattern of the radioactive material (Figure 4b). The elution pattern indicates a hydrodynamic size of more than 200 000 M_r based on elution of protein standards. If the sample was stored at 4 °C (in CHAPS buffer), the amount of radioactivity eluted after fraction 30 increased over time. Analysis by SDS/PAGE indicated that this material was essentially identical (at 45 000 M_r) with the original sample. Preparations with antibodies 18H6, 18E9 and 18B6 all yielded the larger material. The putative receptor isolated with antibody 12B1 was not tested. Neither increasing the concentration of CHAPS (to 0.2%) nor changing the detergent in the column buffer to octyl β -D-glucopyranoside altered the elution of the

Figure 3 Isolation of the putative heparin receptor for biotinylated cells

Antibody 18H6 (lane B) or non-immune mouse IgG (lane A) was used to isolate the putative heparin receptor from biotinylated endothelial cells as described in the Experimental section. The antibody-putative receptor samples were subjected to SDS/PAGE, electrophoretically blotted to nitrocellulose, and developed as described in the Experimental section. The migration of biotinylated M_r standards is indicated on the right.

Figure 4 Gel filtration of antibody-putative heparin receptor on Sephacryl S-300

Antibodies from hybridoma 18H6 ($\blacktriangle$; b) and 18E9 ($\blacksquare$; a) were used to isolate radiolabelled protein from solubilized endothelial cells. The antibody-putative heparin receptor mixtures were analysed by gel filtration on Sephacryl S-300 in 0.2% CHAPS ($\blacksquare$) or 2 M urea/CHAPS ($\blacktriangle$) buffer as described in the Experimental section. The void volume was at fraction 18 and total volume, as marked by Phenol Red, at fraction 73. Arrows indicate the migration of M_r standards (BSA, approx. 66 000; ovalbumin, approx. 45 000).

radiolabelled putative receptor isolates. Lipid analysis of the putative receptor-antibody mixture by TLC suggested the presence of phospholipids in the multimers (results not shown).

Heparin interactions with the putative receptor-antibody mixtures

To provide evidence that the endothelial putative heparin receptor isolated with these hybridoma antibodies is capable of

Figure 5 Heparin-affinity chromatography of the putative heparin receptor

Antibodies from hybridomas 18B6 and 18E9 were used to isolate the putative heparin receptor as described in the Experimental section. The mixtures were prepared for affinity chromatography, applied and eluted as described in the Experimental section. The NaCl gradient was begun at fraction number 1 and ended at number 78. Aliquots of 200 μ l (18B6; **a**) and whole samples (18E9; **b**) were analysed for radioactivity. The dotted line indicates the salt gradient.

Figure 6 Electrophoretic analysis of the 45000- M_r protein

The 45000- M_r polypeptide from the urea elution of an anti-mouse IgG affinity column isolation described in the text was analysed by SDS/PAGE. The gel was analysed by scintillation counting of 2 mm slices as described in the Experimental section.

binding heparin, its ability to bind to heparin-Sepharose affinity columns was determined. When the 35 S-radiolabelled antibody-putative heparin receptor mixtures were warmed to 37 $^{\circ}$ C for 20 min to facilitate antibody exchange and diluted 1:1 with cold column buffer to decrease reassociation, 50–90% of the radiolabelled protein bound to heparin-Sepharose affinity columns. Elution of bound material with a salt gradient resulted in a number of peaks of radioactivity starting somewhat before 0.5 M NaCl (Figure 5), with the last peak eluted close to 1 M

Figure 7 Heparin-affinity-chromatographic analysis of the 45000- M_r protein

The 45000- M_r polypeptide analysed in Figure 6 was also analysed by heparin-affinity chromatography as described in the Experimental section. The NaCl gradient began at fraction 1 and ended at fraction 72. Entire fractions were analysed for radioactivity. The dotted line indicates the salt gradient.

NaCl. This basic profile was observed with putative heparin receptor isolated using antibodies 18E9 and 18B6, although differences in peak height were noted.

Identification of the putative receptor by an alternative affinity isolation

The alternative isolation protocol using anti-mouse IgG resin and 4 M urea to elute any antibody-putative receptor complex resulted in loss of some radiolabelled protein on the column (although antibodies were eluted). Attempts to recover the 35 S-radiolabelled putative heparin receptor for the anti-mouse IgG affinity columns involved batch washing the resin with 4 M urea. This resulted in a preparation of protein which migrated on SDS/identically with the 45000- M_r putative heparin receptor polypeptide (Figure 6).

This 45000- M_r polypeptide also bound to heparin-Sepharose, and was eluted with a gradient of NaCl (Figure 7). The elution produced a single peak at just above 0.5 M NaCl. The peak aligns with the central region of the antibody-putative heparin receptor chromatographic elution shown in Figure 5.

DISCUSSION

Despite evidence of heparin binding to endothelial cells [29,31] and heparin-induced changes in endothelial-cell HSPG synthesis [34–36], identification of a specific heparin receptor in endothelial cells has not been forthcoming. Previous attempts to isolate a heparin-binding protein from PAE cell plasma membranes using heparin-affinity chromatography have been frustrated by the number of proteins that bind to heparin. A recent publication [59] indicates that a very large number of heparin-binding proteins can be identified from epithelial cells when specific heparin affinity of membrane proteins is the criterion employed. Growth factors [11–13,40], antithrombin III [3], extracellular matrix proteins (reviewed ref. in [60]) and platelet/endothelial-cell adhesion molecule [61] are among those proteins known to interact with heparin. The topic of heparin interaction with proteins has been extensively reviewed [1]. Heparin-affinity chromatography has also been used to purify proteins that may

interact with the affinity matrix based on ion-exchange mechanisms or the ability of heparin to act as a nucleic acid analogue (e.g. restriction endonucleases as in ref. [62]).

Evidence obtained by binding of radiolabelled heparin to endothelial cells suggests between 10^6 [31] and 10^7 [29] binding sites per cell. Therefore, to obtain a specific method for identifying putative heparin-receptor proteins, monoclonal antibodies were produced against whole PAE cells. As such a receptor should be among the PAE cell-surface proteins, the hybridomas produced were screened for their ability to produce antibodies that would inhibit the binding of heparin to confluent PAE cells. Nine such hybridomas were identified. The use of confluent cells resulted in low binding of heparin to extracellular matrix. Platelet/endothelial-cell adhesion molecule 1 and other cell adhesion molecules are primarily localized at cell-cell junctions in confluent cells [63] and are probably also unavailable for heparin binding. Therefore antibodies against extracellular matrix proteins or cell adhesion molecules would not be likely to inhibit a significant portion of heparin binding to the confluent cells.

Although the variability for non-blocking antibodies in a single experiment was around 20 % of the average (see Figure 1), some variability in the heparin-binding assay was expected as a result of differences, including the amount of antibody, between the hybridoma supernatants. Differences in [^3H]heparin (between different shipments), PAE cell line and cell age might also contribute to variability between assays. Regardless of the variability between experiments, the reproducibility within each experiment indicates differences between the blocking antibodies and other hybridoma supernatants. Many of the experiments resulted in no identification of blocking antibodies, again indicating that the variability between non-blocking antibodies was low enough to allow recognition of blocking antibodies rather than simple selection of the hybridoma supernatants with the lowest heparin binding from each experiment. The decision to select hybridomas that significantly inhibited heparin binding biased the selection of antibodies towards those recognizing a protein responsible for a significant portion of heparin binding to endothelial cells. Heparin-binding proteins accounting for a low percentage of PAE cell heparin binding would not be identified by this procedure.

Despite variability between the antibody-identification experiments, the values of percentage inhibition by the four antibodies used for protein isolation in this report were quite similar. Although there may be differences in affinity or epitope recognized, the data from the identification experiments suggest that the four antibodies act on the same heparin-binding site, a fact substantiated by the isolation of the same polypeptide by all four antibodies. The inhibition of radiolabelled heparin binding by unlabelled heparin is typically in the same range as the inhibition by these antibodies, suggesting that they act at the primary endothelial heparin-binding site.

The antibody inhibition of heparin binding to the cell surface indicates a cell-surface location for the binding interactions. The biotin labelling of the antibody-isolated putative heparin receptor is further evidence for the cell-surface location of this protein. Activated biotin-containing molecules have been used to label a number of cell-surface proteins in whole cells [64]. The NHS-LC-Biotin used in the present studies was chosen for its water solubility, which would facilitate labelling of whole cells in physiological buffers and make internalization of the biotin very unlikely.

Anti-mouse IgG-agarose-purified antibodies from the supernatants of four of the hybridomas that blocked heparin binding were tested and found to bind a 45000- M_r polypeptide from PAE cells. The data in Figure 2(a) indicate that an IgM antibody

(18C4) which blocks heparin binding does not result in isolation of the 45000- M_r protein band. Therefore the 45000- M_r polypeptide was not isolated as a result of its affinity for the Protein G-Sepharose. In addition, densitometric scans of a control isolation indicate that use of an unrelated [anti-(LH receptor)] IgG antibody does not result in isolation of the 45000- M_r protein recognized by 12B1 analysed in the same experiment. Non-immune mouse IgG also did not isolate the 45000- M_r polypeptide (Figure 3). Therefore the 45000- M_r putative heparin receptor polypeptide is recognized by unique interactions with these four antibodies (which block heparin binding) rather than through non-specific interactions with IgG.

To date, it has not been possible to stain any PAE cell proteins from solubilized whole cells or purified PAE cell membranes in a Western blot using 18H6, 18B6, 12B1 or 18E9 antibodies. Unfortunately, the heparin-binding site in the 45000- M_r polypeptide (which is recognized by these blocking antibodies) may be denatured during electrophoresis and electrophoretic transfer resulting in a structure that cannot be recognized by these antibodies. It has also been impossible to observe [^3H]heparin binding to blotted putative heparin receptor preparations, again suggesting that the heparin-binding site is denatured under these conditions.

The polypeptides obtained by antibody-affinity chromatography are isolated as a large multimer complexed with CHAPS detergent and probably also with phospholipids. Phospholipid in the putative heparin receptor-antibody samples might contribute to its hydrodynamic size and would cause the difficulty of complexing SDS to the protein for gel electrophoresis. The large size of the putative heparin receptor indicated by column chromatography appears to be indicative of large multimers. Neither doubling the concentration of CHAPS nor exchange of CHAPS for octyl glucoside altered the elution behaviour. If there were simply too few detergent molecules for each putative receptor to be surrounded by detergent only, doubling the CHAPS concentration might have resulted in some dissociation of the complexes. Inclusion of urea in the sample buffer to dissociate antibody-putative receptor interactions also failed to decrease the apparent size of the putative heparin receptor; indicating that interactions with the antibody were not responsible for the large apparent size. Long (at least 5 days) 4 °C incubations in CHAPS buffer resulted in the dissociation of some multimers to monomers as measured by gel filtration. The polypeptides still migrated at M_r 45000 as determined by SDS/PAGE and could still bind heparin (K. Gunzel and L. Lowe-Krentz, unpublished work).

The data are consistent with the putative heparin receptor existing as a multimer composed of 45000- M_r polypeptides. The occasional presence of a 30000- M_r polypeptide mentioned in the Results section may result from proteolytic degradation of the 45000- M_r polypeptide, or it may be a contaminant, or minor component not required for heparin binding of the 45000- M_r protein. It will be necessary to purify the 45000- M_r putative receptor and the 30000- M_r polypeptide to resolve their relationship. The epithelial membrane heparin-binding proteins described recently [59] include a minor protein band at M_r 45000 in addition to the 31000- M_r and 18500- M_r major polypeptides and other lower-abundance proteins. The 45000- M_r protein does not appear to be the major protein identified in the epithelial studies, but seemed to be identified by each heparin-affinity method used by the authors.

Specific isolation of the same protein by four different anti-endothelial monoclonal antibodies that block heparin binding to endothelial cells (but not by a variety of control antibodies or Protein G alone) supports the identification of this putative

heparin receptor as a protein responsible for heparin binding to endothelial cells. The ability of the isolated protein to bind to heparin-affinity columns is further support for the identification. The complex elution of the radioactive putative receptor-antibody mixtures from the heparin column may be due to the antibodies remaining in the sample, the lipid component associated with the antibody-putative heparin receptor mixture, the presence of different multimeric forms, or any combination of these factors. Pure 45000- M_r protein (without antibodies) is eluted from heparin-affinity columns with a single peak and small shoulder (Figure 7) which align with the central region of the antibody-putative heparin receptor chromatographic elution (Figure 5). This result is consistent with any of the above explanations proposed for elution of the antibody-putative receptor mixture.

Elution of the putative heparin receptor for the heparin-affinity matrix at around 0.5 M NaCl suggests that heparin binding would occur in physiological salt concentrations. Although some other heparin-binding proteins (e.g. growth factors) bind heparin much more tightly than the putative heparin receptor, the dissociation constants reported for heparin binding to endothelial cells [29,30] are in the micromolar range, suggesting that these sites bind heparin less tightly than growth factors (most of which have dissociation constants in the picomolar range). Furthermore, heparin-induced changes in endothelial cell behaviour occur at heparin concentrations too high to be due only to such high-affinity heparin-binding molecules. In fact, such high-affinity sites might be constantly saturated by endogenous heparan sulphate from one or more of the many endothelial-cell-surface HSPGs [65,66].

Endothelial cells bind heparin [29–31], and saturation of the PAE cell heparin-binding sites results in a change in HSPG synthesis [34–36]. Heparin also modulates endothelial synthesis of extracellular matrix proteins [33]. Responses to heparin binding in a number of cell types include heparin internalization [30] and transport to the nucleus [25,37,38], changes in gene expression [24–27] and inhibition of cell growth [21,22]. Such heparin-induced alterations in cell behaviour may require specific heparin-receptor activity. Although many proteins bind to heparin, the specific protein(s) responsible for mediating heparin effects in cells has not previously been identified.

The heparin responses in endothelial cells may be due to the putative heparin receptor identified in the present report. The identification of a candidate heparin receptor from endothelial cells provides the opportunity to determine whether it plays a role in the control of HSPG synthesis. Significant additional work will be necessary to establish the function(s) of this putative heparin receptor. Antibodies that block heparin binding to endothelial cells through the putative heparin receptor should be useful in determining the involvement of the protein in heparin signalling.

This work was supported by grants from the W. W. Smith Charitable Trust of Philadelphia, PA, U.S.A. to L.J.L.-K. W.A.P., II was an NIH Biotechnology fellow (5T32 GM0835). We gratefully acknowledge the antibody-screening efforts of Karen Harper and preliminary work carried out by Francine Redner, Robert Krukltis, Kelly Thompson and Rebecca Little which made the present research efforts possible. Technical work with the biotin labelling accomplished by Felix Molina is also appreciated.

REFERENCES

- Jackson, R. L., Busch, S. J. and Cardin, A. D. (1991) *Physiol. Rev.* **71**, 481–539
- Colburn, P. and Buonassisi, V. (1982) *Biochem. Biophys. Res. Commun.* **104**, 220–227
- de Agostini, A. I., Watkins, S. C., Slayter, H. S., Youssoufian, H. and Rosenberg, R. D. (1990) *J. Cell Biol.* **111**, 1293–1304
- Marcum, J. A. and Rosenberg, R. D. (1985) *Biochem. Biophys. Res. Commun.* **126**, 365–372
- Marcum, J. A., Atha, D. H., Fritz, L. M. S., Nawroth, P., Stern, D. and Rosenberg, R. D. (1986) *J. Biol. Chem.* **261**, 7507–7517
- Cheng, C.-F., Oosta, G. M., Bensadoun, A. and Rosenberg, R. D. (1981) *J. Biol. Chem.* **256**, 12893–12898
- Sexena, U., Klein, M. G. and Goldberg, I. J. (1991) *J. Biol. Chem.* **266**, 17516–17521
- Saxena, U., Klein, M. G. and Goldberg, I. J. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 2254–2258
- Shimada, K., Gill, P. J., Silbert, J. E., Douglas, W. H. J. and Fanburg, B. L. (1981) *J. Clin. Invest.* **68**, 995–1002
- Williams, M. P., Streeter, H. B., Wusteman, F. S. and Cryer, A. (1983) *Biochim. Biophys. Acta* **756**, 83–91
- Damon, D. H., Lobb, R. R., D'Amore, P. A. and Wagner, J. A. (1989) *J. Cell Physiol.* **138**, 221–226
- Gordon, P. B., Choi, H. U., Conn, G., et al. (1989) *J. Cell. Physiol.* **140**, 584–592
- Klagsbrun, M. (1990) *Curr. Opin. Cell Biol.* **2**, 857–863
- López-Casillas, F., Cheifetz, S., Doody, J., Andres, J. L., Lane, W. S. and Massagué, J. (1991) *Cell* **67**, 785–795
- Kiefer, M. C., Stephens, J. C., Crawford, K., Okino, K. and Barr, P. J. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 6985–6989
- Rapraeger, A. C., Krufka, A. and Olwin, B. A. (1991) *Science* **252**, 1705–1708
- Sakaguchi, K., Yanagishita, M., Takeuchi, Y. and Aurbach, G. D. (1991) *J. Biol. Chem.* **266**, 7270–7278
- Vlodavsky, I., Bar-Shavit, R., Ishai-Michaeli, R., Bashkin, P. and Fuks, Z. (1991) *Trends Biochem. Sci.* **16**, 268–271
- Wang, X.-F., Lin, H. Y., Ng-Eaton, E., Downward, J., Lodish, H. F. and Weinberg, R. A. (1991) *Cell* **67**, 797–805
- Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P. and Ornitz, D. M. (1991) *Cell* **64**, 841–848
- Benitz, W. E., Kelley, R. T., Anderson, C. M., Lorant, D. E. and Bernfield, M. (1990) *Am. J. Respir. Cell Mol. Biol.* **2**, 13–24
- Castellot, J. J., Jr., Addonizio, M. L., Rosenberg, R. and Karnovsky, M. J. (1981) *J. Cell Biol.* **90**, 372–379
- Castellot, J. J., Jr., Wong, K., Herman, B. et al. (1985) *J. Cell. Physiol.* **124**, 13–20
- Au, Y. P. T., Kenagy, R. D. and Clowes, A. W. (1992) *J. Biol. Chem.* **267**, 3438–3444
- Busch, S. J., Martin, G. A., Barnhart, R. L., Mano, M., Cardin, A. D. and Jackson, R. L. (1992) *J. Cell. Biol.* **116**, 31–42
- Majack, R. A. and Borstein, P. (1985) *J. Cell Biol.* **100**, 613–619
- Pukac, L. A., Castellot, J. J., Jr., Wright, R. C., Jr., Caleb, B. L., and Karnovsky, M. J. (1990) *Cell Regul.* **1**, 435–443
- Pukac, L. A., Ottlinger, M. E. and Karnovsky, M. J. (1992) *J. Biol. Chem.* **267**, 3707–3711
- Bärzu, T., Molho, P., Tobelen, G., Petitou, M. and Caen, J. (1985) *Biochim. Biophys. Acta* **845**, 196–203
- Bärzu, T., Van Rijn, J. L. M. L., Petitou, M., Molho, P., Tobelen, G. and Caen, J. P. (1986) *Biochem. J.* **238**, 847–854
- Glimelius, B., Busch, C. and Höök, M. (1978) *Thromb. Res.* **12**, 773–782
- Vannucchi, S., Pasquali, F., Chiarugi, V. and Ruggiero, M. (1986) *Biochem. Biophys. Res. Commun.* **140**, 294–301
- Lyons-Giordano, B., Brinker, J. M. and Kefalides, N. A. (1990) *Exp. Cell Res.* **186**, 39–46
- Morrison, P. and Lowe-Krentz, L. J. (1989) *Exp. Cell Res.* **285**, 304–315
- Nader, H. B., Buonassisi, V., Colburn, P. and Dietrich, C. P. (1989) *J. Cell. Physiol.* **140**, 305–310
- Nader, H. B., Toma, L., Pinhal, M. A. S., Buonassisi, V., Colburn, P. and Dietrich, C. P. (1991) *Semin. Thromb. Hemost.* **17** (Suppl. 1), 47–56
- Fedarko, N. S. and Conrad, H. E. (1986) *J. Cell Biol.* **102**, 587–599
- Ishihara, M., Fedarko, N. S. and Conrad, H. E. (1986) *J. Biol. Chem.* **261**, 13575–13580
- Lowe-Krentz, L. J., Thompson, K. and Patton, W. A., II. (1992) *Mol. Cell. Biochem.* **109**, 51–60
- Imamura, T. and Mitsui, Y. (1987) *Exp. Cell Res.* **172**, 92–100
- Wight, T. N. (1985) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **44**, 381–385
- Kinsella, M. G. and Wight, T. N. (1986) *J. Cell Biol.* **102**, 679–687
- Robinson, J. and Gospodarowicz, D. (1983) *J. Cell. Physiol.* **117**, 368–376
- Robinson, J. and Gospodarowicz, D. (1984) *J. Biol. Chem.* **259**, 3818–3824
- Berenson, G. S., Radhakrishnamurthy, B., Srinivasan, S. R., Vijayagopal, P. and Daiferes, E. R., Jr. (1988) *Arch. Pathol. Lab. Med.* **112**, 1002–1010
- Stevens, R. L., Colombo, M., Gonzales, J. J., Hollander, W. and Schmid, K. (1976) *J. Clin. Invest.* **58**, 470–481

- 47 Völker, W., Schmidt, A., Oortmann, W., Broszey, T., Faber, V. and Buddecke, E. (1990) *Eur. Heart J.* **11** (Suppl. E), 29–40
- 48 Wight, T. N., Curwen, K. D., Litrenta, M. M., Alonso, D. R. and Minick, C. R. (1983) *Am. J. Pathol.* **113**, 156–164
- 49 Cohen, M. P. and Surma, M. L. (1984) *Diabetes* **33**, 8–12
- 50 Kanwar, Y. S., Rosenzweig, L. J., Linker, A. and Jakubowski, M. L. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2272–2275
- 51 Kjellén, L., Bielefeld, D. and Höök, M. (1983) *Diabetes* **32**, 337–342
- 52 Parthasarthy, N. and Spiro, R. G. (1982) *Diabetes* **31**, 738–741
- 53 Rohrbach, D. H., Hassell, J. R., Kleinman, H. K. and Martin, G. R. (1982) *Diabetes* **31**, 185–188
- 54 Lowe-Krentz, L. J. and Joyce, J. G. (1991) *Anal. Biochem.* **193**, 155–163
- 55 Harlow, E. and Lane, D. (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY
- 56 Stamatoglou, S. C. and Keller, J. M. (1983) *J. Cell Biol.* **96**, 1820–1823
- 57 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 58 Towbin, H., Staehelin and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4353
- 59 Raboudi, N., Julian, J., Rohde, L. H. and Carson, D. D. (1992) *J. Biol. Chem.* **267**, 11930–11939
- 60 Kjellén, L. and Lindahl, U. (1991) *Annu. Rev. Biochem.* **60**, 443–475
- 61 DeLisser, H. M., Yan, H. C., Newman, P. J., Muller, W. A., Buck, C. A. and Albelda, S. M. (1993) *J. Biol. Chem.* **268**, 16037–16046
- 62 Pirrotta, V. and Bickle, R. A. (1980) *Methods Enzymol.* **65**, 89–95
- 63 Albelda, S. M., Oliver, P. D., Romer, L. H. and Buck, C. A. (1990) *J. Cell Biol.* **110**, 1227–1237
- 64 Lisanti, M. P., Le Bivic, A., Sargiacomo, M. and Rodriguez-Boulton, E. (1989) *J. Cell Biol.* **109**, 2117–2127
- 65 Kojima, T., Shworak, N. W. and Rosenberg, R. D. (1992) *J. Biol. Chem.* **267**, 4870–4877
- 66 Mertens, G., Cassiman, J.-J., Van den Berghe, H., Vermylen, J. and David, G. (1992) *J. Biol. Chem.* **267**, 20435–20443