Interleukin-8 binds to syndecan-2 on human endothelial cells

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Application of reverse transcription–PCR to total RNA prepared from TNF- α (tumour necrosis factor- α)-stimulated HUVECs (human umbilical vein endothelial cells) revealed that the syndecan-2 mRNA was up-regulated by this inflammatory stimulus. By immunoprecipitation using an anti-syndecan-2 antibody on TNF- α -stimulated HUVEC lysates, inflammation-induced interleukin-8 was found to be an interaction partner of this HS (heparan sulphate) proteoglycan, but not of any other syndecan on these cells. The glycosylated [Syn2_{ect}(+ HS)] and non-glycosylated [Syn2_{ect}(– HS)] forms of Syn2_{ect} (the syndecan-2 ectodomain) were purified from a stably transfected human cell line and from a bacterial expression system respectively. By CD

spectroscopy, Syn2_{ect} was found to adopt an all- β secondary structure. The dissociation constant of Syn2_{ect}(+ HS) with respect to interleukin-8 binding was determined by isothermal fluorescence titrations to be 23 nM. Despite its lack of HS chains, Syn2_{ect}(– HS) exhibited significant binding to the chemokine, with a K_d of $> 1 \mu\text{M}$. Thus, in addition to glycosaminoglycan binding, protein–protein contacts might also contribute to the chemokine–proteoglycan interaction.

Key words: chemokine, heparan sulphate, interleukin-8, syndecan-2, proteoglycan.

INTRODUCTION

Syndecan-2 (fibroglycan) is a cell surface HSPG (heparan sulphate proteoglycan) with a molecular mass of 48 kDa which consists of a short cytoplasmic domain and a single transmembrane domain, both of which are highly conserved among the syndecan family, as well as a large ectodomain (herein called Syn2_{ect}) with three covalently attached HS (heparan sulphate) chains close to the N-terminus [1]. HS belongs to the GAG (glycosaminoglycan) family of polysaccharides, and consists of alternating *N*-acetyl-D-glucosamine and D-glucuronic acid, which can be sulphated at the N, 3-O, and 6-O positions of the glucosamine as well as at the 2-O position of the glucuronic acid, which itself can be epimerized to iduronic acid [2]. The HS chains of the syndecans are responsible for their biological function, for example in extracellular matrix assembly and in growth factor binding [3]. Protein binding to HS is assumed to be facilitated by the occurrence of flexible iduronate residues.

Members of the syndecan family are found on most mammalian cells and tissues [4]. Syndecan-2 is the major HSPG expressed on fibroblasts [5], but is also expressed constitutively on human endothelial cells [6]. It has also been shown to be expressed in human primary osteoblasts [7] as well as in activated macrophages [8]. Syndecan-1 predominates on epithelial cells, syndecan-3 is part of neuronal cell membranes, whereas syndecan-4 is widely expressed [6,9,10]. As a consequence, HSPGs fulfil a great variety of functions, most of them depending upon the interaction between the HS chains of the ectodomain and protein ligands such as protease inhibitors (e.g. antithrombin III), enzymes (e.g. lipoprotein lipase), and growth factors (e.g. fibroblast growth factor). HSPGs are also implicated in signal transduction cascades via their short cytoplasmic domain, which contains two conserved regions separated by a variable region unique to each syndecan [11]. The FYA (Phe-Tyr-Ala) motif at the C-terminus of the con-

served region distal to the membrane interacts with PDZ domain-containing proteins such as syntenin, which is proposed to attach the syndecans to cytoskeletal proteins or cytosolic signalling components [12]. In contrast with syndecan-4, which regulates the activity of protein kinase C, syndecan-2 is a substrate of this enzyme [13].

IL-8 (interleukin-8) is a member of the CXC-chemokine family which is secreted at sites of inflammation by cytokine-activated endothelial cells and which is retained on the cell surface by interactions with HSPGs to establish a local chemokine concentration gradient for the recruitment of neutrophils [14]. The structure of IL-8 has been determined by X-ray crystallography and NMR spectroscopy, which show the chemokine as a homodimer consisting of a six-stranded β -sheet, on top of which the C-terminal α -helices are situated. The amino acids of IL-8 responsible for GAG binding have been identified by site-directed mutagenesis and NMR experiments [15]. Furthermore, Spillmann et al. [16] have proposed a GAG sequence that is specific for IL-8 binding, consisting of two fully sulphated HS hexasaccharides that are linked by a non/under-sulphated dodecasaccharide linker. Our group has recently reported that monomeric IL-8 binds to GAG oligosaccharides with higher affinity than the dimer, suggesting a self-regulatory mechanism of IL-8 presentation *in vivo* [17].

A natural HSPG co-receptor has yet to be identified for any chemokine. We therefore investigated the IL-8-specific HSPG on human endothelial cells by immunoprecipitation. Syndecan-2 was identified to be an interaction partner of IL-8. Syn2_{ect} was cloned, overexpressed and purified in both mammalian and bacterial expression systems. It was found to exhibit native-like secondary structure in the absence of the HS chains, and the structure was shown to be all- β . In addition, both the mammalian and the bacterially expressed proteins were found to bind to IL-8, although with significantly different affinities.

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; GAG, glycosaminoglycan; GST, glutathione S-transferase; HS, heparan sulphate; HSPG, heparan sulphate proteoglycan; HUVEC, human umbilical vein endothelial cell; IL-8, interleukin-8; RT-PCR, reverse transcription–PCR; Syn2_{ect}, ectodomain of syndecan-2; Syn2_{ect}(+ HS), glycosylated form of Syn2_{ect}; Syn2_{ect}(– HS), non-glycosylated form of Syn2_{ect}; TNF- α , tumour necrosis factor- α .

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EXPERIMENTAL

Materials

All chemicals, unless stated otherwise, were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). The antibody against syndecan-1 was kindly donated by Professor Guido David (University of Leuven, Belgium). Antibodies against syndecan-3 and syndecan-4 were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, U.S.A.).

Cloning, bacterial expression and purification of Syn2_{ect}(-HS) (non-glycosylated Syn2_{ect})

Total RNA was prepared from HUVEC (human umbilical vein endothelial cell) lysates by applying TRIreagent (Sigma-Aldrich) to 5×10^6 plated cells. First-strand cDNA was generated by reverse transcription, and the sequence of Syn2_{ect} with restriction site overlaps (*NotI* at the 5' end and *BamHI* at the 3' end) was obtained by PCR using the following primers: forward, 5'-CGGA-ATTCATGCGGCGCGCTGGATCCTGCTCACC-3'; reverse, 5'-GGAAATAAGCGGCCGCTAAAACAGACTGTCTGAGTG-TTCTC-3'.

The Syn2_{ect} gene was digested with the restriction enzymes *NotI* and *BamHI* and was subcloned into the pGEX-4T-1 vector (Amersham Pharmacia Biotech) in-frame with GST (glutathione S-transferase) gene. After transformation of the plasmid into Ca²⁺-competent *Escherichia coli* BL21 cells, the cells were grown in an LB/ampicillin medium to $D_{600} = 0.65$ at 37 °C. Expression of Syn2_{ect}(-HS)-GST was induced by the addition of 1 mM isopropyl β -D-thiogalactoside and further fermentation for 5 h. The cells were harvested by centrifugation (20 min at 4000 g) and the pellet was resuspended in lysis buffer [50 mM NaCl, 50 mM Tris/HCl, pH 8, 50 mM EDTA, 1% (w/v) aprotinin]. After sonication (3 $\times$ 20 s at 150 W on ice) and centrifugation (20 min at 19 000 g), Syn2_{ect}(-HS)-GST was found to be present mainly in the supernatant. For purification, the fusion-protein-containing supernatant was incubated with glutathione-Sepharose (Amersham Pharmacia Biotech) for 1 h at room temperature. The suspension was then suction-filtered and washed three times with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.3). After resuspension in PBS, Syn2_{ect}(-HS) was separated from GST by adding thrombin (20 units/ml). After a 5 h incubation at 37 °C, the supernatant was suction-filtered and the protease activity was inhibited by aprotinin (1%, w/v). The protein solution was concentrated at 13 500 g in microconcentrators (Millipore) using molecular mass cut-off filters of 3.5 kDa. The purity of the protein was analysed by SDS/PAGE followed by Coomassie Blue staining. The protein concentration was determined by the BCA (bicinchoninic acid) method (Pierce).

Stable transfection of full-length syndecan-2 and purification of Syn2_{ect}(+HS) (glycosylated Syn2_{ect})

The full-length syndecan-2 gene was amplified from a first-strand cDNA (see above) using the following primers: forward, 5'-CGGAATTCATGCGGCGCGCTGGATCCTGCTCACC-3'; reverse, 5'-GGAAATTAAGCGGCCGCTTACGCATAAACT-CCTTAGTAGGTGC-3'. The gene was then subcloned into the *NotI* and *BamHI* sites of the mammalian expression plasmid pcDNA3.1 (Invitrogen). For transfection, HEK293 cells at 40% confluency were incubated with DMEM (Dulbecco's modified Eagle's medium; devoid of serum or antibiotics) containing 5 μ g of *PvuI*-linearized plasmid and 10 μ l of Superfect (Qiagen).

After adding full DMEM, the cells were incubated at 37 °C for a total of 50 h before trypsinization and replating in medium containing 1 mg/ml G418 at a density of 5×10^5 cells per plate. After 12 days, resistant colonies were isolated using cloning rings and were transferred to poly-L-lysine-coated plates. Syndecan-2 expression was tested by RT-PCR (reverse transcription-PCR) and by immunoblotting using a commercially available antibody (Santa Cruz).

For protein purification, confluent cell monolayers were mechanically scraped into ice-cold 10 mM Tris/HCl (pH 7.4) containing 0.5 mM EDTA, 0.5 mM PMSF, 5 mM benzamidine, 50 μ g/ml soybean trypsin inhibitor, 1 μ M pepstatin A and 5 mM *N*-ethylmaleimide (all from Sigma). Syn2_{ect}(+HS) was obtained by mild treatment of the cells with 20 μ g/ml porcine pancreatic trypsin (Sigma) for 5 min on ice. The reaction was stopped by adding 200 μ g of soybean trypsin inhibitor, and after centrifugation the supernatant was applied to an affinity column, which was prepared by coupling 200 μ g of anti-syndecan-2 antibody (Santa Cruz) to CNBr-activated Sepharose (Amersham Biosciences). Syn2_{ect}(+HS) was eluted from the column with 2 M NaCl, and its purity was tested by SDS/PAGE followed by Coomassie Blue staining.

Cell culture

HUVECs were prepared according to the procedure of Jaffe et al. [18]. Cells were grown on uncoated dishes in DMEM containing 10% (v/v) fetal calf serum, 4.5 g/l glucose, 0.584 g/l L-glutamine, 25 mM Hepes, 44 mM sodium bicarbonate, 100 units/ml penicillin/streptomycin and 1.25 μ g/ml amphotericin B. HUVECs were serially passaged (1:2 split ratio) and cultivated for a maximum of two passages.

RNA preparation and RT-PCR of syndecan-2

Two culture plates of confluent HUVECs (10^6 cells) were stimulated with TNF- α (tumour necrosis factor- α ; Sigma; 43 ng/ml of medium) for 20 h; another two plates (10^6 cells) were used as controls. After 20 h, the cells were rinsed with pre-chilled PBS, scraped and pelleted. The pellets were then resuspended and lysed in 1 ml of TRIreagent (Sigma) for preparation of total RNA. Purification was accomplished according to the Sigma technical bulletin (MB-205; April 1998). The mRNA was then reverse transcribed using the Prostar First-Strand RT-PCR Kit (Stratagene) into first-strand cDNA, and syndecan-2 gene expression was examined by PCR using the primers described above.

Immunoprecipitation and Western blotting

Two culture plates of confluent cells (10^6 cells) were stimulated with 35.5 ng/ml TNF- α for 24 h; another two plates were used as controls. After 24 h, all cells were rinsed with PBS, scraped and pelleted. The pellets were resuspended in 1 ml of lysis buffer (20 mM Tris/HCl, pH 7.4, 2.5 mM EDTA, 1% Triton X-100), placed on ice for 15 min, and then sonicated for 3 $\times$ 15 s. After centrifugation (25 min at 10 000 g), the supernatant was used for immunoprecipitation.

To bind endogenous syndecan-2 to the anti-syndecan-2 antibody 10H4 (a gift from Professor Guido David, University of Leuven, Belgium), cell lysates of TNF- α -stimulated and unstimulated HUVECs were incubated with 5 μ g of the antibody for 3 h at 4 °C. As a control, stimulated and unstimulated cell lysates were also prepared in the absence of antibody. Immunoprecipitation was achieved by adding 25 μ l of a 50% (w/v) slurry

of Protein A-agarose (Sigma) in PBS to all four batches followed by inverted mixing for a further 3 h at 4 °C. After a short centrifugation (5 s at 13 000 *g*), the supernatant was aspirated and saved for Western blot analysis. The Protein A complexes were washed twice with 500 μ l of washing buffer (50 mM Tris/HCl, pH 8, 500 mM NaCl, 0.1 % Triton X-100). After each wash, they were centrifuged (5 s at 13 000 *g*) and the supernatants were aspirated and put aside for immunoblot analysis. The final pellets as well as all supernatants collected were mixed with equal volumes of Laemmli buffer (denaturing, reducing), heated at 95 °C for 5 min, and fractionated on SDS/10 %-PAGE. After electrophoresis, the proteins were electrotransferred in a semi-dry set-up to Hybond-ECL nitrocellulose membranes (Amersham Pharmacia Biotech); the blots were blocked with 5 % (w/v) skimmed milk and incubated with an anti-IL-8 monoclonal antibody (0.2 μ g/ml; a gift from Dr Antal Rot, Novartis Forschungsinstitut, Vienna, Austria). For detection, a horseradish peroxidase-linked anti-mouse IgG (Sigma) followed by photoluminescent visualization (ECL reagents; Amersham Pharmacia Biotech) were used.

CD measurements and analysis

CD spectra were recorded at a protein concentration of 8 μ M in PBS on a Jasco J-710 spectropolarimeter (Japan Spectroscopic, Tokyo, Japan). Far-UV CD measurements were carried out in the range 195–250 nm using a quartz cuvette with a path length of 0.1 cm, a response time of 0.25 s, and a data point resolution of 0.1 nm. Five scans were recorded to obtain smooth spectra. Mean residue ellipticities of background-corrected spectra were calculated by JASCO standard analysis. Analysis of the spectra with respect to secondary structure content was performed using the algorithm SELCON [19].

Isothermal fluorescence titrations

Steady-state fluorescence measurements were performed on a Perkin-Elmer (Beaconsfield, Bucks., U.K.) LS50B fluorimeter. IL-8 that was fluorescein-labelled at the N-terminus according to published protocols [20] was kindly donated by Dr Amanda Proudfoot (Serono Research Institute, Geneva, Switzerland). The emission of a pre-equilibrated solution of fluorescein-labelled IL-8 (50 nM or 100 nM) in PBS upon excitation at 488 nm was recorded over the range 510–580 nm. Binding isotherms were obtained by coupling the cuvette holder to a thermostat and by the addition of an aliquot of the respective Syn2_{ect} ligand, allowing for an equilibration period of 2 min. The slit width was set at 10 nm and 15 nm for excitation and emission respectively, and the spectra were recorded at 200 nm/min. A 430 nm cut-off was inserted to avoid stray light. The fluorescence spectra were background-corrected and the respective areas were integrated between 510 and 580 nm.

The normalized mean changes in fluorescence intensity ($-\Delta F/F_0$) resulting from three independent experiments were plotted against the ligand concentration. The resulting binding isotherms were fitted by non-linear regression using the program Origin (Microcal Inc., Northampton, MA, U.S.A.) to the following equation describing a bimolecular association reaction, as published previously [17]:

$$F = F_i + F_{\max} \frac{K_d + [\text{IL-8}] + [\text{Syn2}] - \sqrt{(K_d + [\text{IL-8}] + [\text{Syn2}])^2 - 4[\text{IL-8}][\text{Syn2}]}}{2[\text{IL-8}]} \quad (1)$$

where F_i is the initial and $F_{\max}$ is the maximum fluorescence value, K_d is the dissociation constant, and $[\text{IL-8}]$ and $[\text{Syn2}]$ are the total

Figure 1 Western blot of IL-8 co-immunoprecipitated with syndecan-2

Lane 1, unstimulated control (no anti-syndecan-2 antibody); lane 2, syndecan-2 co-immunoprecipitation of unstimulated HUVECs; lane 3, TNF- α -stimulated control (no anti-syndecan-2 antibody); lane 4, syndecan-2 co-immunoprecipitation of TNF- α -stimulated HUVECs; lane 5, IL-8 control; lane 6, second antibody control.

concentrations of IL-8 and syndecan-2 ligand respectively. The fitted parameters were $F_{\max}$ and K_d .

RESULTS

IL-8 has been shown to bind to HS chains on endothelial cells, thereby becoming immobilized, allowing it to be presented to approaching neutrophils [14]. We therefore attempted to identify a HSPG co-receptor of the chemokine on endothelial cells. Since syndecan-2 has been reported to be the major endothelial proteoglycan [6], we have focused in the present study on the interaction of this molecule with IL-8. In Figure 1, a Western blot of IL-8 co-immunoprecipitated using an antibody directed against human syndecan-2 is shown. The rationale behind this experiment was to use the anti-syndecan-2 antibody for 'fishing' for syndecan-2 ligands in TNF- α -treated HUVEC lysates. Detection of IL-8 itself was accomplished by a monoclonal antibody directed against the chemokine. As can be seen from Figure 1, in both TNF- α -stimulated and unstimulated cells IL-8 was found to be an interaction partner of syndecan-2, although in the untreated cells less IL-8 was co-immunoprecipitated. This could be due to the lower concentration of syndecan-2 in these cells (see below) or to a lower concentration of IL-8 in the absence of TNF- α . The apparent molecular mass of IL-8 on the denaturing and reducing SDS gel corresponded to the monomeric form of the chemokine. No higher aggregates or molecular complexes of IL-8 were detected, as was expected under these electrophoretic conditions (the band at 30 kDa corresponds to a non-specific signal of the second antibody; see control in lane 6 of Figure 1). Furthermore, no IL-8 was immunoprecipitated in the control experiments using Protein A-Sepharose devoid of anti-syndecan-2 antibody, indicating that the chemokine did not bind unspecifically to the beads (Figure 1, lanes 1 and 3). Since the washing solutions did not contain IL-8, quantitative binding of the chemokine to syndecan-2 immobilized on the Protein A-Sepharose beads can be assumed. In order to address the specificity of the interaction of IL-8 with proteoglycans on the endothelial cell surface, we also performed the immunoprecipitation with antibodies directed against syndecan-1, syndecan-3 and syndecan-4. No co-precipitation of IL-8 with these proteoglycans was found (results not shown). This does not, however, rule out further interactions of this chemokine with HSPGs other than syndecans on endothelial surfaces.

In order to investigate the response of syndecan-2 to an inflammatory stimulus, total RNA was prepared from HUVECs

Figure 2 Syndecan-2-directed RT-PCR of TNF- α -stimulated (lane 2) and unstimulated (lane 1) HUVECs

In lane 3, a control PCR using the GST-syndecan-2 fusion expression plasmid as template is shown.

that were treated with TNF- α for 20 h before harvesting. RT-PCR was then performed using primers corresponding to Syn2_{ect}. As can be seen in Figure 2, the syndecan-2 mRNA was expressed constitutively in HUVECs, but became significantly up-regulated by a factor of approx. 4.5 (densitometric determination) upon stimulation with TNF- α . An alternative explanation for this finding could be enhanced stability of the syndecan-2 mRNA in the presence of TNF- α . Performing the stimulation/incubation experiment using the non-inflammatory cytokine IL-10, or IL-8 itself, did not result in up-regulation/stabilization of syndecan-2 mRNA (results not shown). Taken together, these experiments suggest the involvement of syndecan-2 in the process of IL-8-mediated inflammation.

With the aim of biophysically comparing the non-glycosylated and glycosylated forms of syndecan-2 *in vitro*, Syn2_{ect} was expressed and purified both from a bacterial and from a eukaryotic expression system. A prokaryotic expression system for the production of the Syn2_{ect}(-HS) was chosen (i) because of the high expression yields (crystallization experiments are still ongoing), and (ii) because of the complete absence of carbohydrate side chains when compared with enzymically removing the HS chains from the eukaryotically expressed protein. The bacterial protein Syn2_{ect}(-HS) was obtained as a GST fusion protein, making a one-step purification with glutathione-Sepharose possible. On-column cleavage of the fusion protein by thrombin gave Syn2_{ect}(-HS) with a purity of > 95%. Despite the high purity, a large part of the protein was found to be insoluble in PBS, which decreased the overall protein yield to 2 mg/litre of bacterial culture and suggested partial structural instability of Syn2_{ect}(-HS). This might partly reflect the conformational dependence of the protein on its HS side chains *in vivo*. For comparison, full-length syndecan-2 was overexpressed in a human cell line and Syn2_{ect}(+HS) was shed from the cell surface, as described in the Experimental section. Affinity purification using a polyclonal antibody against syndecan-2 yielded Syn2_{ect}(+HS) with a purity of > 97% appearing at a molecular mass of approx. 26 kDa (results not shown). This corresponds to a molecular mass of the GAG side chains of 10.1 kDa [the theoretical molecular mass of Syn2_{ect}(-HS) is 15.9 kDa]. The identity of the protein was checked by Western blotting (results not shown). The overall yield in such eukaryotic expression systems is generally quite low (< 100 μ g per litre of cell culture), but in our case it was sufficient to perform spectroscopic experiments.

To investigate the secondary structure content of Syn2_{ect}, far-UV CD spectra were recorded. The CD spectrum of Syn2_{ect}(-HS) (Figure 3) was found to be typical of a β -sheet protein containing only minor amounts of α -helix. Analysis by the SELCON method [20] revealed over 45% of all amino acids to reside in β -sheets and less than 5% in α -helices, with the remaining amino acids being part of loops. Similarly, although giving different absolute values, a secondary structure prediction using PredictProtein [21] resulted in 2.1% α -helix and 15.7% β -sheet (Figure 3, inset). Despite the fact that Syn2_{ect}(-HS) is obviously an all- β protein, the high

Figure 3 Far-UV CD spectrum of 8 μ M Syn2_{ect}(-HS) in PBS

The inset shows the amino acid sequence of Syn2_{ect} and its predicted secondary structure according to PredictProtein [21]. a, α -helix; b, β -sheet; the HS attachment site is boxed. The spectra presented here were corrected for the background signal obtained with buffer alone. Five scans were recorded in order to obtain smooth spectra.

Figure 4 Isothermal fluorescence titrations of fluorescein-labelled IL-8 with Syn2_{ect}(+HS) and Syn2_{ect}(-HS) in PBS

IL-8 was present at 100 nM with Syn2_{ect}(+HS) (main Figure) and at 200 nM with Syn2_{ect}(-HS) (inset). The changes in fluorescence are plotted as the normalized change in fluorescence ($-\Delta F/F_0$, where F_0 is the initial fluorescence intensity before addition of ligand) against the concentration of ligand. The data represent mean values of three independent experiments, and were fitted as described in the Experimental section.

content of loops and other unstructured elements was evident from CD spectroscopy as well as from secondary structure prediction. These parts of the protein might adopt specific structures in the presence of covalently attached HS side chains. However, the far-UV spectrum of Syn2_{ect}(+HS) was dominated by the signal of the HS side chains (results not shown), which rendered a reliable analysis of the protein's secondary structure difficult.

We have recently published the dissociation constants for IL-8 with respect to size-defined GAG oligosaccharides [17]. It was therefore interesting to see how the K_d value of the IL-8-specific HSPG for the chemokine compares with these values. In Figure 4, the fluorescence binding isotherms of fluorescently labelled IL-8 with respect to Syn2_{ect}(+HS) and Syn2_{ect}(-HS) are shown.

Although, due to unspecific binding (most probably as a consequence of ligand-induced chemokine oligomerization), complete saturation could not be reached, fitting by non-linear regression to eqn (1) was reliable ($F_{\max} < 1$), giving a K_d for Syn2_{ect}(+HS) of 22.9 ± 3.9 nM. This value corresponds to the highest affinities found for binding of IL-8 to size-defined GAG oligosaccharides [17]. Interestingly, adding Syn2_{ect}(-HS) to fluorescently labelled IL-8 also led to fluorescence quenching (Figure 4, inset), indicating binding to the chemokine. However, since in these isothermal titration experiments saturation could not be reached, the K_d value for the interaction of Syn2_{ect}(-HS) and IL-8 can only be estimated to be > 1 μ M.

DISCUSSION

The interaction of IL-8 with GAGs is thought to mediate communication between endothelial cells and attracted neutrophils, which finally leads to the extravasation of the neutrophils to the site of inflammation [22]. We have shown recently that the interaction of IL-8 with heparin and HS is dependent upon the quaternary structure of the chemokine as well as upon the length of the GAG oligosaccharide under investigation [17]. Based on these findings, a model was put forward in which monomeric IL-8 is the biologically active form of the chemokine with respect to GAG binding, and that dimerization of IL-8 leads to a lower affinity for GAGs and consequently to dissociation of IL-8 from the endothelium at high chemokine concentrations. This can be regarded as a self-regulatory mechanism of the chemotactic gradient.

The interaction of GAGs with chemokines is therefore important for the biological function of these signalling molecules. Since HS commonly forms a part of cell surface proteoglycans, the aim of the present work was to identify a HSPG responsible for IL-8 binding. This was accomplished by co-immunoprecipitation, which showed that syndecan-2, which is constitutively expressed on endothelial cells, interacts with IL-8. Furthermore, syndecan-2 mRNA was found to be amplified in an inflammatory situation, suggesting the involvement of this proteoglycan in an IL-8-mediated inflammatory response. Our finding represents the first identification of a HSPG co-receptor for chemokine binding. This does not imply that syndecan-2 is the exclusive HSPG co-receptor for IL-8, although for the endothelial surface we have ruled out other syndecans. In order to identify further interaction partners of this chemokine on the endothelium, we are currently setting up a functional proteomic protocol.

In addition, the first structural characteristics of this proteoglycan were unravelled by CD spectroscopy, indicating that Syn2_{ect} adopts an all- β conformation. Comparison with secondary structure predictions revealed that the HS attachment site is not part of the β -sheets, but rather is located on an extended loop structure. Unfortunately, investigation of Syn2_{ect}(+HS) was hampered by the presence of HS chains which yielded a strong CD signal in the far-UV wavelength range. Both Syn2_{ect}(+HS) and Syn2_{ect}(-HS) have failed so far to yield protein crystals suitable for structural determination.

Isothermal fluorescence titration experiments indicated a high affinity of Syn2_{ect}(+HS) for its chemokine ligand, with a K_d value of 23 nM (see Figure 4). Compared with the affinities of IL-8 for size-defined GAG oligosaccharides, the value found for Syn2_{ect}(+HS) corresponds to the affinity of the best IL-8-binding HS octasaccharide fraction [17]. Interestingly, Syn2_{ect}(-HS) also exhibited a weak affinity for IL-8, with a dissociation constant in the micromolar range. In addition to chemokine-GAG

interactions, protein-protein interactions might thus contribute to the binding of IL-8 to syndecan-2. It could be hypothesized that this additional interaction impacts on the specificity of the chemokine-proteoglycan encounter.

Syndecan-2 has been proposed to play a major role in the mitogenic activity of granulocyte/macrophage colony-stimulating factor in osteoblasts [7]. This suggests a more general role for syndecan-2 in cytokine/chemokine signalling. However, this seems to depend upon the nature of the cells/tissue under investigation. Clasper et al. [8] found no indication of syndecan-2 binding to IL-8 on activated human macrophages. Similar to our findings, these authors found syndecan-2 to be transcriptionally up-regulated upon inflammatory stimulation. One explanation for the different results found in our work compared with the work of Clasper et al. [8] with respect to IL-8 binding to syndecan-2 would be that syndecan-2 bears different HS structures depending upon the cell type. In addition to the demonstration of cell-specific HS expression, this hypothesis would support the sequence-specificity of the chemokine-GAG interaction, and would also suggest that chemokines do not accumulate unspecifically on any GAG chains.

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