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Receptor- and heparin-binding domains of basic fibroblast growth factor

(adhesion/angiogenesis/endothelial cell/growth inhibition)

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ABSTRACT **Two functional domains in the primary structure of basic fibroblast growth factor (FGF) have been identified on the basis of their ability to interact with the FGF receptor, bind radiolabeled heparin, and modulate the cellular response to FGF. Peptides derived from these two functional domains can act as partial agonists and antagonists in biological assays of FGF activity. Peptides related to the sequences of FGF-(24–68)-NH_2 and FGF-(106–115)-NH_2 inhibit thymidine incorporation into 3T3 fibroblasts when they are stimulated by FGF but have no effect when the cells are treated with either platelet-derived growth factor or epidermal growth factor. They also possess partial agonist activity and can stimulate DNA synthesis when tested in the absence of exogenous FGF. The active peptides have no effect on the binding of epidermal growth factor to its receptor on A431 cells and they can modulate the effects of FGF, but not fibronectin, on endothelial cell adhesion. The results suggest the possibility of designing specific analogs of FGF that are capable of inhibiting the biological effects of FGF.**

The molecular characterization of basic and acidic fibroblast growth factors (FGFs) has confirmed the existence of two classes of closely related angiogenic factors and helped establish the identity of an unusual family of mitogens (1–7). Acidic and basic FGFs exist in several molecular forms that are products of proteolytic processing at homologous sites (1–3, 8). Both growth factors stimulate a wide spectrum of target cells derived from the primary and secondary mesenchyme as well as from the neural crest. The growth factors bind immobilized heparin with an unusually high affinity, a characteristic that has been exploited for their isolation and characterization from many tissues (4, 9). Because both FGFs also have the capacity to stimulate neovascularization (1–4), their physiological functions have been associated with reproduction, growth, and development. The possible identity of FGF-related proteins with the tumor angiogenic factor activities described by Folkman (10) has suggested that this family of mitogens may also play a critical role in several pathophysiological processes including the growth of tumors, diabetic proliferative retinopathies, and the wound-healing response. The ability of basic FGF to stimulate nerve regeneration (11) and to induce a neovascular response in the chicken chorioallantoic membrane (12), the rat brain (13), the kidney capsule (14), and the carotid artery (unpublished work) has suggested a possible therapeutic application of FGFs in stimulating tissue regeneration, the recovery from episodes of ischemia, and tissue transplantation.

The identification of specific functional domains in the primary structure of basic FGF was examined as a first step in the design of recombinant analogs of basic FGF that have modified heparin-binding activity, increased biological activity, and/or antagonist activity. The results presented here identify two peptide sequences of interest (Fig. 1).

MATERIALS AND METHODS

Synthesis of FGF Fragments. A total of 25 peptides were synthesized with the goal of overlapping the 146 amino acids in the complete primary structure of basic FGF (see Table 1). Peptides were synthesized by solid-phase methodology and purified by gel permeation and ion exchange chromatography (15). Five peptides, FGF-(1–24)-NH_2, FGF-(24–68)-NH_2, [Tyr^{69}]FGF-(69–87)-NH_2, FGF-(93–120)-NH_2, and FGF-(121–146)-NH_2, were synthesized to encompass the major hydrophilic and hydrophobic sequences identified by hydrophilicity analysis of the full sequence. Overlapping sequences included a peptide that corresponds to the N-terminal extension of basic FGF, found in the sequence of γ-FGF, a naturally occurring analog of basic FGF, recently isolated by Ueno *et al.* (16), which was predicted by the cloning of basic FGF (17). The remaining peptides were synthesized to determine the minimal active cores of each functional domain or to test for sequence specificity. With three exceptions, [Tyr^{10}]FGF-(1–10), FGF-(110–118), and FGF-(111–118), the peptides were synthesized in the amidated form to increase stability. Peptides containing the C-terminal sequence of the native molecule [i.e., FGF-(103–146), [Tyr^{139}]FGF-(139–146)] were synthesized as free acids.

Interactions with the FGF Receptor. The effects of the synthetic peptides on the binding of ^{125}I-labeled FGF (^{125}I-FGF) to its receptors were examined in baby hamster kidney (BHK) cells because of their large number of receptors (18, 19). Cells were grown to subconfluence in 48-miniwell plates and washed with cold Ham's F12 medium/0.2% gelatin prior to the binding assay. Cells were incubated for 2 hr in 200 μl of this buffer containing 20–100 fmol of ^{125}I-FGF and various concentrations of peptides. The membranes were solubilized with 0.1% Triton X-100 and labeled FGF bound to the cells was determined. The specific activity of the growth factor was estimated >100,000 cpm/70 fmol and it was stable when stored at 4°C.

Heparin-Binding Sites of Basic FGF. The first attempts to identify the functional domain(s) in the primary sequence of FGF responsible for heparin binding were by chymotryptic mapping of intact FGF. Basic FGF (6 nmol) was incubated with heparin-Sepharose and then digested with chymotrypsin as described by Esch *et al.* (1), to generate a series of heparin-bound FGF fragments. The heparin-Sepharose beads were washed to remove the chymotryptic fragments of basic FGF that did not bind heparin. The chymotryptic fragments that remained bound were eluted with increasing concentrations of NaCl (0.6–2 M) and the eluate was analyzed by reverse-phase HPLC. In no instance were any peptide fragments of FGF recovered by these processes (results

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Abbreviations: FGF, fibroblast growth factor; ^{125}I-FGF, ^{125}I-labeled FGF; EGF, epidermal growth factor.

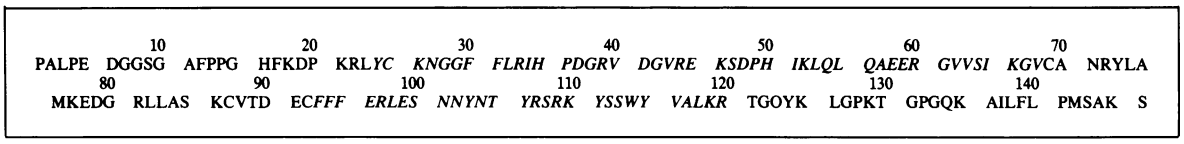

FIG. 1. Structure of basic FGF. FGF-(24–68) and FGF-(93–120) are set in italic type.

not shown) even though identical procedures in the absence of heparin yield specific fragments (1). Instead, the intact molecule was recovered with full intrinsic activity. Binding to heparin thus protects FGF from enzymatic cleavage and it was decided to examine the binding of synthetic peptide fragments.

The capacity of the peptides to bind heparin was tested by drying known amounts of the peptides onto uniform sheets of nitrocellulose that had been prepared with a paper punch. Peptides were first air dried and then further dried for 45 min in a vacuum oven at 80°C. Nitrocellulose papers were then wet with 50 mM Tris-buffered saline, pH 7.4 (TBS), and washed three times. Individual samples were incubated in counting vials for 16 hr with 0.1 μCi of [^{3}H]heparin (1 Ci = 37 GBq; New England Nuclear) in 4% (wt/vol) bovine serum albumin/TBS. Filters were washed four times with TBS and transferred to counting vials and radioactivity was determined in a Beckman β-counter. Background counts of control filters were <100 cpm.

Biological Activities of FGF Peptides. The possibility that the peptide fragments modulate the biological response to FGF was tested by examining their effects on thymidine incorporation by 3T3 cells, on endothelial cell proliferation, and on endothelial cell adhesion. The effects of FGF and these peptides on neurite extension by PC12 cells have been described elsewhere (20). Thymidine incorporation into 3T3 fibroblasts was tested by adding the peptides to serum-starved cells in the absence or presence of basic pituitary FGF for 24 hr. The cells were then incubated with 0.1 μCi of [^{3}H]thymidine (New England Nuclear) for 5 hr and the amount of radioactivity incorporated into the trichloroacetic acid-precipitable, NaOH-solubilized DNA was determined on a Beckman scintillation counter. The effects of the peptides on the growth of vascular and capillary endothelial cells *in vitro* were determined by adding the peptides in the absence or presence of FGF (1 ng/ml) to the cells 8 hr after plating. Cells were grown as described (1, 21) except that they were incubated for 4 rather than 6 days and they received only one treatment of peptide and FGF. At the end of the experiment, the cells were trypsinized and cell number was determined with a Coulter Counter.

The effects of FGF and peptide fragments on endothelial cell adhesion were tested as described (22, 23). Exponentially dividing endothelial cells were dissociated and labeled

Table 1. Receptor binding, heparin binding, and biological activities of basic FGF-related peptides

Peptide (I)	^{125}I-FGF bound, % control (II)	[^{3}H]Heparin bound, cpm × 10^{-3} (III)	Control [^{3}H]DNA, cpm × 10^{-3} (IV)	FGF [^{3}H]DNA, cpm × 10^{-5} (V)	ABAE cell, % basal (VI)	ABAE cell, % FGF (1 ng/ml) (VII)
FGF	100	0.1	1.3	28.9	100	100
[Tyr^{10}]FGF-(1–10)	87	0.1	1.2	26.4	89	97
[Tyr^{12}]FGF-(1–12)-NH_2	94	0.1	1.6	28.9	90	103
FGF-(1–24)-NH_2	100	0.2	1.0	28.0	95	98
FGF-(11–20)-NH_2	86	0.7	1.3	23.2	91	105
FGF-(16–24)-NH_2	83	7.2	2.0	21.7	92	104
FGF-(24–68)-NH_2	21*	212.6*	2.6*	13.5*	39*	70*
FGF-(25–37)-NH_2	92	39.4*	1.4	26.1	97	111
FGF-(30–39)-NH_2	94	0.3	1.9	31.7	99	87*
[Tyr^{50}]FGF-(30–50)-NH_2	78	0.1	3.0*	26.2	84*	88*
FGF-(32–39)-NH_2	85	0.3	1.4	27.6	65*	40*
FGF-(32–53)-NH_2	102	0.3	1.5	14.8*	91	99
FGF-(36–39)-NH_2	104	0.1	1.3	33.3	78*	56*
FGF-(46–53)-NH_2	115	0.4	2.0	24.9	95	92
[Tyr^{87}]FGF-(73–87)-NH_2	85	1.1	1.1	22.4	97	95
FGF-(93–120)-NH_2	0*	200.0*	—	—	—	—
FGF-(97–120)-NH_2	0*	123.8*	4.0*	15.2*	94	103
FGF-(100–120)-NH_2	0*	169.7*	3.5*	20.9*	100	93
FGF-(103–120)-NH_2	0*	135.9*	4.3*	17.7*	97	98
FGF-(103–146)	0*	173.8*	3.8*	9.9*	106	100
FGF-(106–115)-NH_2	10*	39.1*	3.6*	32.5	100	93
FGF-(106–120)-NH_2	0*	131.4*	9.4*	20.9*	96	97
FGF-(110–118)	94	0.1	1.0	—	103	91
FGF-(111–118)	83	0.3	0.5	—	100	—
FGF-(121–146)-NH_2	117	20.2*	2.0*	25.9	102	95
[Tyr^{139}]FGF-(139–146)	92	0.2	1.4	12.8*	101	99

Peptides related to the primary structure of basic FGF (column I) were tested for ability to inhibit ^{125}I-FGF binding to the FGF receptor on BHK cells (column II), to bind radiolabeled heparin (column III), to stimulate thymidine incorporation into quiescent 3T3 fibroblasts (column IV), to inhibit the stimulation of thymidine incorporation in 3T3 cells by FGF (column V), or to modify basal (column VI) and FGF-stimulated (column VII) vascular endothelial cell growth. Peptides were tested at a concentration of 100 μg per well with the exception that FGF-(30–39)-NH_2, FGF-(32–39)-NH_2, and FGF-(36–39)-NH_2 were tested in the proliferation assays (columns VI and VII) at a concentration of 1 mg/ml. ABAE, bovine aortic arch endothelial cells.

*Significantly different from control ($P < 0.05$).

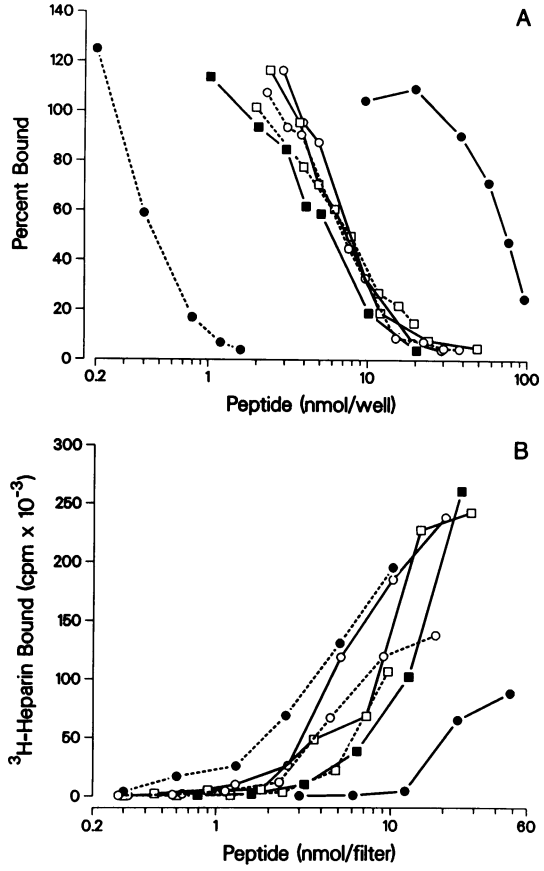

FIG. 2. (*A*) Inhibition of ^{125}I-FGF binding to its receptor by peptide fragments of FGF. FGF-(106–115)-NH_2 (●—●), FGF-(106–120)-NH_2 (□—□), FGF-(103–120)-NH_2 (■—■), FGF-(100–120)-NH_2 (○—○), FGF-(97–120)-NH_2 (□---□), FGF-(103–146) (●---●), and FGF-(24–68)-NH_2 (○---○) were added to cells at the indicated concentrations in 10 μl of 5 mM HCl and tested for ability to compete with 0.1 pmol of ^{125}I-FGF in binding to BHK cells. (*B*) The binding of radiolabeled heparin to these same fragments of FGF was tested after their adsorption onto nitrocellulose filters.

with [^{3}H]leucine overnight in Ca^{2+}/Mg^{2+}-free Dulbecco's modified Eagle's medium (DMEM)/2% calf serum. FGF, fibronectin, or one of the FGF-related peptides was adsorbed onto Petri dishes by incubating them in Hepes-buffered DMEM overnight at 4°C. The following day, the dishes were washed in Hepes-buffered DMEM/0.2% bovine serum albumin. The radiolabeled cells were then washed in this same buffer and pipetted onto the coated dishes for 100 min. Then, the medium was aspirated, the attached cells were dissolved in 3% Triton X-100, and the amount of radioactivity associated with the cells was determined.

RESULTS AND DISCUSSION

Inhibition of FGF Binding. The effects of the FGF-related peptides on the binding of ^{125}I-FGF to its receptor are presented in Table 1. The peptides could be divided into two distinct groups. One group is derived from the C-terminus of FGF, residues 103–146. This functional domain could be reduced to an active core of ten amino acids, residues 106–115. The second group is represented by the sequence of FGF-(24–68)-NH_2. The inhibition of ^{125}I-FGF binding to its receptor by peptides of either group is dose dependent and a potency analysis shows that extension of the C-terminus of FGF-(106–115)-NH_2 increases the potency of the molecule 10- to 100-fold (Fig. 2*A*). FGF-(24–68)-NH_2 has a potency similar to that of FGF-(100–120)-NH_2, FGF-(103–120)-NH_2, and FGF-(106–120)-NH_2 in the binding assay. The inability of smaller sequences within FGF-(24–68)-NH_2 (peptides 7–13 of Table 1) to inhibit binding of ^{125}I-FGF to its receptor has precluded the possibility of determining a smaller active core for this peptide. It is important to note that an identical inhibition of FGF binding by these peptides has been found on all FGF target cells examined to date. These include capillary and vascular endothelial cells, pituitary-derived GH_3 cells, fibroblasts (data not shown), and a neural cell line derived from the rat adrenal medulla, PC12 (20).

The effect of the FGF-related peptides on the binding of FGF to its receptor is specific. This was tested by measuring the ability of the peptides to block the binding of radioiodinated epidermal growth factor (EGF) to the epidermal cell line A431. Neither basic FGF nor any of the related peptides affected the binding of ^{125}I-labeled EGF to its receptor (results not shown).

Heparin-Binding of FGF-Related Peptides. The identification of sequences in basic FGF that bind heparin was attempted because of the important relationship between this glycosaminoglycan and FGF. Heparin-Sepharose affinity chromatography is used in the purification of FGF from many tissues (4). Heparin also modifies the activity of FGFs (24, 25) and heparan sulfate may be the component of the extracellular matrix that stabilizes FGF in the basement membrane (26–28).

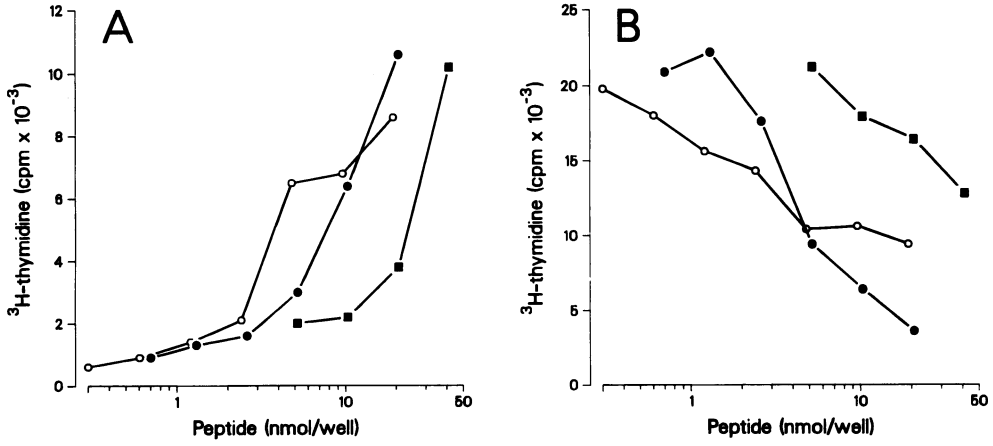

FIG. 3. Effect of FGF-related peptides on incorporation of thymidine by 3T3 fibroblasts. Cells were growth arrested with low serum (0.5%) and incubated with the indicated concentrations of FGF-(100–120)-NH_2 (■), FGF-(103–146) (●), or FGF-(24–68)-NH_2 (○) alone (*A*) or in the presence (*B*) of 60 fmol (1 ng) of basic FGF.

Seven of the 25 peptides tested increased the amount of [^{3}H]heparin that binds the nitrocellulose filters by more than 1000-fold (column III of Table 1). Of these, 6 contain residues 106–115 and the other was the peptide FGF-(24–68)-NH_2. The binding of radiolabeled heparin is dose-dependent (Fig. 2*B*) and can be displaced by unlabeled heparin (results not shown). Peptides containing residues 106–120 have a greater affinity for heparin and an increased capacity for heparin binding when compared to FGF-(106–115)-NH_2. By virtue of this capacity to bind heparin, these peptides are also capable of inhibiting FGF binding to the "nonspecific component" of the radioreceptor assay that has been identified by Moscatelli (19) as being due to the presence of cell-associated glycosaminoglycans (data not shown).

FGF-(16–24)-NH_2 and FGF-(25–37)-NH_2 are fragments of the heparin-binding consensus sequence located at residues 20–30 of basic FGF (1). These peptides bind [^{3}H]heparin, albeit with low capacity and affinity, supporting the observation that sequences near the N terminus of FGF have intrinsic heparin-binding activity. The ability of several peptide fragments derived from the sequence of FGF-(24–68)-NH_2 to bind low levels of [^{3}H]heparin suggests that the high heparin-binding activity of FGF-(24–68)-NH_2 may be due to cumulative effects of smaller sequences with heparin-binding activity rather than to a specific locus. The capacity of FGF-(73–87)-NH_2 to bind low levels of heparin supports the hypothesis of multiple heparin-binding sites in the sequence of FGF-(1–146) and is compatible with the observation described here and reported by others (29) that, when bound to glycosaminoglycans, FGF becomes extremely stable and resistant to enzyme degradation.

Biological Activities of FGF-Related Peptides. The fact that the FGF-related peptides can bind to heparin and interact with the FGF receptor suggests that they might also be capable of modulating the biological response of FGF target cells.

Thymidine incorporation into 3T3 fibroblasts. Several FGF-related peptides have partial agonist activities in mitogenic assays for FGF (Table 1, column IV). The active peptides are identical to those identified in the receptor- and heparin-binding studies described above. FGF itself is a potent stimulator of thymidine incorporation in these cells and can increase thymidine incorporation 25-fold (Table 1, column V). The peptides were also tested for capacity to modify the response to FGF. In these studies, all of the peptides with agonist activity inhibited the stimulation of thymidine incorporation by FGF. A potency analysis of their agonist activity (Fig. 3*A*) showed that the peptides have a similar dose–response relationship as in other assays. When the peptides were tested in the presence of FGF (Fig. 3*B*), they inhibited [^{3}H]thymidine incorporation in a dose-dependent fashion.

The effects of these peptides on the response to FGF are specific. This was established by examining their effects on the capacity of platelet-derived growth factor (PDGF) to stimulate thymidine incorporation. Basal [^{3}H]thymidine incorporation is increased from 1100 ± 200 cpm to 31,800 ± 11,400 cpm with the addition of PDGF (10 ng/ml). The coincubation of FGF-(24–68)-NH_2 (100 μg per well) with PDGF or FGF-(106–120)-NH_2 (100 μg per well) with PDGF gave values of 26,600 ± 3100 and 34,100 ± 3700 cpm, respectively.

Endothelial cell growth. The biological activities of the peptide fragments of FGF were also tested with vascular and capillary endothelial cells. Unlike the results obtained with 3T3 cells, experiments with endothelial cells failed to identify any partial agonist activity of the FGF-related peptides (Table 1, column VI). Instead, the results identify partial antagonist activity. Peptides derived from FGF-(24–68)-NH_2 and with a minimum active core of FGF-(36–39)-NH_2 appear to inhibit basal and FGF-stimulated growth of vascular and capillary (data not shown) endothelial cells (Table 1, columns VI and VII). Although the results with 3T3 cells (above) suggest the antagonist effects of the peptides on FGF-stimulated endothelial cell growth, the observation that the peptides also inhibit basal cell growth (Table 1, column VI) was surprising. The recent demonstration that basal endothelial cell growth may be due to the autocrine release of FGF by endothelial cells (26–28) would explain the inhibitory activity of the peptides. Attempts to demonstrate an

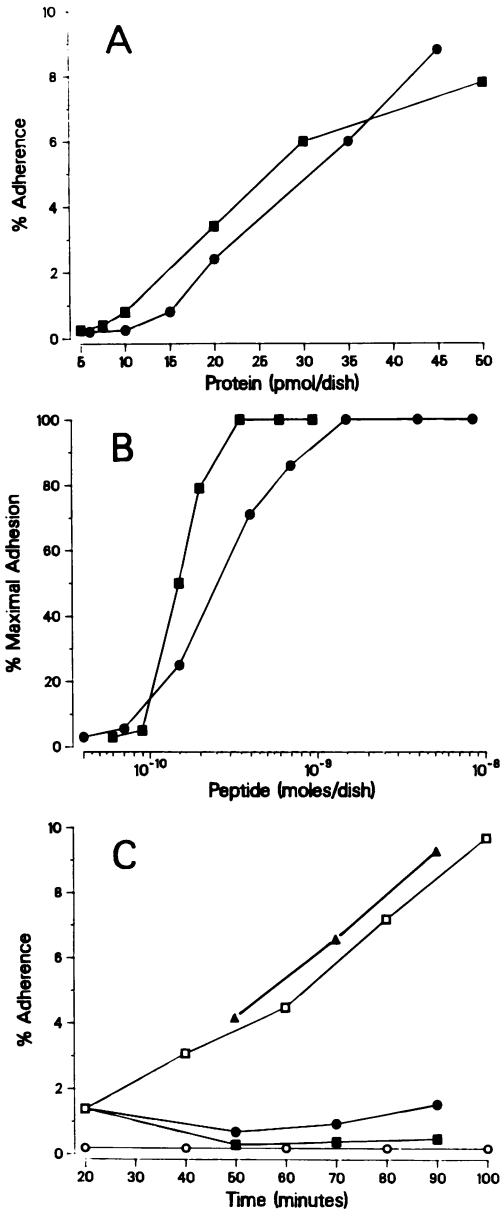

FIG. 4. Effect of FGF-related peptides on adhesion of endothelial cells to FGF and fibronectin. Adhesion of endothelial cells to a FGF (■)- or fibronectin (●)-coated substratum (*A*) or to a FGF-(93–120)-NH_2 (■)- or FGF-(24–68)-NH_2 (●)-coated substratum (*B*) was tested by adsorbing the protein to plastic and examining its capacity to stimulate the cell attachment. Peptides unrelated to these two functional domains were unable to stimulate endothelial cell adhesion. (*C*) Inhibition of endothelial cell adhesion to the FGF-coated substratum by the peptides was tested by adsorbing 50 pmol of FGF to dishes prior to addition of the radiolabeled cells in media alone (□) or in the presence of 18 nmol of FGF-(93–120)-NH_2 (●), 10 nmol of FGF-(24–68)-NH_2 (■), or 19 nmol of FGF-(1–24)-NH_2 (▲). Less than 0.2% cell adhesion was observed in dishes not treated with FGF (○).

inhibitory effect of FGF-(106–115)-NH_2 on vascular or capillary endothelial cells have been unsuccessful, possibly due to the specific requirements of the proliferation assay (i.e., 10% serum, 72- to 96-hr assays at 37°C vs. no serum, 2-hr assays at 4°C in the binding assays).

Cell adhesion and interactions with heparin. FGF, fibronectin, and several other heparin-binding molecules promote cell adhesion when they are adsorbed to a normally nonadhesive plastic surface (Fig. 4*A*). FGF and fibronectin are equipotent in stimulating the adhesion of endothelial cells (Fig. 4*A*), showing that FGF may function as an adhesion molecule, as well as a growth factor, differentiation factor, and morphogen. FGF-(24–68)-NH_2 and FGF-(93–120)-NH_2 are relatively potent stimulators of cell adhesion, a result consistent with their partial agonist activity (Fig. 4*B*). Peptides unrelated to these two domains have no effect. When the active peptides were tested for capacity to modify the biological effects of FGF, they were found to inhibit the adhesion of endothelial cells to FGF-treated plastic surfaces (Fig. 4*C*). Thus, they prevent endothelial cells from binding adsorbed FGF and attaching to the plastic substratum. When this experiment is repeated with a fibronectin substratum, none of the synthetic peptides block endothelial cell attachment (data not shown).

The mechanism through which these peptides inhibit the response to FGF appears to involve their capacity to inhibit the binding of FGF to its receptor and to heparin. Although charge and amphiphilicity might account for their effects, it is important to note that a peptide such as FGF-(121–146) has a high positive charge (+5) and is amphiphilic (residues 123–130) and yet has no effect on the parameters measured here. This is not to say that the charge or amphiphilic nature of the peptides is not involved in the binding of FGF to its receptor. Study of other analogs will be necessary to address this question.

The use of synthetic peptides to probe for functional domains of molecules has been applied by Heath and Merrifield (30), who have recently used solid-phase synthesis to identify the peptide sequences containing the active core of EGF. Similar studies with transforming growth factor type α have identified receptor binding domains (31). Peptide fragments of large proteins have also been used as probes for antigenicity and antibody development (32). Although many higher molecular weight proteins are difficult to map into discrete functional amino acid sequences, a number of successes have been reported including the cell recognition site of fibronectin (33). This molecule, which like basic FGF, is intricately involved in cell growth and adhesion, contains a peptide sequence (Asp-Gly-Arg-Xaa) that mimics the cell recognition site of fibronectin. Peptides with this sequence can inhibit metastases of melanoma cells (34), presumably by virtue of their ability to inhibit fibronectin binding. It may thus be reasonable to propose that the analogs of peptide fragments of FGF described here, some of which are 1000 times more potent than the fibronectin-related peptide, may be capable of inhibiting the biological activities of FGF *in vivo*.

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