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Interactions of putative heparin-binding domains of basic fibroblast growth factor and its receptor, FGFR-1, with heparin using synthetic peptides

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We have examined structure-function relationships that have been proposed to account for the heparin-binding properties of basic fibroblast growth factor and its receptor, FGFR-1, using synthetic peptides, DNA synthesis assays and binding assays in a resonant mirror biosensor. The results suggest that the interaction of FGFR-1 with heparin may not be physiologically relevant and that the site of interaction of the polysaccharide on bFGF is more complex than has been anticipated.

***Keywords*: fibroblast growth factor, FGFR-1, heparin, synthetic peptides**

Introduction

Basic fibroblast growth factor (bFGF) must interact with both its tyrosine kinase receptors (FGFRs) and its heparan sulphate proteoglycan (HSPG) receptor to stimulate cell growth [1]. A variety of approaches, including the analysis of the growth-stimulatory effects of synthetic peptides [2] corresponding to sections of the amino acid sequence of bFGF, implicate Lys 35, Arg 53 and basic residues between Lys 128–Lys 138 as being critical in the bFGF-polysaccharide interaction. It has also been suggested that heparin and hence HS may also bind FGFR-1, the interaction being mediated by a sequence of amino acids, FGFR-1(160–177), situated in the first interloop domain [3]. We have used synthetic peptides to examine the ability of such regions to bind to heparin, using a biosensor-based assay, and to inhibit specifically bFGF-stimulated DNA synthesis in rat mammary fibroblasts.

Materials and methods

Materials and cells

Peptides were synthesized on a Milligen 9050 automated peptide synthesizer and were at least 95% pure. Porcine intestinal mucosal heparin and streptavidin were obtained from Sigma (UK) and *N*-hydroxysuccinimide amino caproate (LC) biotin was from Pierce Warriner (UK). The single-cell-cloned rat mammary (Rama) 27 fibroblast cell line [4] was used to measure DNA synthesis in quiescent cells as described [5]. Epidermal growth factor (EGF) was purified from mouse submandibular glands [6] and human recombinant 18 kDa bFGF was prepared as described [7].

Biotinylation of heparin

Heparin was biotinylated on the free amino groups that occur on some glucosamine residues along the polysaccharide chain [8]. *N*-hydroxysuccinimide amino caproate (LC) biotin (50 µl of a 50 mM solution in dimethyl sulphoxide) was added to 42 mg heparin in 100 µl deionised water and allowed to react overnight at room temperature. Unreacted biotin was removed by precipitation of the heparin with five volumes of 100% ethanol at $-20\,^{\circ}C$.

Binding assays

Streptavidin was coupled to aminosilane and carboxymethyl dextran cuvettes according to the manufacturer's instructions (Affinity Sensors, UK; Application notes 1.1 and 1.2). Biotinylated heparin (100 µl, 1 mg ml^{-1}) was then added to the cuvette. The refractive index of the heparin is below the limit of detection of the IAsys biosensor. The presence of heparin on the surface of the cuvette was thus

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ascertained indirectly, by its ability to bind bFGF. Control, streptavidin-derivatized cuvettes, failed to bind bFGF or the synthetic peptides (result not shown). Binding reactions in the IAsys biosensor (Affinity Sensors) and the subsequent surface regeneration were carried out in carboxymethyl dextran and aminosilane cuvettes at $20 \pm 0.1\,^{\circ}C$ as described (Affinity Sensors, Application note 3.6). Binding data were analysed with the non-linear curve fitting programme FastFit (Affinity Sensors) [9].

Results and discussion

Four of the synthetic peptides used in this study, bFGF (52–61) (numbering is from the initiator met), bFGF(117–126), bFGF(127–140) and FGFR-1(160–178), were designed from published data so as to antagonize specifically the interaction of bFGF and one of its receptors, FGFR-1, with heparin [2, 3, 10–15]. The region encompassed by bFGF(117–126) was originally reported to be involved in binding to heparin [2, 11] but subsequently it has been suggested that this peptide is involved in binding to FGFR-1 [16]. These peptides all contain amino acids with basic side chains. Therefore the peptides bFGF[(−55)–(−43)] and αS2 casein(214–222) were used as controls: bFGF[(−55)–(−43)] because it represents a basic sequence found only in 25 kDa N-terminally extended bFGF [1] which is thus irrelevant to the interaction of 18 kDa bFGF with its cellular receptors and heparin; αS2 casein(214–222) because it possesses the same charge as bFGF(117–126) and an analogous amino acid composition.

The four bFGF-derived synthetic peptides and the FGFR-1(160–178) peptide inhibited DNA synthesis stimulated by 1 ng ml^{-1} bFGF in Rama 27 cells with IC50 values between 10 and 100 μM (result not shown). Two lines of evidence suggest that the inhibitory effects of the peptides on bFGF-stimulated DNA synthesis are not specific to bFGF. Firstly, all but one of the peptides that inhibited bFGF-stimulated DNA synthesis also inhibited EGF-stimulated DNA synthesis (Figure 1). Secondly, the only peptide to inhibit selectively bFGF-, but not EGF-stimulated DNA synthesis was the control peptide bFGF[(−55)–(43)] which does not correspond to a sequence found in 18 kDa bFGF (Figure 1).

One possible reason for the failure of the bFGF-derived synthetic peptides, particularly bFGF(117–126) and bFGF(127–140) to antagonize specifically bFGF-stimulated DNA synthesis in Rama 27 cells is that the peptides do not bind to the same site in heparan sulphate/heparin as bFGF, or do so with too low an affinity. Similar considerations apply to FGFR-1(160–178). The results of the binding experiments indicate that the synthetic peptides that encompass regions of bFGF and FGFR-1 that have been shown by others to interact with heparin, bFGF(52–61), bFGF(117–126), bFGF(127–140) and FGFR-1(160–178) [2, 3, 10], do bind to heparin (Table 1). However, the affinity

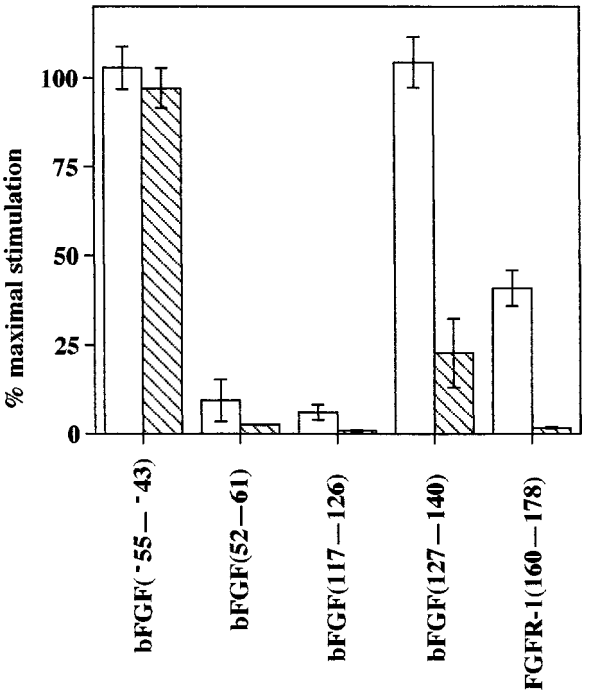

Figure 1. Inhibition of EGF-stimulated DNA synthesis by synthetic peptides. Quiescent Rama 27 fibroblasts were stimulated with 300 pg ml^{-1} EGF in the presence of 80 μM (□) and 200 μM (▨) of the synthetic peptides. Results are expressed as the mean dpm ± SD [^{3}H]-thymidine incorporated into DNA as a percentage of the incorporation observed in the absence of synthetic peptide (23792 ± 1179 dpm). In the absence of any additions the incorporation of [^{3}H]-thymidine into DNA was 1953 ± 43 dpm.

of the peptides for heparin is at least 100-fold lower than the affinity of bFGF for heparin, largely due to a reduction in the association rate constant (Table 1). Nevertheless the affinity of three peptides, bFGF(117–126), bFGF(127–140) and FGFR-1(160–178) for heparin may be sufficiently high (Table 1) to allow the occupation of a significant number of the binding sites in HS/heparin usually occupied by their corresponding protein in the DNA synthesis assays. Therefore the failure of bFGF(117–126), bFGF(127–140) and FGFR-1(160–178) to antagonize bFGF-stimulated DNA synthesis may be due to these peptides binding to a different sequence of saccharides in HS/heparin than that recognised by their corresponding protein.

Competition binding experiments were employed to examine whether the synthetic peptides recognized the same binding sites as bFGF in heparin. In the first set of experiments, the extent of binding of the peptides to immobilized heparin and to pre-formed complexes of bFGF-heparin was measured. In the second type of experiment, the extent of binding of bFGF to immobilized heparin and to preformed complexes of peptide-heparin was measured. In both cases a higher extent of binding of the ligate to the immobilized heparin compared to the preformed complexes of

Table 1. K_ds and rate constants of synthetic peptides and bFGF for immobilized heparin.

	bFGF	*FGFR-1 (160–178)*	*bFGF [(−55)–(−43)]*	*bFGF (52–61)*	*bFGF (117–126)*	*bFGF (127–140)*	*αS2 casein (214–222)*
K_d[a]	84 ± 55 nM	40 ± 18 μM	ND[b]	1.0 ± 0.2 mM	120 ± 50 μM	30 ± 4 μM	ND[c]
k_{ass}(Ms^{-1})[d]	93000 ± 25000	250 ± 120	ND[b]	ND[e]	59 ± 28	400 ± 16	ND[c]
k_{diss} (s^{-1})[f]	0.0068 ± 0.0004	0.0052 ± 0.0004	ND[b]	0.0069 ± 0.0009	0.0035 ± 0.0013	0.014 ± 0.0038	ND[c]
K_d[g]	74 ± 20 nM	20 ± 10 μM	ND[b]	ND[e]	60 ± 36 μM	35 ± 7 μM	ND[c]

[a] K_d values were calculated from the extent of binding observed at five or more different concentrations of ligate in three independent experiments. Two experiments were performed on heparin immobilized on a carboxymethyl dextran surface and one was performed on heparin immobilized on an aminosilane surface; the results are the mean value ± SE of the three independent determinations.
[b] Not determined, the affinity of bFGF[(−55)–(−43)] for heparin was too low to be measured, but was estimated from the amount of binding to heparin observed at 362 μM bFGF[(−55)–(−43) (12 arc seconds) to be at least 10-fold lower than the affinity of bFGF (52–61) for heparin.
[c] Not determined, since no binding of αS2 casein(214N–222) to heparin was observed even at concentrations as high as 1.72 mM.
[d] Results are the mean ± SE of three independent determinations.
[e] Not determined, since the k_{ass} of this peptide for heparin was too slow for it to be measured.
[f] Results are the mean ± SD of at least nine values, determined at three different concentrations of ligate in three independent experiments.
[g] K_d ± SE calculated from the ratio k_{diss}/k_{ass}.

heparin-bFGF or heparin-peptide would indicate competition for a common binding site in the heparin.

FGFR-1(160–178) and bFGF failed to affect each other's binding to heparin (result not shown), so the saccharide sequences that bind FGFR-1(160–178) are likely to be different from the Oligo-H type sequences [17] required by bFGF. Therefore, since Oligo-H type oligosaccharides are able to restore the activity of bFGF in HS-deficient cells [18], the interaction between FGFR-1(160–178) and the polysaccharide may not be directly involved in the interaction between bFGF and its two receptors.

The results of the competitive binding assays between bFGF(117–126) and bFGF are more difficult to interpret, since although bFGF bound equally to immobilized heparin and preformed complexes of bFGF(117–126)-heparin, bFGF(117–126) bound to a greater extent to preformed complexes of bFGF-heparin (1.93 ng mm^{-2}) than to immobilized heparin (1.23 ng mm^{-2}). The observation of changes in extent of binding may suggest that this peptide interacts with bFGF itself or with a region in heparin that is usually close to the saccharide sequences recognized by bFGF and which is more accessible to the peptide once bFGF is bound. In contrast, the extent of binding of bFGF(127–140) to preformed complexes of bFGF-heparin (1.31 ng mm^{-2}) was lower than to heparin alone (1.87 ng mm^{-2}) and the extent of binding of bFGF to preformed complexes of bFGF(127–140)-heparin (2.34 ng mm^{-2}) was lower than the extent of binding of bFGF to immobilized heparin (3.35 ng mm^{-2}). These results indicate that bFGF(127–140) is likely to bind to part of the sequence in heparin recognised by bFGF. Since there is a 10-fold difference in molecular weight between the peptide and bFGF, bFGF bound to heparin will produce a 10-fold higher signal on a molar basis in the IAsys biosensor than the peptides [21, 22]. Therefore the effect of bFGF(127–140) at concentrations comparable to its Kd for heparin in the competitive binding assays is not large. So while the results are consistent with the notion that bFGF(127–140) contains part of the heparin-binding region of bFGF, it seems likely that a substantial part of the heparin-binding site of bFGF may lie in other parts of the polypeptide.

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