Structural domains of heparan sulphate for specific recognition of the C-terminal heparin-binding domain of human plasma fibronectin (HEPII)

Andrew WALKER and John T. GALLAGHER*

CRC Department of Medical Oncology, Christie Hospital NHS Trust, Manchester M20 9BX, U.K.

Heparan sulphate (HS) is an abundant polysaccharide component of the pericellular domain and is found in most soft tissues and all adherent cells in culture. It interacts with a wide spectrum of proteins including polypeptide growth factors and glycoproteins of the extracellular matrix. These interactions might influence fundamental cellular activities such as adhesion, growth and migration. HS might therefore represent a highly adaptive mechanism by which cells respond to their environment. The present study shows that the interaction between fibroblast HS, metabolically labelled with [^{3}H]glucosamine, and the C-terminal heparin-binding domain of human plasma fibronectin (HEPII), is determined by distinct regions of the polysaccharide chain. By using a very sensitive affinity-chromatography method and specific polysaccharide scission it was shown that the HEPII-binding regions of HS reside within sulphated domains that are resistant to degradation by heparinase III. In addition, optimal binding was achieved with specific heparinase III-resistant fragments of 14–16 monosaccharides in length. The affinity of HS for HEPII was significantly decreased when the polysaccharide was cleaved with heparinase I. Chondroitin sulphate and dermatan sulphate were poor competitive inhibitors of [^{3}H]HS binding to HEPII whereas unlabelled HS and heparin gave a strong inhibitory activity, with heparin being the most potent inhibitor. These findings suggest that the interaction between HEPII and HS is specific and requires extended sequences of seven to eight N-sulphated disaccharides in which a proportion of the iduronate residues are sulphated at C-2. The results have important implications for the functions of HS in cell adhesion and migration.

INTRODUCTION

Fibronectin (FN) is produced by many different cell types and can be secreted as a soluble disulphide-linked dimer (molecular mass 500 kDa) or incorporated directly into the extracellular matrix (ECM) as fibrillar aggregates with extremely high molecular masses [1,2]. FN participates in the adhesion, spreading and migration of cells, opsonization, wound healing, thrombostasis, platelet adhesion and the maintenance of normal cell morphology. This diversity of functions is explained in part by the modular structure of the subunits. These can be released by mild proteolysis to yield fragments or domains that retain their ligand-binding properties. Thus FN has been shown to contain domains that specifically interact with components of the cell surface and the ECM. FN must interact with the surface of cells in order to be assimilated into the ECM [3] and to function in many of the activities described above.

Two classes of cell-surface receptor for FN have been described [1,4]. The dimeric, transmembrane integrins form one of these classes and recognize specific peptide sequences within the FN structure. Studies have shown that the recognition of these sequences by the integrins might be highly specific [5] and five independent integrin-binding sites within the FN monomer have been identified [1,4,6]. The second class of cell-surface receptor for FN is represented by the glycosaminoglycan (GAG) heparan sulphate (HS). HS is covalently attached to specific classes of protein to form proteoglycans that are abundant components of most soft tissues and organs *in vivo* and all adherent cells *in vitro*. At least two domains within each FN monomer bind to HS and the chemically related GAG heparin [4]. One of these sites (HEPI), located at the N-terminus, is composed of so-called type I repeat units and can be released by the action of thermolysin to yield a 24 kDa domain [7]. The interaction between heparin and HEPI is considered to be relatively weak, with measurable dissociation constants in the range 10^{-6} to 10^{-5} M [8,9]. This interaction might be inhibited by 0.2 M NaCl [10] or physiological concentrations of Ca^{2+} but not other divalent cations [11].

The other major heparin-binding site of FN (HEPII) is strategically located towards the C-terminus between the RGD-bearing cell-binding site and the variably spliced domain V or IIICS. The estimated molecular mass of the isolated HEPII domain after enzymic cleavage of the intact FN molecule varies from 29 to 38 kDa depending on the conditions and type of protease used [6,8,12–14]. The affinity of HEPII for heparin is the highest seen of the FN-derived heparin-binding fragments, with measurable dissociation constants between 10^{-8} and 10^{-7} M [8,9]. Consequently this fragment binds relatively strongly to heparin–Sepharose and requires NaCl concentrations of 0.3 M [15] or 0.5 M [10] for efficient elution.

Studies to identify clusters of basically charged amino acids within the HEPII domain that may mediate heparin binding have indicated the presence of at least four independent sites located in three type III repeat units (numbers 12–14) [16–18]. However, when the HEPII–heparin interaction was studied in solution, changes in fluorescence polarization suggested a model with only two heparin-binding sites in each HEPII domain [19]. Interestingly, none of the proposed heparin-binding sites overlap with the integrin-binding site identified in the type III repeat by Mould and Humphries [6]. Two main heparin-binding peptides,

Abbreviations used: bFGF, basic fibroblast growth factor; BKHS, bovine kidney heparan sulphate; CS, chondroitin sulphate; dp, degree of polymerization (i.e. for disaccharide dp = 2); ECM, extracellular matrix; FN, fibronectin; GAG, glycosaminoglycan; $GlcNSO_3$(6S), N-sulphate glucosamine 6-sulphate; HEPI, N-terminal heparin-binding domain of human plasma fibronectin; HEPII, C-terminal heparin-binding domain of human plasma fibronectin; HS, heparan sulphate; DS, dermatan sulphate; IdoA, iduronic acid; IdoA(2S), iduronic acid 2-sulphate; pFN, plasma fibronectin; PKC, protein kinase C.

* To whom correspondence should be addressed.

based on sequences in the type III repeat 14, seem to mediate melanoma cell adhesion [14,17]. Furthermore the most potent of these, KNNQKSEPLIGRKKT (termed FN-C/H II), supported the adhesion of a neuroblastoma cell line by an HS-dependent mechanism and also acted in a synergistic manner with the alternatively spliced CS1 peptide of the V domain to promote neurite outgrowth [20].

These studies suggest that specific sequences within the HEPII domain interact with HS and that these might be important for cell interactions with fibronectin. However, there have been few investigations to determine the structural features of HS that interact with this domain of FN. Analyses of HS structure have shown that the repeating disaccharide of *N*-acetylglucosamine and glucuronic acid (GlcNAcα1,4GlcA) is subjected to extensive modification after its initial synthesis and before its expression at the cell surface [21,22]. These modifications include the deacetylation and N-sulphation of GlcNAc, the epimerization of the glucuronic acid to form iduronic acid (IdoA) and finally specific substitution with ester (*O*)-sulphate groups, principally in regions enriched in N-sulphation [23]. In heparin these modifications are very extensive and the polymer has a high and relatively uniform concentration of sulphate groups (approx. 2.4 sulphates per disaccharide), whereas in HS the modifications are restricted and sulphation is targeted to particular regions or 'domains' of the polymer chain. These highly sulphated regions are separated by predominantly unmodified sequences with a low sulphate content [24,25].

The sulphated regions of HS are primarily responsible for its ligand-binding properties [23]. As these are potentially extremely heterogeneous, it follows that modulation of HS structure might represent a mechanism by which cells can respond to FN and other HS-binding components of their pericellular environment. Several studies have suggested that HS and the less commonly occurring GAG heparin are heterogeneous with regard to their ability to bind to either intact FN or the purified HEPII domain [26,27]. Such heterogeneity could be indicative of the presence of specific structures within HS with a high affinity for FN or HEPII. However, detailed analyses of the sequences of HS that mediate this interaction remain unclear. In this study we describe some of the structural features of HS that seem to be important for binding to HEPII.

EXPERIMENTAL

Materials

Human plasma FN (pFN) was purchased from the National Blood Products Laboratory (Elstree, Herts., U.K.). It was isolated from the glycine saline supernatant (intermediate fraction from factor VIII purification process) of expired plasma by gelatin–Sepharose affinity chromatography. Purity by gel filtration was more than 98%, and analysis by PAGE gave a single species. A HEPI fraction of human pFN (referred to as 'HEPI standard'), prepared by the method of Zardi et al. [28] was a kind gift from Professor M. J. Humphries (University of Manchester, Manchester, U.K.). Heparin–Sepharose 6B and CNBr-activated Sepharose 4B were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Bovine kidney HS (BKHS), pig intestinal mucosa heparin, shark cartilage mixed isomer chondroitin sulphate (CS) and thermolysin (protease type X) were supplied by Sigma (Poole, Dorset, U.K.). [^{3}H]Heparin was purchased from New England Nuclear (Boston, MA, U.S.A.). Affi-Gel 10 and BioGel P-10 were supplied by Bio-Rad Laboratories (Richmond, CA, U.S.A.). A mouse IgG_1 monoclonal antibody (MAS 509) raised against the HEPII domain of human plasma FN [29,30] was obtained from Sera-Lab (Crawley Down, Sussex, U.K.). Dermatan sulphate (DS) derived from adult mouse skin was kindly provided by Dr. D. Lane (Department of Haematology, Charing Cross and Westminster Medical School, London, U.K.). Pig mucosal HS was a gift from Organon (Oss, The Netherlands). Heparinases I, II and III were purchased from Seikagaku Kogyo (Tokyo, Japan). Heparinase I and heparinase III were also obtained from Sigma or Grampian Enzymes (Aberdeen, Scotland).

Preparation and depolymerization of HS

Biosynthetically radiolabelled HS was obtained from human skin fibroblasts as previously described [31]. BKHS was depolymerized with heparinase I or heparinase III at a concentration of 0.1 m-units/ml in 0.1 mM calcium acetate, 1.0 mM sodium acetate, 100 μg/ml BSA, pH 7.0, for 8 h at 37 °C. Digestions were monitored by measuring the increase in absorbance at 232 nm and were judged to be complete when no further increase was detected. Enzymes were neutralized by heating the samples to 95 °C for 5 min. Aliquots of depolymerized BKHS were diluted in 0.05 M NaCl, 50 mM Tris/HCl, pH 6.0 (equilibration buffer) to give stock solutions over the range 1.0–1000 μg/ml.

Radiolabelled, fibroblast-derived HS was depolymerized with heparinase III as described above and the resulting oligosaccharides were fractionated by gel filtration on a Bio-Gel P-10 column (1.0 cm × 140 cm) in 0.2 M NH_4HCO_3 at a flow rate of 4.0 ml/h. The oligosaccharides were separated into distinct peaks ranging in size from disaccharides [degree of polymerization 2 (dp2)] to dp18, with a further pool representing oligosaccharides of dp20 and higher [31,32]. The isolated oligosaccharides were freeze-dried and resuspended in equilibration buffer before HEPII binding analyses.

Preparation of HEPII domain from pFN

Human pFN (1.0 mg/ml) was digested for 4 h at room temperature with 10 μg/ml thermolysin in 25 mM Tris/HCl, 0.5 mM EDTA, 50 mM NaCl, 2.5 mM $CaCl_2$, pH 7.6, as described by Zardi et al. [28]. Thermolysin activity was inhibited by the addition of EDTA to a final concentration of 5 mM. Digested samples of pFN were pumped on to a heparin–Sepharose affinity column (2.0 cm × 0.5 cm) at 6.0 ml/h in PBS. After a 10 ml wash, bound material was eluted with a linear NaCl gradient (0.15–0.5 M in 20 mM sodium phosphate, pH 7.2). Gradients were monitored by the measurement of electrical conductance. Fractions (50 × 1.0 ml) were collected and the protein concentration of each was determined by measuring the absorbance at 280 nm. Three peaks were normally detected, being eluted with peak maxima of 0.2 M (fraction I), 0.25 M (fraction II) and 0.32 M (fraction III) NaCl. The HEPI standard was eluted at 0.25 M. Analysis of these fractions by SDS/PAGE showed that fraction I contained three species with apparent molecular masses between 14 and 24 kDa and fraction II contained a major component of 24 kDa that co-migrated with the HEPI standard. Fraction III contained two components of molecular masses 27 and 47 kDa. Repeated digestion and refractionation of this pool enriched the 27 kDa domain with subsequent loss of the 47 kDa species. Apparently homogeneous preparations of the 27 kDa high-affinity fraction stained positively by Western blotting with the specific monoclonal antibody, MAS 509, which recognizes the HEPII domain from human pFN. Importantly the antibody did not react with the lower-affinity fractions isolated from pFN by heparin–Sepharose (results not shown).

Purified HEPII preparations were desalted and concentrated to approx. 1.0 ml in an Amicon Centricon-10 cartridge (Amicon,

Gloucester, U.K.). The protein content of each preparation was determined with a Pierce Coomassie protein assay kit (Pierce, Rockford, IL, U.S.A.) before storage at −20 °C.

Construction of affinity columns

Pre-swollen Affi-Gel 10 was activated by washing with 100 volumes of 20 mM sodium phosphate, 80 mM NaCl, pH 5.5, on a sintered glass pad under vacuum. The activated gel was then combined with a solution of the protein to be coupled (200 μg in total) in 0.5 ml of 80 mM NaCl, 20 mM sodium phosphate, pH 5.5, and agitated at 4 °C for 2 h. Heparin (from pig mucosa) that had been treated with acetic anhydride to acetylate any free amino groups [33] was included in the reaction at a concentration of 1.0 mg/ml to prevent coupling of the protein through the GAG binding site. Any excess active groups present on the matrix were blocked by the addition of 10.0 mg/ml BSA and left for a further 16 h. The affinity gel was poured into a 0.5 cm diameter column to give a bed volume of 0.5-1.0 ml and this was washed with 50 ml of 20 mM Tris/HCl, 1.0 M NaCl, pH 6.0, to remove non-adsorbed protein before use.

Coupling efficiencies were determined by measuring the protein content of a 0.5 ml aliquot of the supernatant before the addition of the BSA and were typically found to be higher than 75%.

Affi-Gel 10 was chosen for these studies after carefully controlled experiments were performed to determine the suitability of a variety of affinity matrix supports. CNBr-activated Sepharose equilibrated in physiological saline bound to HS, heparin, CS and hyaluronic acid and was deemed unsuitable for the present studies.

Gradient and step elution of GAGs from affinity columns

Samples of purified radiolabelled GAG in a maximum volume of 0.5 ml of equilibration buffer (0.05 M NaCl, 50 mM Tris/HCl, pH 6.0) were applied to the affinity column under gravity and left at room temperature for 15 min. The eluate was collected and recycled through the column five times to give maximum binding of GAG. After a 10 ml wash with equilibration buffer, bound material was eluted with a linear gradient (0.05–0.5 M NaCl in 50 mM Tris/HCl, pH 6.0) at a flow rate of 6.0 ml/h and 50 1.0 ml fractions were collected. The formation of gradients was monitored by electrical conductance and aliquots of each fraction were monitored for radiolabel content by liquid-scintillation counting.

Alternatively, GAG samples applied to the columns and washed as described above could be eluted in a stepwise manner. In such cases, 1.0 ml aliquots of NaCl solutions in 50 mM Tris/HCl, pH 6.0, were applied to the column and 1.0 ml fractions collected manually under gravity.

Inhibition studies with affinity chromatography

Inhibition by NaCl

Samples of purified HS derived from human fibroblasts (3000 c.p.m.) were added to 0.5 ml of equilibration buffer containing a known concentration of NaCl, then applied to the affinity column and recycled five times. The column was washed with 4.5 ml of equilibration buffer containing the appropriate concentration of NaCl and this was collected and designated as the unbound fraction. Bound material was then eluted with 5.0 ml of equilibration buffer containing 1.0 M NaCl. Aliquots of 0.5 ml were taken from both the unbound and bound fractions and the percentage of radiolabel in each pool was determined. Maximum binding was the amount of radiolabel that bound to the column when the HS was applied in equilibration buffer only; the amounts of radiolabelled HS bound in the presence of increased concentrations of NaCl were expressed as a percentage of this value. Each data point was performed in triplicate and the results given are means ± S.E.M.

Inhibition by GAGs

The inhibitory effects of unlabelled GAGs on the interaction between HEPII and radiolabelled fibroblast HS were studied as follows. Stock solutions of unlabelled GAGs (heparin, BKHS, bovine lung HS, CS and DS) were prepared in equilibration buffer at concentrations of 0.1–10 mg/ml. A 10 μl aliquot of fibroblast HS GAG containing 5000 c.p.m. was added to 440 μl of equilibration buffer and to this was added 50 μl of unlabelled GAG from the appropriate stock to give a final volume of 500 μl. This was applied to the column and recycled five times. The column was washed with 4.5 ml of equilibration buffer and this was designated as the unbound fraction. Bound material was eluted with 5.0 ml of 1.0 M NaCl in 50 mM Tris/HCl, pH 6.0. Aliquots were taken from each pool to determine the proportion of radiolabel in the bound and unbound fractions. In some experiments the effects of heparinase I and heparinase III on the inhibitory activity of heparin and HS were evaluated.

RESULTS

The interaction of HS with HEPII

When metabolically labelled HS GAG chains from human skin fibroblasts were applied to an Affi-Gel 10 affinity chromatography column coupled to purified HEPII, a small amount of the label passed through the column in the equilibration buffer (50 mM Tris/HCl, pH 6.0, containing 50 mM NaCl). The bound material was eluted with a linear NaCl gradient as a broad peak with the peak maximum at 0.18 M (Figure 1). No qualitative

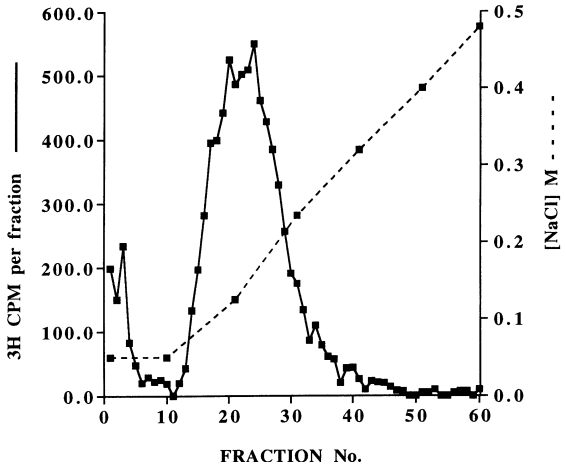

Figure 1 Gradient elution of fibroblast HS GAG chains from HEPII affinity column

A 0.5 ml Affi-Gel 10 affinity column (0.5 cm × 0.7 cm) substituted with 200 μg of purified HEPII was pre-equilibrated with 50 mM NaCl, 50 mM Tris/HCl, pH 6.0 (equilibration buffer). Human skin fibroblast HS chains (3×10^5 c.p.m.) were applied to the column in 0.5 ml of equilibration buffer. Unbound radiolabelled material was eluted with 10 ml of equilibration buffer at a flow rate of 6.0 ml/h and 1.0 ml fractions were collected. Bound material was eluted with a linear NaCl gradient (0.05–0.5 M in 50 mM Tris/HCl, pH 6.0) at 6.0 ml/h and 50 fractions of 1.0 ml each were collected. The formation of the NaCl gradient was monitored by measuring the electrical conductance of selected fractions (broken line). Aliquots were taken from each fraction to determine radiolabel content (solid line).

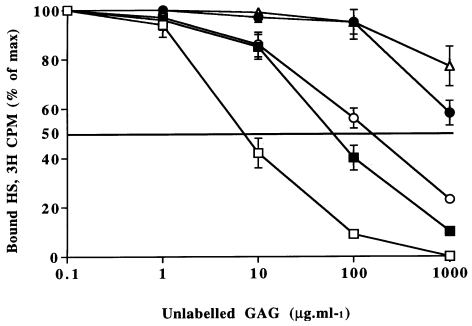

Figure 2 The ability of unlabelled GAG preparations to inhibit the interaction of radiolabelled fibroblast HS with immobilized HEPII

The effects of increasing concentration of different unlabelled GAGs on the binding of human fibroblast HS GAG to an Affi-Gel 10 HEPII affinity column. The column (0.5 cm × 0.7 cm) contained 200 μg of purified HEPII. Labelled HS (3000 c.p.m.) was mixed with a 50 μl aliquot of stock solution of unlabelled GAG, made up to 0.5 ml with equilibration buffer and applied to the column. The eluate was collected and recycled through the column five times. The column was then washed with 4.5 ml of equilibration buffer, which was pooled with the eluent from the sample application and designated the unbound fraction. Bound material was eluted with 5.0 ml of 1.0 M NaCl in 50 mM Tris/HCl, pH 6.0. Aliquots were taken from the unbound and bound fractions and the proportion of labelled HS in each was determined by liquid-scintillation counting. Each data point shows the mean ± S.E.M. for triplicate experiments. The results are expressed as a percentage of the radiolabelled HS that bound to the column in the absence of unlabelled GAG. Symbols: □, pig mucosal heparin; ■, BKHS; ○, Organon pig mucosal HS; ●, DS; △, CS.

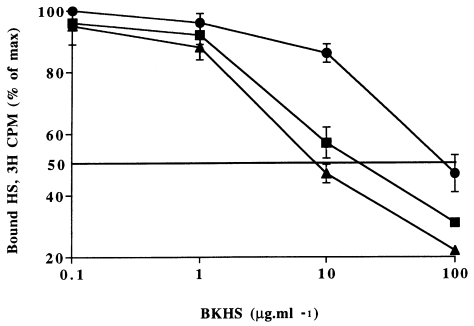

Figure 3 Effects of heparinase I- or heparinase III-treated BKHS on the binding of fibroblast HS to HEPII

BKHS was depolymerized as described in the Experimental section with either heparinase I or heparinase III. Untreated or digested BKHS preparations were assayed to determine their effects on the binding of fibroblast HS to a HEPII affinity column. Aliquots of intact or enzyme-treated BKHS of known concentration were added to labelled fibroblast HS (5000 c.p.m.) in a total volume of 0.5 ml of equilibration buffer. After application to the column, the eluate was collected and reapplied four times before washing with 4.5 ml of equilibration buffer. This was designated the unbound fraction. The bound material was eluted with 5.0 ml of 1.0 M NaCl in 50 mM Tris/HCl, pH 6.0. Aliquots were taken from each pool for liquid-scintillation counting to determine the proportions of bound and unbound label; each data point shows the mean ± S.E.M. for triplicate experiments. The results are expressed as a percentage of the radiolabelled HS that bound to the column in the absence of unlabelled GAG. Symbols: ■, untreated BKHS; ▲, BKHS treated with heparinase III; ●, BKHS treated with heparinase I.

difference was seen in the profile obtained when radiolabelled HS proteoglycan was used in place of the free HS chains (results not shown).

The inhibitory effects of unlabelled GAGs on the interaction of radiolabelled HS with HEPII were then investigated (see the Experimental section). As expected, when unlabelled GAGs were included with the labelled HS sample before application to the affinity column, the amount of label recovered in the bound pool decreased (Figure 2). IC_{50} values were calculated as a comparative indicator of relative inhibitory potency of the unlabelled GAGs. Pig mucosal heparin was found to be the most effective inhibitor of the fibroblast HS–HEPII interaction with an IC_{50} of 9.0 μg/ml. BKHS and pig mucosal HS were less effective inhibitors, with IC_{50} values of 90 and 200 μg/ml respectively in this experiment. CS and DS were found to be poor inhibitors of the interaction and IC_{50} values could not be derived directly for these GAGs even at concentrations as high as 1.0 mg/ml (Figure 2).

Effects of heparinase I and heparinase III

To gain some information about the structures within the BKHS that participate in the interaction with HEPII, the GAG was treated with heparinase I or heparinase III, which cleave HS only at specific linkages within the polymer chain [34]. The digested preparations were then assessed over a range of concentrations for their ability to compete with radiolabelled HS and immobilized HEPII (Figure 3). In these experiments the treatment of BKHS with heparinase I effectively increased the IC_{50} of the polysaccharide from 33.0 to 88.0 μg/ml, indicating that the interaction of the heparinase-resistant fragments with HEPII was considerably weaker than that of the native GAG. Conversely the treatment of BKHS with heparinase III shifted the resulting inhibition curve to the left, thereby reducing the IC_{50} compared with the intact HS from 33.0 to 9.5 μg/ml in this set of experiments (Figure 3). This suggested that the interaction of the comparatively sulphate-rich heparinase III-resistant fragments

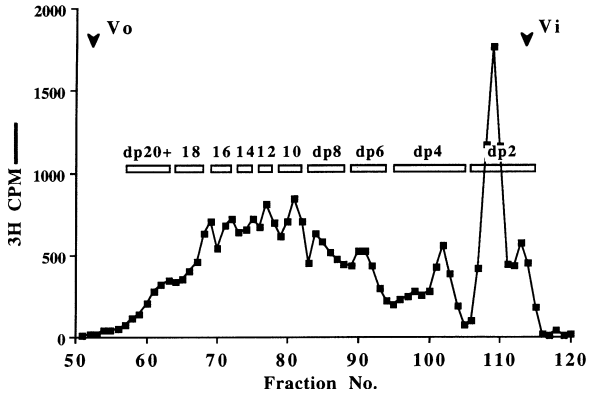

Figure 4 Bio-Gel P-10 gel-filtration chromatography of fibroblast HS digested with heparinase III

A 0.5 ml aliquot of biosynthetically labelled fibroblast HS containing 3×10^6 c.p.m. was treated with 0.1 unit/ml heparinase III in 0.1 mM calcium acetate, 0.1 mM sodium acetate, 100 μg/ml BSA, pH 7.0, at 37 °C for 16 h. The digest was applied directly to a Bio-Gel P-10 column (1.0 cm × 140 cm) and eluted with 0.2 M NH_4HCO_3 at a flow rate of 4.0 ml/h. Fractions of 1.0 ml were collected and 10 μl aliquots were taken for liquid scintillation counting. The void and included volumes (V_0 and V_i respectively) were indicated by the inclusion of Dextran Blue and sodium dichromate respectively as described previously [32]. The peaks were pooled as indicated.

with the HEPII domain was of greater strength than that of the intact BKHS. The effects of treating BKHS with the heparinase enzymes indicate that the HEPII-binding domains are located within specific sequences of the HS chain.

The relationship between HS oligosaccharide size and HEPII binding

To carry out more detailed studies of the interaction between HS oligosaccharides and HEPII, radiolabelled HS was treated with

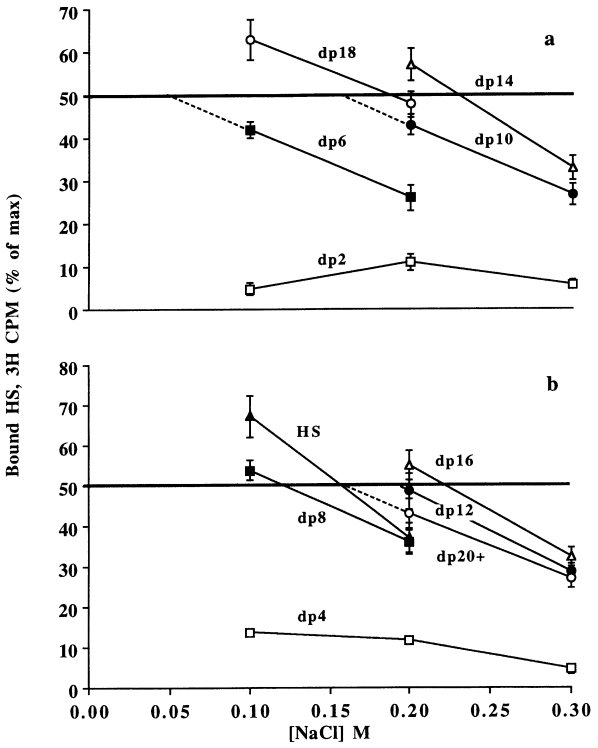

Figure 5 Investigation of the relationship between heparinase III-resistant HS oligosaccharide size and HEPII binding

Human fibroblast heparinase III-resistant HS oligosaccharides were fractionated by gel filtration (see Figure 4), freeze-dried and resuspended in 100 μl of distilled water. Samples of oligosaccharide (5–20 μl), containing 5000 c.p.m., were added to Tris-buffered NaCl solution (0.1, 0.2 or 0.3 M) to give a total volume of 0.5 ml and applied to a pre-equilibrated HEPII affinity column (0.7 cm × 0.5 cm, substituted with 200 μg of HEPII). The eluate was collected and recycled through the column five times. Unbound material was eluted with 4.5 ml of the same buffered NaCl solution used during sample application. Bound material was eluted with 5.0 ml of 1.0 M NaCl in 50 mM Tris/HCl, pH 6.0. Aliquots (five of 0.5 ml each) were taken from bound and unbound fractions for liquid-scintillation counting. Each data point represents the mean ± S.E.M. for triplicate experiments. The results are expressed as a percentage of the amount of intact HS that bound to the HEPII column in 50 mM Tris/HCl, pH 6.0. The binding curves are shown in two separate panels for clarity. Symbols: (**a**) □, dp2; ■, dp6; ●, dp10; △, dp14; ○, dp18; (**b**) □, dp4; ■, dp8; ●, dp12; △, dp16; ○, dp20+; ▲, intact HS.

heparinase III and the fragments were separated on the basis of molecular size by chromatography in BioGel P-10 (Figure 4). These oligosaccharides were assayed to determine the relative affinities of their binding with HEPII by using NaCl to suppress the strength of the interactions. Selected analyses were performed at appropriate NaCl concentrations to obtain both mean and standard error values from triplicate results. For each oligosaccharide preparation repeat experiments were performed to obtain data at or near the IC_{50} (Figure 5). All NaCl solutions were made up in 50 mM Tris/HCl, pH 6.0. (The results of these experiments are shown in two separate panels in the interests of clarity.) Oligosaccharides of dp2 and dp4 bound very weakly to the HEPII column; IC_{50} values could not be derived for these samples. In contrast, the hexasaccharide (dp6) interacted with the affinity column, and extrapolation of the data indicated an IC_{50} of 0.05 M NaCl (Figure 5). As the fragment length increased from dp6 to dp14, higher concentrations of NaCl were required to reduce the binding of the ^{3}H-labelled saccharides to 50% of the recovered material. This is clearly evident from the plots in Figure 5 and is summarized in Table 1. The estimated IC_{50} values for dp14 and dp16 were 0.23 and 0.225 M NaCl respectively and

Table 1 Summary of data in Figure 5: relationship between HS oligosaccharide size and relative affinity for HEPII

For comparative purposes the concentration of NaCl that allowed 50% of the radiolabelled oligosaccharides to bind to the HEPII column was determined (IC_{50}).

Oligosaccharide	IC_{50} (M)
2	0.0
4	0.0
6	0.05*
8	0.120
10	0.15*
12	0.193
14	0.226
16	0.223
18	0.193
20+	0.16*
Intact HS	0.158

* IC_{50} obtained indirectly by extrapolation.

represented the highest values recorded in this experiment. Higher-molecular-mass oligosaccharides, dp18 and dp20+, achieved half-maximal binding at 0.185 and 0.16 M NaCl respectively. The intact fibroblast HS had an IC_{50} of 0.158 M, which was comparable with the binding strength of the dp10 fraction. This study shows that the fractions of HS that bind most strongly to HEPII are dp14 and dp16 oligosaccharides. Furthermore all fragments containing 12–18 monosaccharides bound with greater strength to the column than the intact HS chains from which they were derived. This reinforces the previous finding (Figure 3) that unfractionated, heparinase III-treated BKHS was a better inhibitor of radiolabelled HS binding to HEPII than the native BKHS chains.

DISCUSSION

In 1977 Stathakis and Mosesson [35] reported that heparin could precipitate purified human pFN at 4 °C and demonstrated that this process was due to the interaction of the GAG with the protein. Since this important discovery, the interaction between heparin or HS and FN has been implicated in a diverse range of cellular functions (reviewed in [1]). In an attempt to elucidate the structural requirements of this interaction many studies have focused on the components of FN, particularly HEPII, that interact with GAGs [1] but to our knowledge this is the first study of the structure of the HEPII-binding site in the HS polysaccharide.

The findings presented in this paper demonstrate an interaction between HS and HEPII that is sustained at physiological ionic strength and seems to be specific for the N-sulphated GAGs: HS and the chemically related heparin (Figures 1 and 2). The binding of radiolabelled HS to a HEPII affinity column was inhibited by these polysaccharides but not by CS or DS even though the latter have a higher sulphate content than most HS preparations. Several studies have described an interaction between CS and HEPII and it has been proposed that a specific HEPII-derived peptide binds to CS [18]. Our results neither confirm nor refute the presence of a CS-binding site in the HEPII domain but the very weak inhibitory activity of CS in the HS-binding assay suggests that if CS does bind to HEPII it interacts at a site sufficiently remote from that recognized by HS not to interfere in a competitive manner. In addition, a recent study confirms that CS binds to HEPII in hypotonic salt only [36].

HS binding to HEPII seems to be determined by sulphated

domains of the polysaccharide that are resistant to heparinase III and can be released from the GAG chain by scission with this enzyme. The released saccharides were highly effective competitive inhibitors of the interaction between intact radiolabelled HS and the HEPII affinity column. Furthermore a subfraction of the heparinase III-resistant fragments, of size dp12–dp18, displayed a higher apparent affinity for HEPII than the original HS (Table 1). One possible explanation for these results is that such saccharide sequences are constrained in a less favourable binding conformation in the native chain than they can attain when free.

Heparinase III (also known as heparitinase) acts on regions of low sulphation in HS [24]. It cleaves the linkage between amino sugars and glucuronic acid, but disaccharides that contain iduronic acid or iduronate-2-sulphate [IdoA(2S)] are resistant to the enzyme [34]. Glucuronic acid is found mainly in linkage to GlcNAc, and scission of the GlcNAc (or $GlcNSO_3$)–GlcA link causes extensive breakdown of HS (as shown in Figure 4) and generates resistant saccharides enriched in N-sulphates and iduronate residues [23,24,37]. The general formula (excluding O-sulphates) for the sugar residue sequence of heparinase III-resistant saccharides is GlcA-$GlcNSO_3$-(IdoA-$GlcNSO_3$)$_n$-IdoA-GlcNAc. The smallest of these saccharides that showed binding activity for HEPII was a hexasaccharide ($n = 1$) although the requirement for O-sulphation, if any, in this minimal binding sequence is currently unknown. However, as N-sulphate groups are essential for the interaction of heparin with FN [38], it is likely that N-sulphation is also important for the HEPII-binding activity of HS fragments.

Heparinase III-resistant fragments displayed a progressive size-dependent increase in HEPII affinity from hexasaccharide up to structures containing a maximum of five or six internal iduronic acid repeat units (i.e. dp14–dp16 fragments). Previous work has shown that the iduronic acid residues in sequences of this length in fibroblast HS are commonly sulphated at C-2 [39]. More direct evidence for the involvement of iduronic acid 2-sulphate in HEPII binding was obtained with the enzyme heparinase I, which caused a marked decrease in the inhibitory activity of BKHS (Figure 3). As this enzyme acts specifically on the $GlcNSO_3(\pm 6S)$–IdoA(2S) linkage [34], this suggests that sulphated iduronate residues are present within the HEPII-binding region of HS and are important for protein recognition. From consideration of the optimum size of the binding saccharides and the actions of specific enzymes, the general structure of the highest-affinity sites in fibroblast HS for interaction with HEPII may be written as GlcA-$GlcNSO_3$-[IdoA($\pm$2S)-$GlcNSO_3$]$_{5-6}$-IdoA-GlcNAc. These are among the longest N-sulphated domains found in HS (Figure 4). The minimum number and arrangement of sulphated iduronates required for optimum binding is unknown, as is the influence, if any, of C-6 sulphation of $GlcNSO_3$. Nevertheless some of the major structural features of HS that mediate strong binding to HEPII have been identified in this study, the relatively large size of the binding sequences perhaps reflecting the need to span the two HS-recognition sites in the HEPII fragment [19].

HEPII was initially identified as a heparin- rather than HS-binding domain of FN but it is not clear whether the highly sulphated heparin, which is synthesized only by connective tissue mast cells [40,41], has any natural physiological role that involves binding to FN. The heparin structure [major disaccharide $GlcNSO_3$(6S)-IdoA(2S)] is replete with the structural features identified here as being important for the binding of HS to HEPII; however, in HS, the high-affinity HEPII-binding motifs (represented by the dp12–dp16 heparinase III-resistant fragments in Figure 4) comprise only approx. 20% of the HS chain and their O-sulphate concentration is significantly less than that of heparin [23].

Ingham et al. [19] calculated the dissociation constant (K_d) for the interactions of heparin oligosaccharides and the HEPII domain in solution. They found that the K_d decreased from 18 μM for hexasaccharides to approx. 1.0 μM for fragments containing 14, 16 and 18–20 saccharides. The dissociation constants of two different heparin preparations (molecular masses 15 and 30 kDa) were calculated at 2.5 μM. Thus, as in the results presented here with HS, the interaction between the intact heparin GAG and HEPII was weaker than that of the defined smaller oligosaccharides.

Ogamo et al. [38] had demonstrated a linear relationship between heparin oligosaccharide size and affinity for FN but with no apparent enhancement of affinity for dp14–18 fragments when compared with the polymeric heparin. Subsequently [41], FN was immobilized by coupling to a CNBr-activated Sepharose affinity support. However, we have found that HS, heparin and CS bind non-specifically to CNBr-activated Sepharose at 0.5 M NaCl or less (A. Walker, unpublished work). Such non-specific interactions might have adversely influenced the findings of Ogamo et al. [38]. Neither HS, heparin nor CS interacts with Affi-Gel 10 used in the present studies, even at very low salt concentrations (50 mM NaCl). Thus, on the basis of our findings and the results of Ingham et al. [19], we conclude that the interaction between polysaccharide and HEPII is determined mainly by reasonably well defined sulphated regions of the polymer chain.

It is interesting to compare the structural features of the HEPII-binding sites in HS with the binding sites for anti-thrombin III and basic fibroblast growth factor (bFGF), which have been studied in greater detail. In common with HEPII these proteins recognize the modified regions of the HS chain and require specific arrangements of N-sulphate and O-sulphate groups and iduronate residues. The anti-thrombin III site is a pentasaccharide with a highly distinctive sequence including a rare 3-O-sulphated $GlcNSO_3$ residue [42]. In contrast, high-affinity sequences for bFGF are considerably longer structures (dp12–14), are resistant to heparinase III, and contain an internal repeat sequence of IdoA(2S)-$GlcNSO_3$ [39]. The iduronic acid 2-sulphate residue is particularly important for recognition of bFGF [39,43]. High-affinity HS sequences are essential for the biological activation of bFGF through the signal-transducing receptors [44]. However, the actual contact region for bFGF in HS might only extend over five sugar residues [45], with the additional sugars perhaps being required to confer a specific conformation on the GAG structure [46]. The HEPII-binding site in HS has close similarities to the bFGF-binding sequence (similar in size and requirement for N-sulphation and iduronic acid 2-sulphate). Therefore the two proteins may compete for the same domains in the HS chain, the nature of the interaction being determined by the balance between protein affinity (higher for bFGF; 1–3 nM) [47] and protein concentration, which would normally be higher for HEPII in a typical FN-rich pericellular matrix. Competing mechanisms of this kind are likely to play a key role in the control and integration of cellular responses to growth factors and the ECM.

The apparent specificity of the interaction between fragments of HS and the HEPII domain is of importance because cell-surface interactions with this portion of the FN structure are thought to mediate the adhesion of melanoma cells and lymphocytes via the integrin cell-surface receptors [48–51]. In addition, fibroblast cell spreading on FN after the development of stress fibres and terminating in focal adhesions seems to involve both specific integrins and HS, the latter binding to the HEPII domain

[52–54]. As the binding of HS (or heparin) to FN induces or stabilizes conformational changes in the protein structure [55–57], it is possible that the binding of HS to the HEPII domain facilitates other functions of the protein *in vivo*, such as the interaction with the integrin cell-surface receptors [58,59].

Activation of protein kinase C (PKC) is a prerequisite for the formation of focal adhesions [60], and a membrane HS proteoglycan, syndecan IV, is concentrated in these adhesion sites and has been implicated in PKC activation [60]. Perhaps specific recognition of HEPII by the HS chains of syndecan IV initiates a signalling mechanism to activate PKC. Furthermore the relatively high apparent affinity of HS-saccharides for HEPII (Figure 5 and Table 1) suggests a mechanism by which heparanases in the pericellular matrix could modulate cell adhesion to FN by releasing HS-fragments that then act as strong competitive inhibitors of the binding activity of membrane-bound HS [61,62].

We thank Dr. Malcolm Lyon for his helpful advice and Professor Martin Humphries for providing a standard HEPI fragment. This research is supported by grants from the Cancer Research Campaign.

REFERENCES

1 Hynes, R. O. (1990) in Fibronectins, pp. 301–334, Springer-Verlag, Berlin
2 Moria, A., Zhang, Z. and Ruoslahti, E. (1994) Nature (London) **367**, 193–196
3 McDonald, J. A., Quade, B. J., Broekelmann, T. J., La Chance, R., Forsman, K., Hasegawa, E. and Akiyama, S. (1987) J. Biol. Chem. **262**, 2957–2967
4 Yamada, K. M. (1991) in Cell Biology of the Extracellular Matrix (Hay, E. D., ed.), pp. 111–139, Plenum Press, London
5 Humphries, M. J. (1990) J. Cell Sci. **97**, 585–592
6 Mould, A. P. and Humphries, M. J. (1991) EMBO J. **10**, 4089–4095
7 Sekiguchi, K and Hakomori, S. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 2661–2665
8 Benecky, M. J., Kolvenbach, C. G., Amrani, D. L. and Mosesson, M. W. (1988) Biochemistry **30**, 4298–4306
9 Bentley, K. L., Klebe, R. J., Hurst, R. E. and Horovitz, P. M. (1985) J. Biol. Chem. **260**, 7250–7256
10 Gold, L. I., Frangione, B. and Pearlstein, E. (1983) Biochemistry **22**, 4113–4119
11 Hayashi, M. and Yamada, K. M. (1982) J. Biol. Chem. **257**, 5263–5267
12 Hayashi, M. and Yamada, K. M. (1983) J. Biol. Chem. **258**, 3332–3340
13 Woods, A., Couchman, J. R., Johansson, S. and Hook, M. (1986) EMBO J. **5**, 665–670
14 McCarthy, J. B., Skubitz, A. P., Zhao, Q., Yi, X. Y., Mickelson, D. J., Klein, D. J. and Furcht, L. T. (1990) J. Cell Biol. **110**, 777–787
15 Yamada, K. M., Kennedy, D. W., Kimata, K. and Pratt, R. M. (1980) J. Biol. Chem. **225**, 6055–6063
16 Barkalow, F. J. B. and Schwarzbauer, J. E. (1991) J. Biol. Chem. **266**, 7812–7818
17 McCarthy, J. B., Chelberg, M. K., Mickelson, D. J. and Furcht, L. T. (1988) Biochemistry **27**, 1380–1388
18 Iida, J., Skubitz, A. P. N., Furcht, L. T., Wayner, E. A. and McCarthy, J. B. (1992) J. Cell Biol. **118**, 431–444
19 Ingham, K. C., Brew, S. A. and Atha, D. H. (1990) Biochem. J. **272**, 605–611
20 Haughen, P. K., McCarthy, J. B., Skubitz, A. P. N., Furcht, L. T. and Letourneau, P. C. (1990) J. Cell Biol. **111**, 2733–2745
21 Kusche, M., Hannesson, H. H. and Lindahl, U. (1991) Biochem. J. **275**, 151–158
22 Lidholt, K. and Lindahl, U. (1992) Biochem. J. **287**, 21–29
23 Gallagher, J. T., Turnbull, J. E. and Lyon, M. (1992) Int. J. Biochem. **24**, 533–560
24 Turnbull, J. E. and Gallagher, J. T. (1991) Biochem. J. **273**, 553–559
25 Lyon, M., Deakin, J. A. and Gallagher, J. T. (1994) J. Biol. Chem. **269**, 11208–11215
26 Lark, M. W. and Culp, L. A. (1984) J. Biol. Chem. **259**, 6773–6782
27 Lee, M. R. and Lander, A. D. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 2768–2772
28 Zardi, L., Carnemolla, B., Balza, E., Borsi, L., Castellani, P., Rocco, M. and Siri, A. (1985) Eur. J. Biochem. **146**, 571–579
29 Engvall, E. and Ruoslahti, E. (1977) Int. J. Cancer **20**, 1–5
30 Pierschbacher, M. D., Hayman, E. G. and Ruoslahti, E. (1981) Cell **26**, 259–267
31 Turnbull, J. E. and Gallagher, J. T. (1990) Biochem. J. **265**, 715–724
32 Gallagher, J. T. and Walker, A. (1985) Biochem. J. **230**, 665–674
33 Fraenkel-Conrat, H. (1957) Methods Enzymol. **4**, 247–269
34 Linhardt, R. J., Turnbull, J. E., Wang, H. M., Loganathan, D. and Gallagher, J. T. (1990) Biochemistry **29**, 2611–2617
35 Stathakis, N. E. and Mosesson, M. W. (1977) J. Clin. Invest. **60**, 855–865
36 Barkalow, F. J. and Schwarzbauer, J. E. (1994) J. Biol. Chem. **269**, 3957–3962
37 Gallagher, J. T., Turnbull, J. E. and Lyon, M. (1990) Biochem. Soc. Trans. **18**, 207–209
38 Ogamo, A., Nagai, A. and Nagasawa, K. (1985). Biochim. Biophys. Acta **841**, 30–41
39 Turnbull, J. E., Fernig, D. G., Ke, Y., Wilkinson, M. C. and Gallagher, J. T. (1992) J. Biol. Chem. **267**, 10337–10341
40 Nader, H. B. and Dietrich, L. P. (1989) in Heparin (Lane, D. and Lindahl, U., eds.), pp. 81–96, Edward Arnold Ltd., London
41 Kolset, S. O. and Gallagher, J. T. (1990). Biochim. Biophys. Acta Rev. Cancer **1032**, 191–211
42 Lindahl, U., Thunberg, L., Backstrom, G., Reisenfield, J., Nordling, K. and Bjorg, I. (1984) J. Biol. Chem. **259**, 12368–12376
43 Habuchi, H., Suzuki, S., Saito, T., Tamura, T., Harada, Y., Yoshida, K. and Kimata, K. (1992) Biochem. J. **285**, 805–813
44 Walker, A., Turnbull, J. E. and Gallagher, J. T. (1994) J. Biol. Chem. **269**, 931–935
45 Maccarana, M., Casu, B. and Lindahl, U. (1993) J. Biol. Chem. **268**, 23898–23905
46 Kan, M., Wang, F., Xu, J., Crabb, J. W., Hou, J. and McKeehan, W. L. (1993) Science **259**, 1918–1921
47 Burgess, W. H. and Maciag, T. (1989) Annu. Rev. Biochem. **58**, 575–606
48 Humphries, M. J., Akiyama, J. K., Komoriya, A., Olden, K. and Yamada, K. M. (1986) J. Cell Biol. **103**, 2637–2647
49 Humphries, M. J., Komoriya, A., Akiyama, J. K., Olden, K. and Yamada, K. M. (1987) J. Biol. Chem. **262**, 6886–6892
50 Garcia-Pardo, A., Rostagno, A. and Frangione, B. (1987) Biochem. J. **241**, 923–928
51 Mould, A. P., Wheldon, L. A., Komoriya, A., Wayner, E. A., Yamada, K. M. and Humphries, M. J. (1990) J. Biol. Chem. **265**, 4020–4024
52 Izzard, C. S., Radinsky, R. and Culp, L. A. (1986) Exp. Cell Res. **165**, 320–326
53 Woods, A., Couchman, J. R., Johansson, S. and Hook, M. (1986) EMBO J. **5**, 665–670
54 Woods, A., McCarthy, J. B., Furcht, L. T. and Couchman, J. R. (1993) Mol. Biol. Cell **4**, 605–613
55 Osterlund, E., Eronen, I., Osterlund, K. and Vuento, M. (1985) Biochemistry **24**, 2661–2667
56 Narasimhan, C. and Lai, C. S. (1991) Biopolymers **31**, 1159–1170
57 Novokhatny, V., Schwarz, F., Atha, D. and Ingham, K. (1992) J. Mol. Biol. **227**, 1182–1191
58 Woods, A. and Couchman, J. R. (1988) Collagen Relat Res. **8**, 155–182
59 Johansson, S. and Hook, M. (1984) J. Cell Biol. **98**, 810–817
60 Woods, A. and Couchman, J. R. (1992) J. Cell Sci. **101**, 277–290
61 Vlodavsky, I., Kuks, Z., Bar-Ner, M., Ariav, Y. and Schirrmacher, V. (1983) Cancer Res. **43**, 2704–2709
62 Nakajima, M., Irimura, T. and Nicolson, G. L. (1988) J. Biol. Chem. **36**, 157–167

Received 17 January 1996/21 March 1996; accepted 3 April 1996