Eur. J. Biochem. *241*, 827–834 (1996)

Expression and characterization of human perlecan domains I and II synthesized by baculovirus-infected insect cells

Alexander J. A. GROFFEN[1], Carin A. F. BUSKENS[1], Karl TRYGGVASON[2], Jacques H. VEERKAMP[3], Leo A. H. MONNENS[1] and Lambert P. W. J. VAN DEN HEUVEL[1]

[1] Department of Pediatrics, University of Nijmegen, The Netherlands
[2] Department of Medical Biochemistry and Biophysics, Karolinska Institute, Stockholm, Sweden
[3] Department of Biochemistry, University of Nijmegen, The Netherlands

(Received 22 May/9 August 1996) – EJB 96 0752/2

We present the *in vitro* expression and purification of N-terminal fragments of human perlecan in insect cells. Three tailored fragments of human perlecan cDNA were introduced into the polyhedrin locus of baculovirus expression vectors (BEVs) encoding amino acids 1–196 (domain I), 1–404 (domain I+II^{a}) and 1–506 (domain I+II^{ab}). The integrity of the BEVs was checked by DNA sequencing, polymerase chain reaction, restriction enzyme analysis and Southern blotting. Northern hybridization and metabolic labeling with [^{35}S]methionine showed that expression of the perlecan-(1–404)- and the -(1–506)-peptide was successful, but in the case of the perlecan-(1–196)-peptide no recombinant protein was produced. Immunoblotting showed that both the (1–404)-peptide and (1–506)-peptide are recognized by 95J10, a monoclonal antibody that was previously raised against perlecan-(24–404)-peptide expressed in *Escherichia coli*. Gel permeation and anion-exchange chromatography were applied to purify the recombinant proteins. Glycosaminoglycans were demonstrated to be present. Deglycosylation with chondroitinase ABC showed that the perlecan-(1–404)-peptide was glycosylated with chondroitin sulfate residues. Consistent with these results, glycosaminoglycans isolated from the perlecan-(1–404)-peptide were identified as chondroitin sulfate by agarose gel electrophoresis. Furthermore the perlecan-(1–404)-peptide showed affinity to immobilized basic fibroblast growth factor. The availability of baculovirus-derived recombinant perlecan fragments will facilitate domain-specific investigation of the structural and functional properties of perlecan in the future.

Keywords: perlecan; structure; baculovirus expression; chondroitin sulfate; basic fibroblast-growth factor.

Heparan sulfate proteoglycans are essential components of cell surface and basement membranes serving a diversity of functions. Perlecan (HSPG2) is a large proteoglycan of basement membranes consisting of a 467-kDa core protein with three heparan sulfate chains attached to its N-terminal domain. The other domains share similarity with the low-density lipoprotein receptor (domain II), laminin α1, β1 and γ1 short arms (domain III), neural cell-adhesion molecule (domain IV) and the globular end of laminin α1 (domain V) [1–3]. The perlecan gene is mapped to the short arm of chromosome 1 and comprises over 120 kb, consisting of 94 exons [4, 5]. Consistent with its multidomain structure, the core protein of perlecan serves multiple functions. The proteoglycan is anchored within the basement membrane through binding to laminins (an interaction that is enhanced in presence of nidogen/entactin), fibronectin and collagen types IV and VI(α2) [6–8]. These interactions may increase the rigidity of the basement membrane and immobilize perlecan within the matrix. Up to date, little information is available regarding the functionality of the individual domains of the core protein. However, some insight into the roles of domain III and I has been gained by previous *in vitro* expression studies. Domain III of mouse perlecan was produced by HT1080 cells [9]. The recombinant protein binds to the cell surface through interaction of RGD peptide sequences with integrins [10]. Although this tripeptide is not conserved in human perlecan an alternative cell binding mechanism may exist. In another study, domain III of human perlecan was expressed successfully in *Escherichia coli*. A monoclonal antibody was raised and used to demonstrate the ubiquity of perlecan in human basement membranes [3]. Furthermore, expression of murine domain I in Chinese hamster ovary cells provided evidence for the location of the glycosaminoglycan attachment sites near the N-terminus of perlecan [11].

The presence of the strongly anionic heparan sulfate is essential for the charge-selective permeability of the glomerular basement membrane [12]. Heparan sulfate has also been shown to bind a wide range of growth factors like heparin-binding epidermal growth factor, interferon-γ, granulocyte-macrophage colony stimulating factor, interleukins IL-3, IL-8 and basic fibroblast-growth factor (bFGF) [13–16]. The biological activity of bFGF requires the presence of a specific oligosaccharide se-

Correspondence to L. P. W. J. van den Heuvel, Dept. of Pediatrics, University of Nijmegen, P.O. Box 9101, NL-6500 HB Nijmegen, The Netherlands

Abbreviations. AcNPV, *Autographa californica* nuclear polyhedrosis virus; BEV, baculovirus expression vector; bFGF, basic fibroblast growth factor; GBM, glomerular basement membrane; HSPG, heparan sulfate proteoglycan.

Enzymes. Peroxidase (EC 1.11.1.7); polynucleotide kinase (EC 2.7.1.78); DNA polymerase (EC 2.7.7.7); type II site-specific DNase (EC 3.1.21.4); proteinase K (EC 3.4.21.64); chondroitin ABC lyase (EC 4.2.2.4); DNA ligase (EC 6.5.1.1).

quence within heparan sulfate or heparin [14, 17]. Binding to this oligosaccharide is essential for the growth factor to trigger its cell surface receptor. It was demonstrated that bFGF activation *in vivo* is solely dependent on the heparan sulfate chains of perlecan [13].

Together with cell-surface heparan sulfate proteoglycans (HSPGs) of the vascular endothelium, perlecan also plays a role in the regulation of the coagulative response. Antithrombin III binding to heparan sulfate increases its anti-coagulant activity [18, 19]. Finally, perlecan may be involved in lipoprotein metabolism. Heparan sulfate chains are known to bind lipoprotein lipase, a key enzyme in lipoprotein metabolism [20, 21]. Regarding the strong similarity between domain II and the low-density lipoprotein receptor it can be speculated that the heparan sulfate residues, lipoprotein lipase, low-density lipoprotein and domain II might associate somehow into a large complex [22]. However, no experimental evidence has been obtained thus far to support this hypothesis. In the present study we have used recombinant baculovirus expression vectors (BEVs) to produce truncated perlecan fragments that are both glycosylated and expressed at high level. The baculovirus expression system utilizes the extremely strong polyhedrin promoter by substituting the polyhedrin gene for a cDNA sequence encoding the protein of interest [23, 24]. Many post-translational modifications are well described in insect cells, including O-glycosylation [25], N-glycosylation, palmitoylation and myristoylation. Although chondroitin sulfate and heparan sulfate proteoglycans have been observed in the fruit fly *Drosophila melanogaster* [26], no evidence is available yet for the correct synthesis of HSPGs during late stages of baculovirus infection.

MATERIALS AND METHODS

Materials. Vent polymerase, T4 polynucleotide kinase, *Xcm*I and *Dra*III endonuclease (New England Biolabs), *Bam*HI, *Not*I, *Pst*I endonuclease, T4 DNA ligase, bFGF, methionine-deficient Grace's medium, fetal bovine serum (Life Technologies), *Srf*I endonuclease, pCR-Script plasmid (Stratagene Cloning Systems), pVL1393 plasmid (Invitrogen), *Spodoptera frugiperda* SF21 cells, wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV) strain C6, transfection reagent, BacPAK6 linearized viral DNA (Clontech Laboratories), SDS-6B biotinylated marker, Brij35, TNM-FH insect culture medium (Sigma Chemical), RNAzol (Biotecx Laboratories), [^{35}S]methionine (ICN Biomedicals), [γ-^{32}P]ATP, Hybond N$^+$, photographic film (Amersham), EN$_3$HANCE (NEN), SDS (Fluka Chemie), PhastSystem, FPLC system, MonoQ, Superose 12 column, DEAE-Sepharose CL-4B (Pharmacia Biotech), secondary antibody conjugates (Dakopatts), Immobilon-P membrane (Millipore), MultiMark prestained marker (Novex) and chondroitinase ABC from *Proteus vulgaris* (Seikagaku).

Construction of transfer vectors. Three transfer vectors were constructed designated pVL1393-P1 (encoding amino acids 1−196 of human perlecan), pVL1393-P12^a (amino acids 1−404) and pVL1393-P12^{ab} (1−506). The initiation codon is supplied by the insert; the open reading frame is placed out of the original polyhedrin reading frame to avoid aberrant initiation. To introduce translation termination codons, mutagenic primers (Fig. 1) were used with clones Hpe2 and P5 [1] as template and Vent polymerase. The PCR products were inserted into the *Srf*I cloning site of pCR-Script according to the suppliers protocol. Transformants (prepared according to [27]) were screened to contain the unique *Bam*HI site at the 5′ orientation and the *Not*I site at the 3′ orientation from the cDNA fragments. The cDNA sequences were checked completely by sequencing

Product	Nucleotides	Primer set
1	41-628	atggggtggcgggcgccgg (forward)
		tcatgggaactggggcactgtgc (reverse)
2	543-1252	tcatctccagcggctctgt (forward)
		ttacatgcagccaaactcgtc (reverse)
3	985-1560	tgagctagactgtggccccc (forward)
		tcaggggcctgctcgttgtgg (reverse)

Fig. 1. Primers used for introduction of translation stop codons into sequences of human perlecan. All sequences are in 5′ to 3′ direction; underlined residues indicates mismatch in mutagenic primer. Nucleotide numbering corresponds to the cDNA sequence available from GenBank by accession number X62515 [1].

(ABI apparatus type 37717). The *Bam*HI-*Not*I fragment containing PCR product 1 (Fig. 1) was subcloned into pVL1393, yielding pVL1393-P1. The *Xcm*I−*Not*I fragment containing the major 3′ part of PCR product 2 (Fig. 1) was subcloned into pVL1393-P1, yielding pVL1393-P12^a. The *Dra*III−*Not*I fragment containing the major 3′ part of PCR product 3 (Fig. 1) was subcloned into pVL1393-P12^a, yielding pVL1393-P12^{ab}.

Tissue culture and isolation of recombinant baculoviruses. The general tissue culture methods were adapted from [23]. *Spodoptera frugiperda* SF21 cells were cultured at 27°C in TNM-FH medium supplemented with 10% fetal bovine serum. Except where stated, monolayer cultures were used. Recombinant viruses were derived from BacPAK6 by liposome-mediated cotransfection. The culture supernatant was harvested 4 days after cotransfection and individual plaques were obtained by a soft-agar overlay method. Well-isolated plaques were picked and left to diffuse overnight in 500 µl normal medium at 4°C. Pure plaques were propagated by infecting 5×10^5 SF21 cells in a 35-mm dish with 100 µl of the plaque pick and harvesting the medium 4 days after infection. For further propagation of baculovirus strains during a maximum of two passages, suspension cultures of 50 ml were inoculated with 5×10^5 cells/ml and incubated until logarithmic growth. Cells were then infected with a multiplicity of 0.1 and harvested 96 h post-infection, at which time the cell densities had reached a plateau of about 2×10^6 cells/ml. Cell densities were monitored in a hemocytometer (Bürker). Virus stocks were titrated by plaque assays. For expression of recombinant proteins, cells were infected at semi-confluence with a multiplicity of 5 and harvested at 50−60 h post-infection.

Genomic analysis of BEVs. Independently isolated baculovirus strains were analyzed for correct integration locus and purity. Baculovirus genomic DNA was isolated from non-occluded virions as described [28]. Shortly, the medium was cleared from cells (15 min at 300×*g*) and non-occluded virions were collected by ultracentrifugation for 1 h at 100000×*g*. The virions were incubated overnight at 37°C in presence of 0.1 mg/ml proteinase K and 1% SDS. The DNA was further purified following standard procedures [29] and stored at 4°C. After endonuclease *Pst*I digestion, 1 µg fragmented virus DNA was applied to each lane of a 0.35% agarose gel and separated overnight at 4°C. Southern blot transfer to a Hybond N$^+$ membrane was performed overnight by capillary force. The filter was hybridized at 42°C with an oligonucleotide (35 nucleotides) similar to exon 3 of human perlecan (domain I), and washed under medium stringent conditions (75 mM NaCl, 7.5 mM sodium citrate, 0.1% SDS, pH 7.0 at 42°C). For autoradiography, a photographic film was developed after 4 days exposure.

[^{35}S]Methionine incorporation and autoradiography. This procedure was adapted from [23]. A suspension culture of insect cells was grown to a cell density of 5×10^5 cells/ml and infected

with recombinant baculovirus with a multiplicity of 10. 48 h after infection, 1.5 ml cell suspension was centrifuged (5 min at 500×*g*, 24°C) and the cells were resuspended in 1.5 ml methionine-deficient Grace's medium. The cells were further incubated for 60 min; all incubations were at 27°C under frequent inversion. The cells were collected and incubated for 60 min in 1.5 ml methionine-deficient Grace's medium supplemented with 50 μCi/ml [^{35}S]methionine (specific activity >1000 Ci/mmol, Amersham Life Science). The labeling mixture was removed and cells were washed twice with NaCl/P_i (1.4 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4, pH 7.5). Finally, the cells were incubated for 90 min in 200 μl NaCl/P_i and centrifuged (5 min 500×*g*). The supernatant was stored at −80°C. Due to the host shut-off in the baculovirus infection cycle, the supernatant contains a limited set of extracellular proteins, synthesized at 50 h post-infection and labelled with [^{35}S]methionine. Proteins were analyzed by SDS/PAGE (12.5%) and processed for autoradiography with EN^{3}HANCE according to the manufacturer's instructions.

Size-exclusion and ion-exchange chromatography. For size-exclusion chromatography, a Superose 12 column (height 30 cm, V_t = 23.7 ml, V_0 = 7.6 ml as determined for blue dextran-2000) was calibrated with molecular mass marker proteins and contained 208 plates on average in the range of 14–440 kDa. Sample volume was 200 μl, flow rate 0.5 ml/min and elution was performed in NaCl/P_i + 0.01% Brij35 + 0.01% NaN_3.

For ion-exchange chromatography, sample volumes varying from 200 μl to 10 ml were applied to a MonoQ column with a bed volume of 1 ml. A linear gradient from 0 to 1 M NaCl in 10 mM Tris pH 6.8, 0.1% Zwittergent 3–12 and 0.01% NaN_3 was used for elution with a flow rate of 0.5 ml/min. Aliquots of each sample were dissolved in UltimaGold scintillation mix (Packard) and counted for 4 min in a liquid scintillation analyzer.

Chondroitinase ABC treatment. Samples were incubated with 0.5 U/ml chondroitinase ABC (protease-free) for 16 h at 37°C in 0.1 M Tris pH 8.0 containing 0.025% NaN_3. Control reactions were included containing 0.1 mg/ml chondroitin sulfate in the presence and absence of chondroitinase ABC. The effiency of the deglycosylation was measured by a 1,9-dimethylmethylene blue assay [30].

Immunological methods. For immunoblotting and ELISA procedures we followed standard methods [31] using 95J10, a perlecan-specific mAb that was previously raised in recombinant *E. coli* (unpublished results). In summary, recombinant perlecan-(24–404)-peptide was produced in the bacterial pGEX expression system [32] and treated with thrombin. The recombinant fragment was used for production of mAbs in mice according to previously described procedures [33]. Hybridoma cell lines were screened in ELISA against the recombinant fusion protein and in indirect immunofluorescence studies on human kidney cortex sections. The glomerular staining pattern observed with 95J10 matched the results obtained with the anti-perlecan mAb 7B5 [3]. Immunological detection was based on chemiluminescent (immunoblotting) or chromogenic (ELISA) reactions driven by horseradish peroxidase.

bFGF binding assay. Quantities of 24, 100, 250 and 500 ng bFGF and BSA were dissolved in 100 μl 50 mM sodium carbonate pH 9.6 and transferred into 96-well plates incubated overnight at 4°C for coating. After thorough washing, the wells were incubated with with 2.5 μg of the perlecan-(1–404)-peptide in NaCl/P_i containing 3% BSA, partially purified by ion-exchange chromatography (fractions eluted between 0.4–0.6 M NaCl from MonoQ). After thorough washing with NaCl/P_i, bound perlecan-(1–404)-peptide was detected as in the above described ELISA.

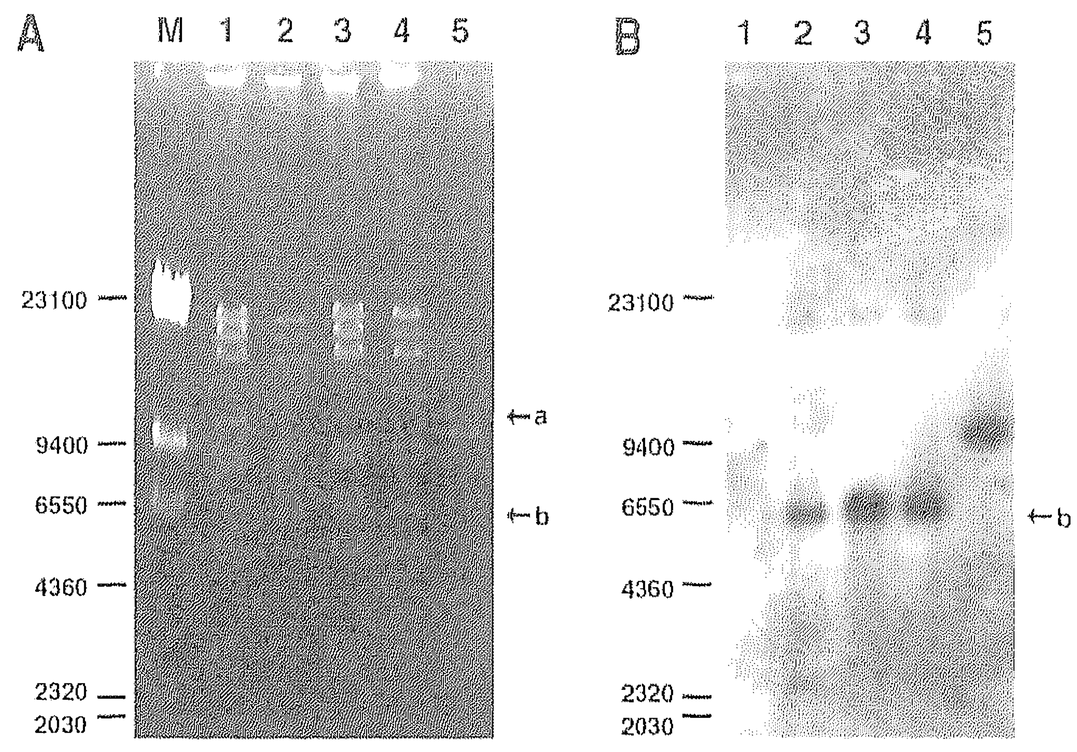

Fig. 2. (A) Restriction enzyme analysis of wild-type (lane 1) and recombinant (lanes 2–4) baculovirus strains and (B) Southern hybridization confirming the insertion of perlecan cDNA constructs into the polyhedrin locus of the baculovirus genome. Lane 1, wild-type AcNPV; lane 2, recombinant AcNPV encoding perlecan-(1–196)-peptide; lane 3, AcNPV encoding perlecan-(1–404)-peptide; lane 4, AcNPV encoding perlecan-(1–506)-peptide; lane 5, linearized pVL1393-P1 plasmid DNA as positive control for hybridization. Genomic DNA was digested with *Pst*I and fragments were separated by electrophoresis on a 0.35% agarose gel (A). The same gel was used for Southern blot transfer and hybridization with a domain-I-specific probe against human perlecan (B). Arrows indicate (a) wild-type *Pst*I-D fragment carrying the polyhedrin locus; (b) recombinant *Pst*I fragments carrying the inserted cDNA constructs.

Additional methods. Glycosaminoglycan electrophoresis was performed according to [34]. Protein concentrations were measured by the Bradford method with BSA as reference.

RESULTS

Isolation of recombinant baculovirus strains. To allow expression of perlecan fragments in *Spodoptera frugiperda* SF21 insect cells, three recombinant baculovirus strains were generated as follows. Truncated cDNA fragments were prepared encoding amino acids (1–196), (1–404) and (1–506) of human perlecan. The cDNA fragments were inserted into the transfer vector pVL1393. The integrity of the cDNA constructs was confirmed by restriction enzyme analysis and the complete coding regions and non-translated borders were verified by sequence analysis.

In the pVL1393-derived transfer vectors, the cDNA inserts are flanked by regions that are similar to AcNPV sequences surrounding the polyhedrin gene. These flanking sequences allow homologous recombination, resulting in substitution of the wild-type polyhedrin gene by the cDNA constructs of interest. To counterselect against non-recombinant virus replication, we employed a parental virus specially designed for this purpose, BacPAK6. A soft-agar overlay plaque assay yielded only polyhedrin-negative plaques. Well-isolated plaques were picked to isolate recombinant baculovirus strains. PCR amplification techniques were used for a primary screening of the virus strains (data not shown).

For more thorough investigation, the baculovirus isolates were propagated and genomic DNA (134 kb circular) was purified and digested with *Pst*I endonuclease (Fig. 2A). The *Pst*I digestion pattern can discriminate between different loci of integration because an additional restriction site is introduced jux-

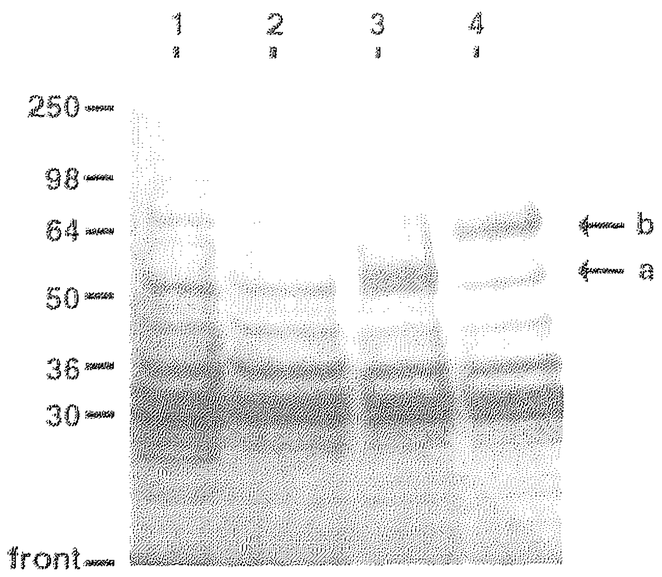

Fig. 3. Extracellular protein production by baculovirus-infected insect cells after metabolic labeling with [^{35}S]methionine. *Spodoptera frugiperda* SF21 cells were infected with wild-type AcNPV (lane 1), AcNPV encoding perlecan-(1–196)-peptide (lane 2), AcNPV encoding perlecan-(1–404)-peptide (lane 3) or AcNPV encoding perlecan-(1–506)-peptide (lane 4). Arrows a and b indicate perlecan-(1–404)-peptide and perlecan-(1–506)-peptide respectively. Molecular mass markers are indicated on the left (MultiMark prestained marker, Novex). At 49 h post-infection, cells were incubated for 60 min in the presence of [^{35}S]methionine, washed, and extracellular proteins were collected during 90 min. The supernatants were harvested and analyzed without further manipulation by SDS/PAGE (12.5%) and autoradiography.

taposing the inserted cDNA sequence. Consequently, the polyhedrin-containing *Pst*I fragment (arrow a in Fig. 2A) of wild-type AcNPV is further degraded into smaller products in recombinant strains (arrow b in Fig. 2A). The observed fragment sizes were in full concordance with the expected values, calculated from the nucleotide sequences of AcNPV (GenBank L22858) and of the introduced cDNA constructs. Furthermore, the presence of perlecan cDNA in these subfragments was demonstrated by Southern blotting with a probe similar to an internal fragment of domain I (arrow b in Fig. 2B). From these data we conclude that perlecan cDNA constructs encoding perlecan-(1–196)-, -(1–404)- and -(1–506)-peptide were successfully introduced into the correct *Pst*I fragment of the baculovirus genome.

Transcription and expression of perlecan fragments. To confirm the presence of recombinant mRNA, a northern hybridization was performed with a cDNA probe specific for domain I of human perlecan (data not shown). No mRNA was detected in wild-type cells whereas high levels were produced in recombinant baculovirus-infected SF21 cells. As discussed below, the size of the mRNA observed for perlecan-(1–196)-peptide was substantially larger than expected (approx. 4 kb). Transcription was observed at 36 h and 64 h post-infection but not at 0 or 12 h, which corresponds with the characteristics of late-phase baculovirus promoters.

Production of the recombinant perlecan peptides was studied by metabolic labeling with [^{35}S]methionine. Best results were obtained by labeling at 50 h post-infection, when only a limited set of virus-encoded proteins is secreted from baculovirus-infected cell cultures. The supernatant was harvested and analyzed without further manipulation by SDS/PAGE and autoradiography (Fig. 3). Comparison of recombinant and wild-type baculovirus-encoded proteins revealed the presence of recombinant perlecan-(1–404)-peptide and perlecan-(1–506)-peptide. In contrast, no additional band was visible in case of the perlecan-(1–196)-peptide. The perlecan-(1–404)- and -(1–506)-peptide migrate in SDS/PAGE as molecules of about 53 kDa and 62 kDa, respectively, as estimated by standard curve interpolation. Based on the primary amino acid sequences, molecular masses of the core proteins are predicted as 42 kDa and 53 kDa

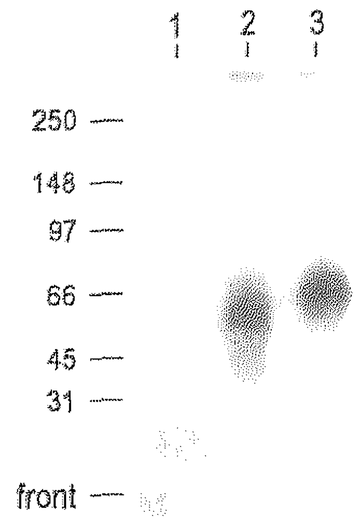

Fig. 4. Western blot detection of recombinant perlecan fragments. SF21 cells were infected with wild-type AcNPV (lane 1), AcNPV encoding perlecan-(1–404)-peptide (lane 2) or AcNPV encoding perlecan-(1–506)-peptide (lane 3). At 64 h post-infection, the supernatant was harvested and separated by SDS/PAGE on a 10–15% gradient gel (PhastSystem). After blot transfer, recombinant proteins were detected with the mAb 95J10. Marker proteins are indicated on the left (SDS-6B biotinylated marker and MultiMark prestained marker).

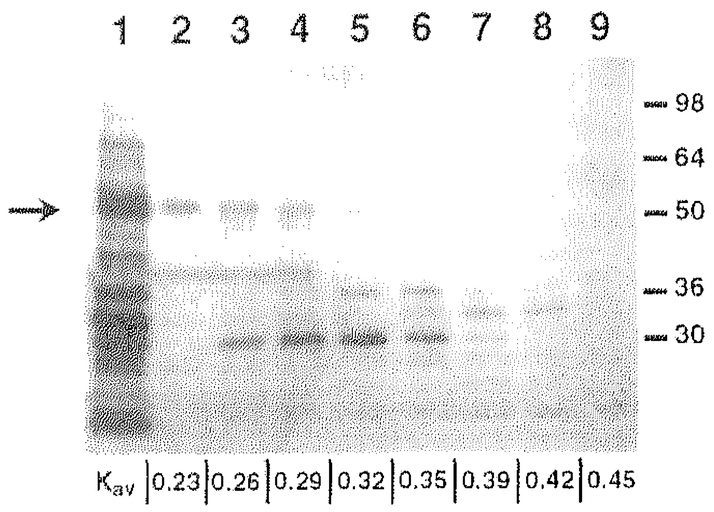

Fig. 5. Purification of the perlecan-(1–404)-peptide by size exclusion chromatography. Crude culture supernatant obtained after metabolic labeling with [^{35}S]methionine (lane 1) was applied onto a Superose 12 column under non-denaturing conditions. Individual fractions (lanes 2–9) were analyzed by 12.5% SDS/PAGE and autoradiography. K_{av} values are noted for each fraction. The arrow indicates the perlecan-(1–404)-peptide. Molecular mass markers are indicated on the right (MultiMark prestained marker).

respectively, taking removal of the signal peptides into account. To investigate the integrity of the two produced proteins, immunological studies were performed with the mAb 95J10 (raised against domain I and II^a of human perlecan expressed in *E. coli*). Western blot analysis showed that the perlecan-(1–404)- and -(1–506)-peptide are immunologically related to human perlecan (Fig. 4). As a control, the 95J10 epitope was not detected in insect cells infected with wild-type AcNPV (lane 1) or recombinant AcNPV encoding perlecan-(1–196)-peptide (not shown). Consistent results were found in ELISA (data not shown). The obtained yield was between 1–5 mg recombinant protein/10^9 cells.

Purification of recombinant perlecan fragments. [^{35}S]Methionine-labelled supernatants (Fig. 3, lane 3 and 4) were used to investigate the behaviour of the perlecan-(1–404)- and -(1–506)-peptide on chromatographic media. For size-exclusion chromatography, a Superose 12 column was utilized. Individual fractions were collected and aliquots were separated by SDS/PAGE. Radioactive proteins were visualized by autoradiography (Fig. 5). Both the perlecan-(1–404)- and -(1–506)-peptide (not shown) eluted at K_{av} = 0.23–0.29, whereas these bands did not appear in case of the perlecan-(1–196)-peptide or after infection with wild-type AcNPV (data not shown).

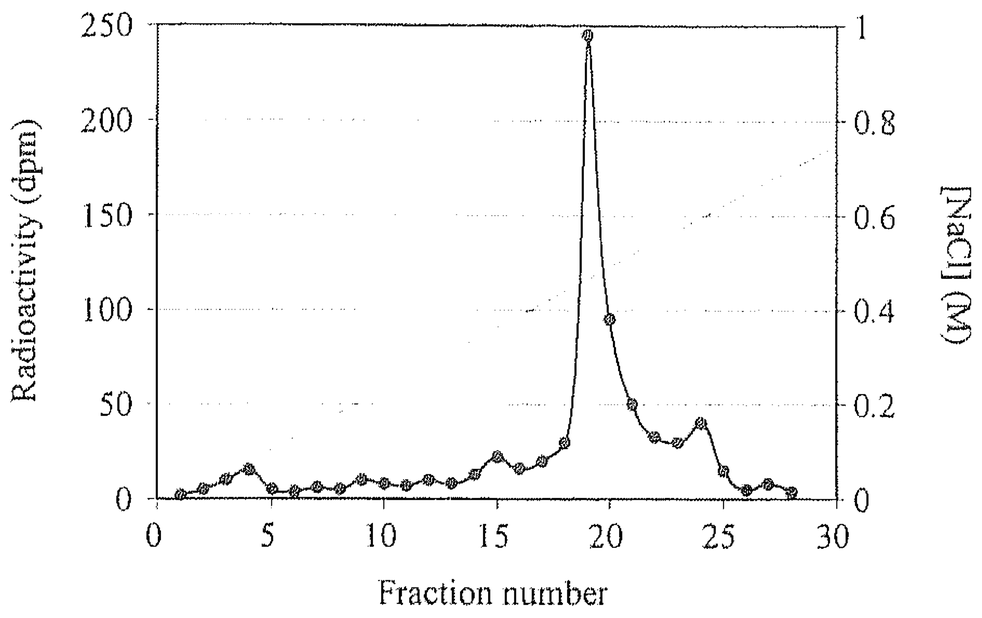

Fig. 6. Anion-exchange chromatography of perlecan-(1–404)-peptide. [^{35}S]Methionine-labelled perlecan-(1−404)-peptide, purified by size-exclusion chromatography as shown in Fig. 5 (lane 2), was applied onto a MonoQ anion-exchange column. The protein strongly bound to the matrix at pH 6.8 and eluted in a linear NaCl gradient at a concentration of 0.4−0.5 M.

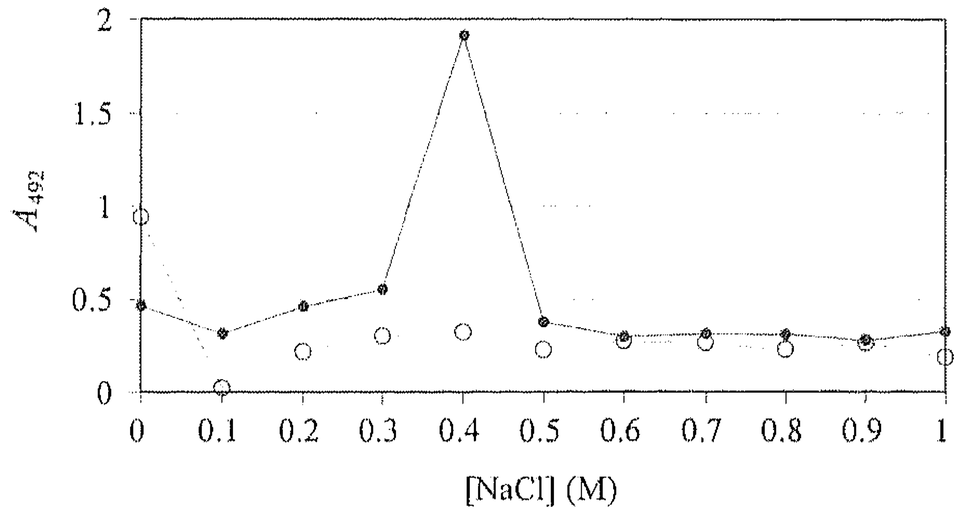

Fig. 7. Ion-exchange chromatography of intact and chondroitinase-ABC-digested perlecan-(1–404)-peptide. (●) Intact perlecan-(1−404)-peptide; (○) perlecan-(1−404)-peptide digested with protease-free chondroitinase ABC. The proteins were eluted from DEAE-Sepharose beads using a stepwize NaCl gradient. Aliquots of each fraction were analyzed *in duplo* by an ELISA using the perlecan-specific mAb 95J10. The relative perlecan-(1−404)-peptide concentrations were measured as absorbance at 492 nm.

An essentially pure fraction of [^{35}S]methionine-labelled perlecan-(1−404)-peptide obtained by gel permeation (Fig. 5, lane 2) was applied onto a MonoQ anion-exchange column to study its binding characteristics. The recombinant protein strongly bound to the matrix at pH 6.8. A linear salt gradient was used for elution and radioactivity of the fractions was quantified by liquid scintillation counting (Fig. 6). The protein eluted from the column at a concentration of 0.4−0.5 M NaCl.

A similar procedure was followed with non-radioactive perlecan-(1−404)-peptide, using immunological detection procedures (Fig. 7). In this case, the perlecan-(1−404)-peptide was incubated with DEAE-Sepharose (a matrix carrying the same active groups as MonoQ). After elution with a NaCl gradient increasing stepwise from 0 to 1.0 M, all fractions were analyzed by an ELISA procedure using 95J10. Consistent with the previous results, the majority of the protein was detected in the fraction containing 0.4 M NaCl.

Characterization of glycosaminoglycan residues. The binding characteristics of the perlecan-(1−404)-peptide to anion-exchange beads suggest that exposed negative charges are present on the molecule. We therefore studied the glycosylation characteristics of the perlecan-(1−404)-peptide produced by baculovirus-infected insect cells. After treatment with chondroitinase ABC (protease-free), its binding capacity to DEAE-Sepharose was abolished (Fig. 7) and the protein was found in the flow-through. Further evidence for the presence of chondroitin sulfate residues on the recombinant perlecan-(1−404)-peptide is shown in Fig. 8. Glycosaminoglycans were extracted from the perlecan-(1−404)-peptide by alkaline borohydride treatment and analyzed by agarose gel electrophoresis. A single band was visible, comigrating with chondroitin sulfate.

The above data suggest that the perlecan-(1−404)-peptide produced by recombinant AcNPV, is glycosylated with one or

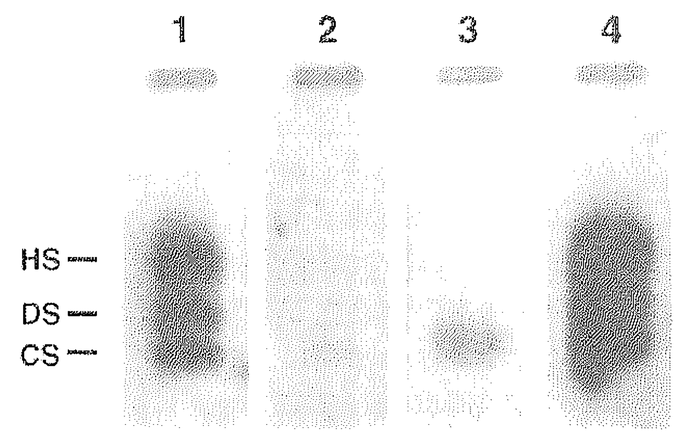

Fig. 8. Characterization of glycosaminoglycans extracted from recombinant perlecan-(1–404)-peptide. Lanes 1 and 4, marker glycosaminoglycans (5 and 20 ng, respectively) heparan sulfate (HS), dermatan sulfate (DS) and chondroitin sulfate (CS); lanes 2 and 3, glycosaminoglycans extracted from recombinant perlecan-(1−404)-peptide (fivefold diluted and undiluted, respectively). Glycosaminoglycans were separated by agarose gel electrophoresis and stained with combined Azure A/silver [34].

Table 1. The recombinant perlecan-(1–404)-peptide binds to immobilized bFGF. Increasing quantities of bFGF or BSA were coated on ELISA plates. Wells were incubated with supernatant from perlecan-(1−404)-peptide expressing cells, washed, and bound perlecan-(1−404)-peptide was measured by ELISA with mAb 95J10.

Amount coated	Peptide bound to	
	BSA	bFGF
ng	A_{492}	
0	0.225	0.225
24	0.226	0.202
100	0.225	0.639
250	0.225	1.690
500	0.226	3.254

Table 2. Free glycosaminoglycans compete for binding of perlecan-(1–404)-peptide to bFGF. A constant amount of bFGF (250 ng) was coated on ELISA plates and binding of perlecan-(1−404)-peptide was assayed in the presence of increasing concentrations of free heparan sulfate (HS) or chondroitin sulfate (CS).

Competitor concentration	Competitor type	
	HS	CS
μg/ml	A_{492}	
0	1.690	1.690
0.25	0.675	1.564
1	0.159	1.149
5	0.058	0.573
25	0.066	0.159
100	0.023	0.213

more chondroitin sulfate residues. The proteoglycan-like properties of the perlecan-(1−404)-peptide conform with its ability to bind bFGF (Table 1). This was demonstrated by coating increasing quantities of bFGF on ELISA plates, incubating with culture supernatant of baculovirus-infected insect cells, and determining the amount of immobilized perlecan-(1−404)-peptide in an ELISA-like procedure with mAb 95J10. This interaction between the perlecan-(1−404)-peptide and bFGF is inhibited in the presence of free glycosaminoglycans, heparan sulfate being a stronger competitor than chondroitin sulfate (Table 2). This is not surprising since bFGF has been reported to have a higher affinity for heparan sulfate than for chondroitin sulfate. The results indicate that the binding of the perlecan-(1−404)-peptide to immobilized bFGF is mediated by the glycosaminoglycan moieties of the recombinant proteoglycan.

DISCUSSION

No recombinant protein is synthesized by BEVs encoding the perlecan-(1–196)-peptide. The inability of AcNPV-infected insect cells to produce the recombinant perlecan-(1−196)-peptide is poorly understood. The possibility of a frame shift or point mutation in the cDNA construct was excluded by sequence analysis. Baculoviruses carrying the cDNA construct encoding the perlecan-(1−196)-peptide were convincingly shown to contain the insert correctly in the polyhedrin locus, and the size of the cDNA fragment was confirmed by PCR. These results clearly indicate that the recombinant virus offers an intact open reading frame. Transcriptional analysis by northern blotting showed that an unexpectedly long mRNA (approx. 4 kb) was present, induced between 12−36 h post-infection. However, no recombinant protein was produced.

One possible explanation for the deficiency in recombinant perlecan-(1−196)-peptide production is the inefficient use of the polyadenylation signal. In late phases of baculovirus infection, transcription is mediated by a virus-encoded RNA polymerase [35] and not all factors that influence termination of transcription by this enzyme are known [36]. For the AcNPV polyhedrin gene, multiple transcripts (1.2, 3.4 and 4.9 kb in size) have been observed with a common 5′ end [37, 38]. Therefore the elongated mRNA might result from read-through transcription, and we speculate that this may cause instability of the mRNA.

Another reason for the lack of perlecan-(1−196)-peptide expression could be the activity of an additional late-phase promoter positioned approximately 2 kb downstream of the polyhedrin coding sequence [37]. This promoter drives the transcription of a 3.2-kb mRNA in the opposite orientation, comprising the antisense strand of the inserted cDNA. Since the probe used for northern hybridization was double-stranded, the observed signal may represent the antisense transcript. In wild-type AcNPV, the level of this transcript is down-regulated by transcription from the polyhedrin promoter [37]. Possibly the consensus generated in this BEV influences the balance between sense and antisense transcription, in favour of the antisense product.

Successful expression and purification of perlecan-(1–404)- and -(1–506)-peptide. The recombinant perlecan-(1−404)- and -(1−506)-peptide, comprising domains I and II of human perlecan, were successfully synthesized in the baculovirus expression system. This was shown by metabolic labeling (Fig. 3) and immunological assays with the mAb 95J10 (Fig. 4). The signal peptide (originating from human perlecan cDNA) was recognized and the proteins were transported to the extracellular compartment. During electrophoresis on a 12.5% SDS/polyacrylamide gel, the [^{35}S]methionine-labelled recombinant proteins migrate as 53-kDa and 62-kDa proteins, respectively, which is 10 kDa larger than the calculated mass of their core proteins. As argued below, this difference can be explained by post-translational modifications, since the electrophoretic mobility of glycosylated proteins does not correlate to molecular mass in a linear logarithmic manner. In contrast, elution on a gel permeation column calibrated with globular marker proteins takes place at $K_{av} = 0.23-0.26$ which coincides with an apparent mass of 180−250 kDa. This discrepancy illustrates that molecular mass determinations based on relative mobility should be considered very carefully, especially when proteoglycans are involved. The yield of between 1−5 mg recombinant protein/10^9 cells is in agreement with the results obtained with other recombinant proteins [23].

Identification of glycosaminoglycans as chondroitin sulfate. The presence of chondroitin sulfate glycosaminoglycans on baculovirus-derived perlecan-(1−404)-peptide is supported by the following findings. Firstly, the perlecan-(1−404)-peptide strongly binds to the anion-exchange matrices DEAE-Sepharose CL-4B and MonoQ. The observed elution pattern resembles the characteristic behaviour of proteoglycans previously isolated from basement membranes [39, 40]. Secondly, the binding capacity of the perlecan-(1−404)-peptide to anion-exchange columns is abolished by chondroitinase ABC treatment. Furthermore, glycosaminoglycans isolated from the perlecan-(1−404)-peptide were convincingly identified as chondroitin sulfate (Fig. 8) by agarose gel electrophoresis. Finally, the perlecan-(1−404)-peptide binds to immobilized bFGF (Table 1). This interaction is inhibited in presence of free glycosaminoglycans (Table 2), suggesting that binding of the perlecan-(1−404)-peptide is mediated through its side chains.

Although the interaction between bFGF and the perlecan-(1−404)-peptide provides evidence for attachment of glycosaminoglycans, it can hardly be compared to the binding event that occurs in the basement membrane. The binding of bFGF *in vivo* is known to require specific oligosaccharide sequences that are present solely in heparan sulfate and heparin [13, 41], involving a dissociation constant of $10^{-8}-10^{-9}$ M [16]. In our ELISA-based binding assay the affinity must be lower, since free chondroitin sulfate acts as a potent competitor. We therefore conclude that baculovirus-derived perlecan-(1−404)-peptide binds to immobilized bFGF with relatively low affinity through its chondroitin sulfate chains.

The appearance of chondroitin sulfate residues on the perlecan-(1−404)-peptide is unexpected since the predominant *in vivo* form of perlecan carries heparan sulfate chains. Some efforts have been made to elucidate the factors influencing glycosylation of proteoglycans. The composition of glycosaminoglycan chains has been found to depend firstly on the amino acid sequence of the core protein [42]. The primary structure of perlecan domain I permits both attachment of heparan sulfate and chondroitin sulfate [11]. A second determinant for glycosylation is the cell type in which the proteoglycan is synthesized. Hybrid heparan/chondroitin sulfate forms of perlecan have been observed in Engelbreth-Holm-Swarm tumor cells [43], in cultured human glomerular visceral epithelial cells [44] and Chinese hamster ovary cells [11]. It is therefore comprehensible that the perlecan-(1−404)-peptide is expressed as a chondroitin sulfate proteoglycan. However, the exact determinants for this type of glycosylation remain unclear.

Application of recombinant fragments of human perlecan. Expression of perlecan fragments in the baculovirus system provides an important tool to study the functions of this proteogly-

can in a domain-specific manner. In the present study we described the expression and purification of perlecan-(1−404)- and -(1−506)-peptide, comprising the two N-terminal domains of perlecan. Given the spatial separation of the structural domains as visualised by electron microscopy [45], it is likely that the perlecan-(1−506)-peptide will have identical properties as the corresponding globulus in the whole perlecan molecule. The included domains are likely candidates to play a role in lipoprotein metabolism. Domain II of perlecan shows strong similarity with the ligand binding domain of the low-density lipoprotein receptor [1, 2]. A synergic action has been proposed for the combined binding of lipoprotein together with lipoprotein lipase, a complex that could be stabilized by a favourable configuration of heparan sulfate in the proximity of the low-density lipoprotein receptor-like domain [20, 22]. These and other hypothetical functions of the N-terminal core domains of perlecan will be assessed in the future.

The authors wish to acknowledge Elly M. M. Versteeg and Toin H. M. S. M. van Kuppevelt for glycosaminoglycan gel electrophoresis. This study was supported by grant C93.1309 of the Dutch Kidney Foundation. The laboratory participates in a concerted action *Alterations in extracellular matrix components in diabetic nephropathy and other glomerular diseases*, which is financially supported by the EC with the Biomed I program (BMH1-CT92-1766)

REFERENCES

1. Kallunki, P. & Tryggvason, K. (1992) Human basement membrane heparan sulfate proteoglycan core protein: a 467-kDa protein containing multiple domains resembling elements of the low density lipoprotein receptor, laminin, neural cell adhesion molecules, and epidermal growth factor, *J. Cell Biol. 116*, 559−571.
2. Murdoch, A. D., Dodge, G. R., Cohen, I., Tuan, R. S. & Iozzo, R. V. (1992) Primary structure of the human heparan sulfate proteoglycan from basement membrane (HSPG2/perlecan). A chimeric molecule with multiple domains homologous to the low density lipoprotein receptor, laminin, neural cell adhesion molecules, and epidermal growth factor, *J. Biol. Chem. 267*, 8544−8557.
3. Murdoch, A. D., Liu, B., Schwarting, R., Tuan, R. S. & Iozzo, R. V. (1994) Widespread expression of perlecan proteoglycan in basement membranes and extracellular matrices of human tissues as detected by a novel monoclonal antibody against domain III and by in situ hybridization, *J. Histochem. Cytochem. 42*, 239−249.
4. Dodge, G. R., Kovalszky, I., Chu, M. L., Hassell, J. R., McBride, O. W., Yi, H. F. & Iozzo, R. V. (1991) Heparan sulfate proteoglycan of human colon: partial molecular cloning, cellular expression, and mapping of the gene (HSPG2) to the short arm of human chromosome 1, *Genomics 10*, 673−680.
5. Cohen, I. R., Grassel, S., Murdoch, A. D. & Iozzo, R. V. (1993) Structural characterization of the complete human perlecan gene and its promoter, *Proc. Natl Acad. Sci. USA 90*, 10404−10408.
6. Battaglia, C., Mayer, U., Aumailley, M. & Timpl, R. (1992) Basement-membrane heparan sulfate proteoglycan binds to laminin by its heparan sulfate chains and to nidogen by sites in the protein core, *Eur. J. Biochem. 208*, 359−366.
7. Heremans, A., de Cock, B., Cassiman, J. J., Van den Berghe, H. & David, G. (1990) The core protein of the matrix-associated heparan sulfate proteoglycan binds to fibronectin, *J. Biol. Chem. 265*, 8716−8724.
8. Laurie, G. W., Bing, J. T., Kleinman, H. K., Hassell, J. R., Aumailley, M., Martin, G. R. & Feldmann, R. J. (1986) Localization of binding sites for laminin, heparan sulfate proteoglycan and fibronectin on basement membrane (type IV) collagen, *J. Mol. Biol. 189*, 205−216.
9. Chakravarti, S., Horchar, T., Jefferson, B., Laurie, G. W. & Hassell, J. R. (1995) Recombinant domain III of perlecan promotes cell attachment through its RGDS sequence, *J. Biol. Chem. 270*, 404−409.
10. Hayashi, K., Madri, J. A. & Yurchenco, P. D. (1992) Endothelial cells interact with the core protein of basement membrane perlecan through beta 1 and beta 3 integrins: an adhesion modulated by glycosaminoglycan, *J. Cell Biol. 119*, 945−959.
11. Kokenyesi, R. & Silbert, J. E. (1995) Formation of heparan sulfate or chondroitin/dermatan sulfate on recombinant domain I of mouse perlecan expressed in Chinese hamster ovary cells, *Biochem. Biophys. Res. Commun. 211*, 262−267.
12. Kanwar, Y. S., Linker, A. & Farquhar, M. G. (1980) Increased permeability of the glomerular basement membrane to ferritin after removal of glycosaminoglycans (heparan sulfate) by enzyme digestion, *J. Cell. Biol. 86*, 688−693.
13. Aviezer, D., Hecht, D., Safran, M., Eisinger, M., David, G. & Yayon, A. (1994) Perlecan, basal lamina proteoglycan, promotes basic fibroblast growth factor-receptor binding, mitogenesis, and angiogenesis, *Cell 79*, 1005−1013.
14. Gallagher, J. T. (1994) Heparan sulphates as membrane receptors for the fibroblast growth factors, *Eur. J. Clin. Chem. Clin. Biochem. 32*, 239−247.
15. Schlessinger, J., Lax, I. & Lemmon, M. (1995) Regulation of growth factor activation by proteoglycans: what is the role of the low affinity receptors? *Cell 83*, 357−360.
16. Guillonneau, X., Tassin, J., Berrou, E., Bryckaert, M., Courtois, Y. & Mascarelli, F. (1996) In vitro changes in plasma membrane heparan sulfate proteoglycans and in perlecan expression participate in the regulation of fibroblast growth factor 2 mitogenic activity, *J. Cell. Physiol. 166*, 170−187.
17. Turnbull, J. E., Fernig, D. G., Ke, Y., Wilkinson, M. C. & Gallagher, J. T. (1992) Identification of the basic fibroblast growth factor binding sequence in fibroblast heparan sulfate, *J. Biol. Chem. 267*, 10337−10341.
18. Pejler, G., Backstrom, G., Lindahl, U., Paulsson, M., Dziadek, M., Fujiwara, S. & Timpl, R. (1987) Structure and affinity for antithrombin of heparan sulfate chains derived from basement membrane proteoglycans, *J. Biol. Chem. 262*, 5036−5043.
19. Mertens, G., Cassiman, J. J., Van den Berghe, H., Vermylen, J. & David, G. (1992) Cell surface heparan sulfate proteoglycans from human vascular endothelial cells. Core protein characterization and antithrombin III binding properties, *J. Biol. Chem. 267*, 20435−20443.
20. Eisenberg, S., Sehayek, E., Olivecrona, T. & Vlodavsky, I. (1992) Lipoprotein lipase enhances binding of lipoproteins to heparan sulfate on cell surfaces and extracellular matrix, *J. Clin. Invest. 90*, 2013−2021.
21. Olivecrona, G. & Olivecrona, T. (1995) Triglyceride lipases and atherosclerosis, *Curr. Opin. Lipidol. 6*, 291−305.
22. Iozzo, R. V. (1994) Perlecan: a gem of a proteoglycan, *Matrix. Biol. 14*, 203−208.
23. O'Reilly, D. R., Miller, L. K. & Luckow, V. A. (1992) *Baculovirus expression vectors: a laboratory manual*, Freeman and Company, New York.
24. Miller, L. K. (1993) Baculoviruses: high-level expression in insect cells, *Curr. Opin. Genet. Dev. 3*, 97−101.
25. Thomsen, D. R., Post, L. E. & Elhammer, A. P. (1990) Structure of O-glycosidically linked oligosaccharides synthesized by the insect cell line Sf9, *J. Cell Biochem. 43*, 67−79.
26. Graner, M., Stupka, K. & Karr, T. L. (1994) Biochemical and cytological characterization of DROP-1: a widely distributed proteoglycan in Drosophila, *Insect. Biochem. Mol. Biol. 24*, 557−567.
27. Inoue, H., Nojima, H. & Okayama, H. (1990) High efficiency transformation of Escherichia coli with plasmids, *Gene 96*, 23−28.
28. Smith, G. E. & Summers, M. D. (1978) Analysis of baculovirus genomes with restriction endonucleases, *Virology 89*, 517−527.
29. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular cloning, a laboratory manual*, 2nd edn, Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY.
30. Reubsaet, F. A., Langeveld, J. P. & Veerkamp, J. H. (1985) Glycosaminoglycan content of glomerular and tubular basement membranes of various mammalian species, *Biochim. Biophys. Acta 838*, 144−150.
31. Harlow, E. & Lane, D. (1988) *Antibodies, a laboratory manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY.

32. Smith, D. B. & Johnson, K. S. (1988) Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione-S-transferase, *Gene 67*, 31–40.
33. Van den Heuvel, L. P., Van den Born, J., Veerkamp, J. H., Van de Velden, T. J., Schenkels, L., Monnens, L. A., Schröder, C. H. & Berden, J. H. (1990) Heparan sulfate proteoglycan from human tubular basement membrane. Comparison with this component from the glomerular basement membrane, *Biochim. Biophys. Acta 1025*, 67–76.
34. Van de Lest, C. H., Versteeg, E. M., Veerkamp, J. H. & Van Kuppevelt, T. H. (1994) Quantification and characterization of glycosaminoglycans at the nanogram level by a combined azure A-silver staining in agarose gels, *Anal. Biochem. 221*, 356–361.
35. Huh, N. E. & Weaver, R. F. (1990) Identifying the RNA polymerases that synthesize specific transcripts of the *Autographa californica* nuclear polyhedrosis virus, *J. Gen. Virol. 71*, 195–201.
36. Westwood, J. A., Jones, I. M. & Bishop, D. H. (1993) Analyses of alternative poly(A) signals for use in baculovirus expression vectors, *Virology 195*, 90–99.
37. Ooi, B. G. & Miller, L. K. (1990) Transcription of the baculovirus polyhedrin gene reduces the levels of an antisense transcript initiated downstream, *J. Virol. 64*, 3126–3129.
38. Pham, D. Q. & Sivasubramanian, N. (1992) *In vivo* transcriptional analysys of three baculovirus genes: evidence of homology between viral and host transcripts, *Virology 190*, 288–297.
39. Van den Heuvel, L. P., Van den Born, J., Van de Velden, T. J., Veerkamp, J. H., Monnens, L. A., Schroder, C. H. & Berden, J. H. (1989) Isolation and partial characterization of heparan sulfate proteoglycan from the human glomerular basement membrane, *Biochem. J. 264*, 457–465.
40. Van de Lest, C. H., Versteeg, E. M., Veerkamp, J. H. & Van Kuppevelt, T. H. (1995) Digestion of proteoglycans in porcine pancreatic elastase-induced emphysema in rats, *Eur. Respir. J. 8*, 238–245.
41. Aviezer, D., Levy, E., Safran, M., Svahn, C., Buddecke, E., Schmidt, A., David, G., Vlodavsky, I. & Yayon, A. (1994) Differential structural requirements of heparin and heparan sulfate proteoglycans that promote binding of basic fibroblast growth factor to its receptor, *J. Biol. Chem. 269*, 114–121.
42. Zhang, L., David, G. & Esko, J. D. (1995) Repetitive Ser-Gly sequences enhance heparan sulfate assembly in proteoglycans, *J. Biol. Chem. 270*, 27127–27135.
43. Danielson, K. G., Martinez Hernandez, A., Hassell, J. R. & Iozzo, R. V. (1992) Establishment of a cell line from the EHS tumor: biosynthesis of basement membrane constituents and characterization of a hybrid proteoglycan containing heparan and chondroitin sulfate chains, *Matrix 12*, 22–35.
44. Van Det, N. F., Van den Born, J., Tamsma, J. T., Verhagen, N. A., Van den Heuvel, L. P., Berden, J. H., Bruijn, J. A., Daha, M. R. & Van der Woude, F. J. (1995) Proteoglycan production by human glomerular visceral epithelial cells and mesangial cells *in vitro*, *Biochem. J. 307*, 759–768.
45. Paulsson, M., Yurchenco, P. D., Ruben, G. C., Engel, J. & Timpl, R. (1987) Structure of low density heparan sulfate proteoglycan isolated from a mouse tumor basement membrane, *J. Mol. Biol. 197*, 297–313.