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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Fibroblast growth factors (FGFs) are a family of 23 heparin-binding polypeptides with diverse biologic functions. FGF-2 is stored in an immobile, functionally inactive form in the extracellular matrix. Since FGF-2 exerts its biologic roles by binding to cell surface receptors, a major interest is how FGF-2 reaches the cell surface from its inactive pool in the extracellular matrix. While heparinases and proteases can cleave components of the extracellular matrix and thus release FGF-2, an alternative strategy is the secretion of a soluble binding protein, FGF-Binding Protein (FGF-BP). Using a number of investigative techniques, our laboratory is studying the protein-protein interactions enabling this binding protein to function in its hypothesized role, that FGF-BP can release FGF-2 from its inactive reservoir in the extracellular matrix, making FGF-2 functionally available. We have produced two recombinant FGF-BP1 proteins, have documented reversible binding to FGF-2, and have extensively studied their protein-protein interactions. We have demonstrated that FGF-BP1 and heparansulfate compete for binding to FGF-2, and that FGF-2 can bind its receptor in the presence of FGF-BP1. We have also shown that, in numerous biologic systems, FGF-BP1 can positively modulate FGF-2 biologic activity.

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Introduction

Fibroblast Growth Factors (FGF's) are a major family of proteins which mediate physiologic processes including angiogenesis, tumorigenesis, and neuroprotection. The extracellular activity of these proteins, especially that of FGF-2, the major FGF studied, is not well-correlated with their concentrations, largely because FGF's are sequestered in the extracellular matrix by binding to heparan sulfate proteoglycans. A novel protein has been described which binds, protects, and presents FGF-2 and possibly other FGF's to their extracellular cell-surface receptor. We hypothesize that it is the regulation of this protein, FGF-Binding Protein (FGF-BP1), which controls the physiologic concentration of FGF-2. We produced two recombinant FGF-BP1 proteins with which we have studied the binding properties and biology of FGF-BP1. We have confirmed that FGF-BP1 specifically and reversibly binds FGF-2 as well as FGF-1. Additionally, we have been able to examine the role of FGF-BP1 in models of angiogenesis and anchorage independent growth. We have used phage display technology to look for peptides which bind FGF-BP1 or FGF-2 with superior specificity and affinity. In summary, we have demonstrated some of the binding characteristics and kinetics of FGF-BP1, and found that it can positively modulate the angiogenic and tumorigenic properties of FGF-2.

Body

FGF-Binding Protein 1 (FGF-BP1) was first described in 1991 as a binding protein for FGF-1 and FGF-2 and was suggested to reversibly bind these proteins[1]. This initial report presented data which also suggest that FGF-BP1 modulates the activity of FGF-1 and FGF-2, and that this inhibition, as well as the binding, can be abolished by heparin.

A series of studies in our laboratory have demonstrated that FGF-BP1 plays a crucial role physiologically in embryogenesis and pathologically during epithelial carcinogenesis[2-4]. FGF-BP1 expression at the mRNA and protein levels are highly regulated through these processes. Most significantly, studies using transfection and ribozyme-targeting of FGF-BP1 mRNA indicate that FGF-BP1 might actually be rate-limiting in angiogenesis and carcinogenesis in a number of *in vitro* and *in vivo* models, proposing FGF-BP1 as the "angiogenic switch" for human cancer.

In addition to human FGF-BP1, homologues have been identified in mouse, rat, xenopus frog, and cow. Additionally, FGF-BP2 has been described, as has the EST for FGF-BP3. Having identified FGF-BP1 as a component of bovine prepartum mammary gland secretions, Lametsch et al. found that bovine FGF-BP1 shares all 10 conserved cysteine amino acids with mouse and human FGF-BP1 homologues, suggesting similar tertiary structure across species, as well as raising the possibility that this protein plays crucial roles in physiologic and pathologic processes in mammalian biology.[5]

Heparan sulfate proteoglycans (HSPGs) bind FGF-2 and are present either as cell-surface bound molecules or in the extracellular matrix. Binding of FGF-2 to the basement membrane is due to the HSPGs contained therein. Moreover, binding of FGF-2 to its high-affinity receptors requires cell-surface HSPGs, and several of FGF-2's functional effects appear to be dependent on presentation through heparan sulfate. Heparan sulfate and other HSPGs thus function in the sequestration of FGF-2 in the extracellular matrix and its presentation to its receptors on the cell surface.

Our research is the first systematic analysis of the direct effects of recombinant FGF-BP1 in *in vitro* and *in vivo* systems (See Appendix I). Our data confirm earlier studies by Wu et al. that FGF-BP1 binds FGF-2 in a specific, reversible manner, as well as earlier studies by our laboratory which suggested that FGF-BP1 serves as the angiogenic switch in human cancer.[1, 4]

To evaluate the direct effects of FGF-BP1 on FGF-2 dependent activities, we produced two recombinant FGF-BP1 proteins, one in each prokaryotic and eukaryotic systems. A GST-FGF-BP1 fusion protein was made in bacterial cells, and a bilaterally histidine-tagged FGF-BP1 recombinant protein (His-FGF-BP1) was made through baclovirus infection of Sf-9 insect cells. To assess if these proteins were purified to homogeneity we analyzed them on polyacrylimide gel under denaturing conditions and visualized with silver staining technique. A primary polyclonal antibody generated against a peptide sequence of FGF-BP1 recognized FGF-BP1 protein as a discrete band of appropriate molecular size in a Western Blot of this protein gel.

In order to determine whether these recombinant FGF-BP1 proteins retained ability to bind to FGF-2 as described, we immobilized recombinant FGF-BP1 on wells, blocked non-specific binding sites, and tested for binding of ^{125}I-FGF-2 to these wells, compared to binding of radiolabeled ligand to wells not coated with FGF-BP1. We found that ^{125}I-FGF-2 binds both recombinant FGF-BP1 proteins in a dose-dependent, saturable

manner, and that excess non-radiolabeled FGF-2 could abolish binding of the radiolabeled ligand to FGF-BP1.

Since the histidine-tagged recombinant FGF-BP1 protein contains a smaller modification, and both recombinant proteins exhibited reversible binding to FGF-2, we proceeded to use only the histidine-tagged recombinant protein for further experimentation.

In order to further evaluate the binding characteristics of FGF-BP1, we evaluated the ability of other ligands to compete with ^{125}I-FGF-2 for binding to FGF-BP1. We tested other members of the FGF family (FGF-1, 4, 5, 6, and 9), another heparin-binding proteins (platelet-derived growth factor), a non-heparin binding growth factor (EGF), FGF structural analogs (IL-1α and IL-1β), as well as heparansulfate proteoglycans and related molecules (heparansulfate, heparin, and pentosanpolysulfate). We found that FGF-1 could compete with ^{125}I-FGF-2 for binding to immobilized recombinant FGF-BP1, suggesting that FGF-BP1 binds FGF-1 at a site on FGF-BP1 which overlaps with its FGF-2-binding site. However, we found that FGF-4, 5, 6, and 9 could not compete with ^{125}I-FGF-2 for binding to recombinant FGF-BP1, suggesting that these other FGF's either do not bind FGF-BP1, or do not bind at a site which overlaps with the FGF-2-binding site. Furthermore, neither EGF nor IL-1α and IL-1β were able to compete for binding to FGF-BP1, nor could platelet-derived growth factor, the latter suggesting that FGF-BP1 does not bind ligands simply by binding to a heparin binding site. Interestingly, heparin and heparan sulfate could compete with ^{125}I-FGF-2 for binding to FGF-BP1, suggesting that FGF-2 can bind either FGF-BP1 or heparan sulfate (or heparin), but not both simultaneously. Furthermore, pentosanpolysulfate (PPS), a heparinoid used as an anti-angiogenic agent in clinical trials, was able to inhibit binding of ^{125}I-FGF-2 to FGF-BP1 at concentrations of PPS lower than those found in serum of patients in clinical trials. These competition experiments using HSPG's suggest that FGF-BP1 can effectively displace FGF-2 from heparan sulfate in the extracellular matrix, strongly supporting its role as a secreted carrier protein which mobilizes FGF-2 from its inactive pool in the extracellular matrix, to the cell surface.

Since we hypothesize that FGF-BP1's activity as a secreted carrier protein can positively modulate FGF-2 biologic activity, we tested the ability of FGF-BP1 to enhance FGF-2's angiogenic effects in a chick chorioallantoic membrane (CAM) assay. We found that FGF-BP1 enhances FGF-2-induced angiogenesis, resulting in increased and directed neovasculature.

FGF-2 binds to cell-surface tyrosine kinase receptors, FGF-Receptors 1 through 4, to achieve biologic effects. In order to determine whether FGF-BP1 would have detrimental effects on this protein-protein interaction, we tested FGF-2 binding to FGF-R1 in a cell-free system, and found that FGF-2 can bind FGF-R1 in the presence of excess recombinant FGF-BP1. This finding confirms that, acting as a secreted carrier protein, FGF-BP1 would not interfere with FGF-2 binding its cell-surface receptors after FGF-BP1 solubilizes FGF-2 from the extracellular matrix.

We have performed numerous experiments with phage display in an effort to identify novel peptides that can bind FGF-2, FGF-BP1, and other proteins that function in the angiogenic process. Our laboratory has successfully designed and produced phage peptide libraries that contain peptide fragments from three cancer cell lines: MDA-MB-231 breast carcinoma, 1205LU melanoma, and LS174T colon carcinoma. We have

performed numerous experiments where we have attempted to identify peptide fragments which demonstrate selective binding to recombinant FGF-BP1, in an effort to identify novel binding partners which could be either (1) FGF-2 fragments which bind FGF-BP1, (2) other angiogenic molecules which bind FGF-BP1, or (3) peptide fragments which bind FGF-BP1 and could potentially inhibit binding of FGF-BP1 to FGF-2 (and thus inhibit FGF-BP1 biologic and pathologic effects). We have been able to perform sequential biopanning rounds, with positive (immobilized FGF-BP1) and negative (control immobilized protein) targets, and have found some phage enrichment. Sequencing of the inserted phage DNA has not identified any recognized or consistent sequences.

We have also pursued biopanning using random peptide phage libraries with seven amino acids presented in a cyclic manner (flanking cysteine residues forming a disulfide bond), and have also performed experiments with successive biopanning rounds. We are still attempting to identify peptide sequences with enhanced binding to target. We have additionally performed experiments on monolayer cells where we attempt to select for peptides with enhanced binding to cancer (glioblastoma or breast cancer), compared to control, cells.

In conclusion, we are aggressively pursuing our investigation of protein-protein interactions which drive breast cancer growth, and have made significant findings regarding the protein-protein interactions of a major angiogenic molecule, FGF-2, with its secreted carrier protein, FGF-BP1. Knowledge of the interaction of FGF-2 with FGF-BP1 is of major importance because there is increasing evidence that suggests that FGF-BP1 acts as an "angiogenic switch," such that immobilized FGF-2 in the extracellular matrix is made functionally available through the secretion of the carrier protein, FGF-BP1. We have purified two recombinant FGF-BP1 proteins, have documented saturable, reversible binding to FGF-2, demonstrated that FGF-BP1 and heparan sulfate proteoglycans can compete for binding to FGF-2, that FGF-2 can still bind to its cell-surface receptor in the presence of FGF-BP1, and that FGF-BP1 can positively modulate FGF-2 biologic activity in an *in vivo* model of angiogenesis.

Key Research Accomplishments

--We produced and purified two recombinant FGF-Binding Protein (FGF-BP1) proteins in procaryotic and eucaryotic systems;

--we determined the binding characteristics and kinetics of FGF-BP1 binding to FGF-2, FGF-1, heparin sodium, heparan, and Pentosan Polysulfate (PPS);

--we demonstrated that recombinant FGF-BP1 does not interfere with FGF-2 binding to FGF-R1, its tyrosine-kinase cell-surface receptor;

--we demonstrated that recombinant FGF-BP1 added exogenously to an *in vivo* model of angiogenesis (chorioallantoic membrane assay) can positively modulate the effects of FGF-2.

Reportable Outcomes

(Although there are no Reportable Outcomes at this time, we have submitted a paper to the *Journal of Biologic Chemistry* for publication; it is currently being revised, and a copy is included in Appendix I.)

Conclusions

We demonstrate both *in vitro* and *in vivo* that FGF-BP1 positively modulates FGF-2 biologic activity. We document that FGF-BP1 binds FGF-2 reversibly, that this binding can be disrupted in a dose-dependent manner by FGF-1, heparin, heparan sulfate, and PPS. Moreover, structural analysis using mass spectroscopy confirm binding of FGF-BP1 and FGF-2 at the biochemical level. *In vitro* studies show that FGF-BP1 positively modulates the tumorigenic and angiogenic effects of FGF-2 at physiologic concentrations of both proteins. We found in an *in vivo* model of angiogenesis, the CAM assay, that maximal stimulation of angiogenesis required the addition of FGF-BP1.

Both heparin and FGF-BP1 have been shown to bind FGF-2 in the extracellular matrix, However, the relationship between the roles of heparin and FGF-BP1 remains unclear. While it is established that FGF-2 is sequestered in the extracellular matrix predominantly bound to heparin moieties in the basement membrane, the solubilization of FGF-2 appears to be due to the arrival of either heparinases or of FGF-BP1. The added function of protection is afforded by FGF-BP1 and heparin, whereas heparinases and other proteases may serve equally to digest FGF-2 *in vivo*..

It therefore appears as though FGF-BP1 and heparin-like molecules act in surrogate roles in the binding, protection, and possibly even the presentation of FGF-2. It is consistent with all reported studies on FGF-BP1 that it serves, when present, to shuttle FGF-2 from the heparin in the extracellular matrix to that on the cell surface, therefore ushering in the enhanced activity of FGF-2-dependent functions, as detected by our various assays. This model is well-supported by hybridization studies which demonstrate that FGF-BP1 mRNA expression is highly regulated and its presence is strongly correlated with the effects of FGF-2-dependent activity. Whereas most adult tissues have no basal FGF-BP1 mRNA production, a pre-malignant focus can, by production of FGF-BP1, mobilize the extracellular, heparin-bound FGF-2 and thus switch on the angiogenic machinery essential for malignant growth.

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Appendices

I. Al-Attar, A.*, Tassi, E.*, Aigner, A., Swift, M., McDonnell, K., Karavanov, A., Wellstein, A. Enhancement of fibroblast growth factor (FGF) activity by an FGF-binding protein. *manuscript submitted.*

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Enhancement of fibroblast growth factor (FGF) activity by an FGF-binding protein

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Abstract

Fibroblast growth factor-binding protein 1 (FGF-BP1) is a secreted protein, which can bind FGF-1 and FGF-2. These FGFs are typically stored on heparansulfate proteoglycans in the extracellular matrix in an inactive form and it has been proposed that FGF-BP1 functions as a chaperone molecule that can mobilize locally-stored FGF and present the growth factor to its tyrosine kinase receptor. FGF-BP1 is upregulated in squamous cell, colon and breast cancers and can act as an angiogenic switch during malignant progression of epithelial cells. For the present studies we focused on FGF-2 and investigated its interactions with recombinant human FGF-BP1 protein as well as the effects on signal transduction, cell proliferation and angiogenesis. We show that FGF-BP1 specifically recognizes and binds FGF-2. Furthermore, FGF-2 dependent ERK2 phosphorylation and proliferation of NIH-3T3 fibroblasts is enhanced by addition of FGF-BP1. Finally, in the chicken chorioallantois membrane angiogenesis assay FGF-BP1 synergizes with FGF-2. We conclude that FGF-BP1 directly binds to FGF-2 and positively modulates the biological activities of this growth factor.

Introduction

Fibroblast Growth Factors (FGFs) represent a family of at least 20 distinct proteins that are widely expressed in various tissues. FGFs have been reported to be involved in both development and adult tissue homeostasis, as well as in angiogenesis and cancer progression. FGF-2 (basic FGF), a 16 to 18 kDa protein is amongst the best-studied of this family and has been shown to have a variety of biological effects in different cells and organ systems, including embryonic development, tumorigenesis and angiogenesis (for a review see e.g.1,2).

FGF-2 interacts with low affinity cell surface and extracellular matrix heparan sulfate proteoglycans (HSPGs), which enable the growth factor to bind and activate its high affinity tyrosine kinase receptors (FGFRs), thereby forming a trimolecular active complex (3-6). It has been reported that cell surface HSPGs can modulate the action of FGF-2 by increasing its affinity for FGFRs (7). Moreover, HSPGs seem to protect FGF-2 from degradation by proteases in the extracellular environment (8,9) and to modulate the bioavailability of FGF-2, generating a local reservoir for the growth factor (10). The binding of FGF-2 to the cell surface receptor induces receptor tyrosine-kinase dimerization and autophosphorylation (11). The phosphorylated FGFRs associate and subsequently activate SH2 domain-containing downstream signaling molecules, such as PLCγ (12,13) and Src (14,15). Moreover, upon ligand-dependent receptor autophosphorylation, adaptor proteins, such as Grb2 and Shc, link the FGFRs to the Ras/MAP kinase signaling cascade (16-18). Grb2 and Shc form a complex with the GDP/GTP-exchange factor Son of Sevenless (Sos), which results in the translocation of Ras to the plasma membrane and its further activation by the exchange of GDP for GTP

by Sos. Thus, activated Ras leads to the consecutive activation of a cascade of protein kinases involving Raf, MEK and the $p42/44^{MAPK}$, also known as extracellular signal-regulated kinase 1 and 2 (ERK-1 and ERK-2) (16,18).

FGF-2 lacks the classic leader sequence, which targets intracellular proteins for secretion to the extracellular environment and several reports indicate that FGF-2 secretion occurs via ER- and Golgi-independent passive processes (19-21). In addition to the requirement for extracellular secretion, FGF-2 needs to be released and solubilized from the extracellular matrix (ECM) to act on its receptor. In comparison to other members of the FGF family, FGF-2 is tightly bound to the ECM and is a relatively abundant protein in numerous adult tissues from which it can be extracted as a biologically active growth factor (22). In addition to FGF-2, several other less-abundant members of this growth factor family are also stored in the ECM, although, they have a lower affinity for glycosaminoglycans and are released easier. Two distinct mechanisms have been described, by which locally stored FGF-2 can be released from the ECM. One mechanism involves digestion of the protein backbone of the proteoglycans that carry the heparansulfates and other glycosaminoglycans. In particular, it has been described that heparinases or other glycosaminoglycan-degrading enzymes can fulfill this function (23-25). Binding of FGF-2 to an extracellular chaperone protein represents a separate mechanism for FGF-2 release and solubilization from the ECM. Studies from our laboratory showed earlier that the binding of FGF-2 to a secreted binding protein (FGF-BP) might represent such a mechanism (26,27). Wu et al (1991) initially described FGF-BP as a low-affinity, heparin-binding protein, isolated from the human epidermoid carcinoma A431 cells. FGF-BP has been shown to bind to FGF-1 and FGF-2 in a non-

covalent, reversible manner. Moreover, FGF-BP protects and presents FGF-2 to its high-affinity cell-surface receptor (26-28) and a recent paper demonstrates the interaction of FGF-BP with perlecan, a member of the heparan sulfate proteoglycans of the basement membrane (29). This most likely represents a local reservoir for FGF-BP. A related protein, designated FGF-BP2, has been recently identified by our laboratory (2) and we will thus refer to the original protein as FGF-BP1.

FGF-BP1 is expressed below detection by Northern blotting in normal adult human tissues, whereas its expression is significantly elevated in various tumors, including head and neck, skin, cervix, and lung squamous cell carcinomas (26,29). In addition, FGF-BP1 is upregulated in some colon cancers and breast adenocarcinomas (27). Furthermore, we have recently shown that phorbol esters as well as epidermal growth factor (EGF) can upregulate FGF-BP1 gene transcription (30-32). We previously reported that expression of the human FGF-BP1 cDNA in the FGF-2 positive SW-13 cells led these cells to grow anchorage-independently (26). Likewise, whereas wild type SW-13 cells did not form tumors in nude mice, FGF-BP1-overexpressing SW-13 cells grew into highly vascularized tumors. Finally, we showed earlier that the depletion of FGF-BP1 from squamous cell carcinoma (SCC) and colon adenocarcinoma cell lines by ribozyme-targeting resulted in a significant reduction of tumor growth and angiogenesis. In summary, these regulation, expression and depletion experiments supports a role of FGF-BP1 as a pro-angiogenic molecule in human tumors (27).

In the present study we used recombinant FGF-BP1 protein to directly evaluate its binding to FGF-2 *in vitro* and study the functions of the protein. We found that FGF-BP1 was able to bind ^{125}I-FGF-2 in a dose-dependent and specific manner and can be

competed by FGF-1 and FGF-2. Furthermore, we studied the role of FGF-BP1 on the activation of the Ras/MAP kinase signaling pathway and on the mitogenic response of FGF-1- and FGF-2-treated NIH-3T3 fibroblasts. We demonstrate that FGF-2-induced ERK2 phosphorylation and proliferation were enhanced by addition of FGF-BP1. Finally, in chorioallantoic membrane (CAM) assays we found a significant FGF-BP1-dependent increase of FGF-2-mediated angiogenesis. Thus, our results indicate that the FGF-BP1 protein positively modulates the biochemical and biological activity of FGF-2 in multiple models.

Materials and Methods

Cell Cultures

NIH-3T3 cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies Inc., Gaithersburg, MD) supplemented with 10 % (v/v) calf serum. Sf-9 cells (BD PharMingen, San Diego, CA) were cultured in EX-Cell 400 media (JRH Bioscience, Lenexa, KS), supplemented with 5% (v/v) fetal calf serum in a humidified incubator at 27°C in the absence of CO_2.

Recombinant Histidine-tagged FGF-BP1 protein purification

The his-tagged FGF-BP1 protein was produced by infecting Sf-9 cells with a baculovirus vector that contains an expression cassette for human FGF-BP1 (BAC-TO-BAC Baculovirus Expression System, Life Technologies Inc., Gaithersburg, MD). The baculovirus construct contains the nucleotides 197-799 of the human FGF-BP1 cDNA, flanked bilaterally by cDNAs encoding six histidine residues. The FGF-BP1 cDNA fragment was inserted into pFASTBAC HTb donor plasmid that was then transformed into the bacmid-containing DH10BAC competent cells. LacZ negative clones, containing the recombinant bacmid, were identified. The bacmid DNA was isolated, and then transfected into Sf-9 cells to generate baculovirus. Infected Sf-9 cells were grown for five days, pelleted, and lysed in a buffer containing 6 M Guanidine-HCl, 0.01 M Tris-HCl, 0.1 M sodium phosphate, pH 8.0. Cell lysates were homogenized and then incubated for one hour on ice. Cellular debris was removed by centrifugation at 10,000 x g for 15 minutes. Supernatant was loaded onto a Ni-NTA sepharose column (Qiagen, Hilden, Germany). The column was sequentially washed with buffers containing 30 mM sodium citrate, 300

mM NaCl and decreasing pH-values of 8, 6.3, 5.9, and 5.7 respectively. His-tagged FGF-BP1 was then eluted with three aliquots of 0.5 ml of the buffer at pH 4.5. Eluates were neutralized immediately and stored at 4°C.

Silver Staining and Western Blot analysis

30 µl of recombinant FGF-BP1 were resuspended with 5X Laemmli's buffer, boiled at 95°C for 5 minutes and separated by electrophoresis on a 4-20% gradient polyacrylamide gel. The protein was then detected by silver staining and after immunoblotting. Silver staining was performed as suggested by the manufacturer (Biorad Laboratories, Hercules, CA). The his-tagged protein was detected with a rabbit polyclonal anti-FGF-BP1 (27) or with a mouse monoclonal anti-His antibody (Invitrogen Corporation, Carlsbad, CA) that were then visualized by enhanced chemiluminescent detection (ECL) using horseradish peroxidase-linked donkey anti-rabbit or anti-mouse immunoglobulin G as the secondary antibodies, respectively (Amersham Pharmacia Biotech, Piscataway, NJ).

Phosphorylation studies

50% confluent NIH-3T3 cells were serum-deprived overnight and treated for 5 minutes with 2 and 10 ng/ml of FGF-2 (Invitrogen Corporation, Carlsbad, CA) and recombinant FGF-BP1. Controls were left untreated. Cells were then washed with cold phosphate-buffered saline (PBS), pH 7.4 and subsequently lysed at 4°C in a buffer containing 50 mM Tris HCl, pH 8, 150 mM NaCl, 40 mM β-glycerophosphate, 1 mM EGTA, 0.25% sodium deoxycholate, 1% Nonidet P-40, 50 mM sodium fluoride, 20 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 2 µg/ml leupeptin, 2 µg/ml aprotinin, 1

μg/ml pepstatin, 100 μg/ml pefabloc. Cellular debris was removed by centrifugation at 14,000 rpm for 15 minutes. Phosphorylated proteins were immunoprecipitated from the cleared lysates by incubation with agarose-conjugate anti-phosphotyrosine (4G10) (Upstate Biotechnology, Lake Placid, NY) monoclonal antibody for 2 hours at 4°C. Immunocomplexes were recovered by centrifugation and washed five times with cold lysis buffer. Samples were then resuspended with 15 μl of 2x Laemmli's buffer and boiled for 5 minutes at 95°C. Alternatively, 10-50 μg of total protein cell extracts were resuspended with 5X Laemmli's buffer and heated for 5 minutes at 95°C. Both immunoprecipitates and total cell lysates were separated on 10% SDS-polyacrylamide gels, transferred onto PVDF membranes and analyzed by immunoblot analysis. Tyrosine phosphorylated proteins and ERK2 were detected with the corresponding mouse monoclonal antibody and then visualized by enhanced chemoluminescent detection (ECL), using horseradish peroxidase-linked goat anti-mouse antibody (Amersham Pharmacia Biotech, Piscataway, NJ), respectively. Monoclonal anti-pan ERK antibody was purchased from BD Transduction Laboratories (Lexington, KY).

FGF/FGF-BP1 binding assays

100 ng/ml of His-BP1 diluted in Tris Buffered Saline (TBS, 50 mM Tris HCl, 150 mM NaCl, pH 7.5) was incubated overnight in 96 well plates (EIA/RIA Strip Plate, Corning Inc., Corning, NY) at 4°C with constant rocking. Excess of unbound His-BP1 was removed by washing the wells twice with TBS. Non-specific binding was blocked by the addition to the wells of 300 μl of Luria Bertani (LB) medium (Bio 101, Carlsbad, CA) for one hour at room temperature. Wells were then washed five times with TBS. ^{125}I-FGF-2

(1-20 ng/ml) was added to the wells and incubated for two hours at room temperature with constant rocking. Unbound ^{125}I-FGF-2 was removed by washing the wells five times with TBS containing 2% Tween-20. In the competition assays, different amounts of FGF-1, FGF-2, or His-BP1 were simultaneously added with ^{125}I-FGF-2. Binding of radiolabeled FGF-2 to His-BP1 was measured by counting radioactive emission from the individual wells. ^{125}I-FGF-2 was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Human recombinant FGF-1 and FGF-2 were purchased from Life Technologies, Inc. (Gaithersburg, MD).

Protein / protein interaction studies on protein arrays ("protein chip assay").

The analysis was performed with a SELDI (Surface-Enhanced Laser Desorption / Ionization) (33) (Protein Biology System I, Ciphergen, Palo Alto, CA). The different FGF-BP1-containing preparations (1 µl of a 20 mg/ml solution) were placed on a normal-phase protein array, which was then washed and 1 µl of a-cyano-4-hydroxy cinnamic acid (2 mg/ml) in 50% (v/v) acetonitrile and 0.5% (w/v) trifluoroacetic acid was added to the spot. The retained proteins were then subjected to mass spectrometry. For the analysis of the interaction of FGF-BP1 with FGF-2, 3 µl of a 240 mg/ml solution of FGF-BP1 in phosphate-buffered saline (PBS) were applied to a preactivated protein array, which was then incubated overnight in a humidified chamber at 4°C. The protein solution was removed, 3 ml of 1 M ethanolamine (pH 8.2) were added to each spot, and the array was incubated for an additional 30 min at room temperature. Further details in Reference (34).

Proliferation Assay

5×10^3 NIH-3T3 fibroblasts were seeded in 3 replicates in 96-well plates for 8 hours. Cells were serum deprived for 16 hours and then treated with human recombinant FGF-1 (10 ng/ml), FGF-2 (5 ng/ml), anti-FGF-2 (15 μg) and His-BP1 (6 ng/ml). The proliferation rate was evaluated after 48 hours by the addition of 10 μl/well of WST-1 reagent, as suggested by the manufacturer (Boheringer Mannheim GmbH, Germany). Rabbit polyclonal anti FGF-2 was purchased from R&D Systems (Minneapolis, MN).

Chorioallantoic Membrane (CAM) assay.

The CAM assay was carried out as originally described (35). Briefly, two-day-old, fertilized chicken eggs were broken open into 35 mm x 10 mm petri dishes, and incubated at 37°C for 48 hours. Sterile Whatmann filter disks (8 mm diameter) were pre-wetted in TBS solution and placed peripherally on the CAM of viable embryos, in between adjacent visible blood vessels. FGF-2 and His-BP1 were placed on the disks as indicated. CAM's were photographed using a digital camera at 0, 12, 24, and 36 hours following disk placement. The degree of angiogenesis around each disk was measured using a score from 1 (minimal angiogenesis) to 4 (maximal angiogenesis, with directional growth of new vasculature towards the disk). Scoring was carried out blinded and results were averaged. Baseline (no treatment) was subtracted from the average score, where indicated.

Results

Generation of recombinant FGF-BP1 protein

In previous studies we investigated FGF-BP1 effects by overexpression experiments in FGF-BP1-negative cells or by depletion of the endogenous FGF-BP1 from cells using ribozyme-targeting (26,27). Since FGF-BP1 is secreted from cells and acts as an extracellular chaperone, we sought to examine the biological effects of a human recombinant FGF-BP1 when added to the extracellular milieu. For this purpose, two recombinant human FGF-BP1 proteins were produced *in vitro*. A bilaterally hexahistidine-tagged FGF-BP1 protein ("His-BP1") was purified from Sf-9 insect cells infected with a baculovirus construct containing the nucleotides 197-799 of the human FGF-BP1 cDNA (GenBank # M60047). This protein was used for the functional assays and its purification is shown in Fig. 1. In addition a Glutathione-S-transferase (GST)-tagged FGF-BP1 ("GST-BP1") was generated in BL21 bacteria using a pGEX-2TK construct that contains the same nucleotides (not shown). This protein was used to generate antibodies and in some of the initial protein/protein interaction studies (see below). To determine whether the protein generated in the Sf-9 cells indeed represented FGF-BP1, serial elutions obtained from the final affinity chromatography were separated by SDS-PAGE and an apparently 34 kDa protein was detected by silver staining and Western blotting with anti-BP1 as well as anti-histidine tag antibodies (Fig. 1A, B and C). The BP1 protein ran at 34 kDa apparent molecular mass and the silver staining of a pooled sample shows that the purity of the preparation is >90% (Fig. 1A). The electrophoretic mobility of the full-length human BP1 protein is slower than predicted by

its molecular mass most likely due to its basic nature, a feature also reported for the bovine BP1 protein (see Ref. 36).

FGF binding to FGF-BP1 in cell-free assays

We next sought to assess whether the GST-BP1 and His-BP1 recombinant proteins retained the ability to bind to FGF-2 *in vitro*. A binding assay was performed in a cell-free system, in which the binding of increasing concentrations of ^{125}I-FGF-2 to the immobilized recombinant proteins was measured. As shown in Fig. 2A, both GST-BP1 (upper panel) and His-BP1 (lower panel) bound to the radiolabeled growth factor in a dose-dependent manner. The bacterial GST-BP1 displayed less specific and more non-specific binding to ^{125}I-FGF-2 per unit of protein in comparison to the eukaryotic His-BP1, and we thus decided to use the latter as the molecular tool for our further studies. To further support the specificity of His-BP1 binding to the FGF-2 radioligand, competition assay were performed with excess cold FGF-2, FGF-1 or His-BP1 as well as EGF as a non-specific growth factor control. As shown in Fig. 2B, increasing concentrations of His-BP1 were able to prevent the binding of ^{125}I-FGF-2 to the immobilized His-BP1 and an approximately 10-fold excess of the His-BP1 in solution completely inhibited the FGF-2 binding. FGF-2 also competed for FGF-2 radioligand binding (Fig. 2D). From a series of competition assays, we calculated an appared dissociation constant (K_d-value) of approximately 10 nM for FGF-2 binding to His-BP1. Furthermore, in support of the original report on FGF-BP1 by D. Sato's laboratory (37), we found that FGF-1 also competed with FGF-2 for its binding to His-BP1 (Fig. 2C). Finally, even 10 μg/ml of

EGF did not inhibit FGF-2 binding to the immobilized His-BP1 (bar in Fig. 2D) supporting a specific interaction of FGF-BP1 and FGF-2.

Obviously, these binding studies will show whether or not the labeled FGF-2 binds to FGF-BP1, but they will not reveal as to whether other unidentified proteins present in the preparation also bind to FGF-2. To address this question, we used protein chip technology coupled to mass spectrometry (33) to assess FGF/FGF-BP protein interactions qualitatively (Fig. 2E). This approach has been applied by us recently to characterize ligand/receptor interaction when studying pleiotrophin and its receptor, anaplastic lymphoma kinase (ALK) (34). For this, the His-BP1 protein was immobilized on a protein chip and incubated with FGF-2 that had been spiked into growth media containing 10% fetal calf serum. FGF-2 comprised only a very small portion of the overall protein preparation used as the input (arrow head in Fig. 2E, *lane 1*, *input*). Still, FGF-2 was specifically recognized by the immobilized His-BP1 in this mixture of proteins (arrow head in Fig. 2E, *lane 2*) and only non-specific binding was observed without the immobilized His-BP1 (Fig. 2E, *lane 3*). From this we concluded that FGF-2 indeed binds specifically to FGF-BP1 even when the growth factor is only present at very low abundance in a complex mix of proteins.

FGF-BP1 enhances FGF-2-induced proliferation in NIH-3T3 fibroblasts.

After we demonstrated the ability of the recombinant human FGF-BP1 to specifically bind FGF-2 in a cell-free system, we sought to determine the effect of this interaction on FGF-2 biological activity *in vitro*. FGF-2 is a potent mitogen for a number of cell lines, including fibroblasts and endothelial cells (17) and we used NIH-3T3

fibroblasts as an experimental model system to study the effect of His-BP1 on FGF-2-induced proliferation (Fig. 3). In order to control the effectiveness of FGF-2 mitogenic action, we first assessed the ability of the growth factor to induce cell proliferation. NIH-3T3 cells were serum deprived overnight and grown for 48 hours in the presence or absence of different concentrations of FGF-2 to establish a dose-response curve (data not shown). We then stimulated the cells with a sub-maximally effective concentration of FGF-2 (5 ng/ml) and added equimolar concentrations of His-BP1. Whilst no proliferation was detected when cells grew in the presence of His-BP1 alone, addition FGF-2 enhanced the mitogenic activity of the growth factor. To rule out the possibility that interaction of His-BP1 with a mitogenic factor other than FGF-2 was responsible for the His-BP1-mediated effect, we included an FGF-2 specific antibody. The anti-FGF-2 antibody blocked the FGF-2-dependent cell growth and completely inhibited the synergistic effect of His-BP1 and FGF-2. As a control, the anti-FGF-2 antibody failed to reduce FGF-1-dependent cell growth and, in addition, synergistic effects between His-BP1 and FGF-1. Taken together, these results establish that the synergistic interaction between His-BP1 and FGF-1- or FGF-2-stimulated NIH-3T3 mitogenesis is specific and dependent on the respective growth factor.

FGF-BP1 positively modulates FGF-2-induced ERK2 activation in a dose-dependent fashion.

ERK2 plays an important role in transducing proliferative signals from receptor tyrosine kinases (RTKs) (18). In particular, engagement of FGFRs by their extracellular ligands, such as FGF-1 and FGF-2, has been extensively reported to induce activation of the

Ras/ERK2 cascade (16,38). Consistent with these observations and in light of our results, we next decided to examine the early signaling events elicited by the specific interaction between FGF-2 and His-BP1 (Fig. 4). Interestingly, we found that FGF-2-dependent ERK2 activation, determined by immunoblot analysis with an anti-phosphotyrosine antibody, was significantly enhanced in NIH-3T3 fibroblasts when cells were co-stimulated for 5 minutes with different concentrations of FGF-2 and His-BP1. In particular, 3 and 6 ng/ml of His-BP added to FGF-2 (2 ng/ml) exhibited an enhancement of phosphorylation of 1.8 and 2.5 fold respectively, when compared to the levels obtained with FGF-2 alone (Fig. 4, top panel). The same results were obtained in immunoprecipitation studies (not shown). In addition, as shown in the bottom panel of Figure 4, the levels of expression of ERK2 were not affected by these treatments and, consistent with the phosphoMAP kinase blots, a mobility shift of the lower ERK2 band due to phosphorylation was obvious. From these findings we conclude that FGF-2-dependent phosphorylation of ERK2 is synergistically modulated by His-BP1.

FGF-BP1 enhances FGF-2-dependent angiogenesis in vivo.

FGF-2 has been shown to be a powerful inducer of angiogenesis both *in vitro* and *in vivo* (2,21,39). As an experimental approach to investigate the effects of FGF-BP1 on FGF-2-mediated angiogenesis *in vivo*, we used the chick embryo chorioallantoic membrane (CAM) assay. As shown in Fig. 5, FGF-2 as well as His-BP1 induce an angiogenic response on their own. The baseline effect of FGF-BP1 on its own is likely due to locally-stored FGFs from the chicken embryo. However, the simultaneous stimulation with both FGF-2 and FGF-BP1 resulted in a significant enhancement of this

response. These findings support the notion that FGF-BP1, by its cooperative interaction with FGF-2, is a positive regulator of FGF-2-mediated angiogenesis *in vivo*.

Discussion

FGF-BP1 is a secreted protein, which binds FGF-2 and is hypothesized to mobilize FGF-2 from its storage in the ECM (37). Previous studies have found that endogenous FGF-BP1 is overexpressed in several cancers (26,27,29). Additionally, depletion of FGF-BP1 mRNA has been shown to abrogate the angiogenesis-dependent growth of ME-180 squamous cell carcinoma and Ls174T colon cancer cells when implanted into athymic nude mice (27). Since endogenous FGF-BP1 plays a critical role in tumor growth and angiogenesis, we here set out to explore the effects of exogenously added FGF-BP1 protein on FGF-2 dependent cellular responses.

For our experiments, we produced and purified human recombinant, polyhistidine-tagged FGF-BP1 and showed that it retains the ability to bind radiolabeled FGF-2 *in vitro*. The binding is specific as it is competed by excess concentrations of either cold FGF-2 or recombinant FGF-BP1. There have been conflicting reports on whether FGF-BP1 binds FGF-1. In the original description of FGF-BP1, Wu et al. found that FGF-BP1, purified from the media of A431 human epidermoid carcinoma cell cultures, was also able to bind FGF-1 (37). In contrast, Lametsch et al. reported that FGF-BP1, purified from bovine prepartum mammary secretion, binds FGF-2, but not FGF-1 (36). Nevertheless, in our experiments, FGF-1 completely inhibited FGF-2 binding to the human recombinant FGF-BP1, thus demonstrating an interaction of FGF-1 with FGF-BP1.

The MAPK pathway has been extensively studied in the stimulation of quiescent cells with mitogenic factors and is generally considered to be responsible for the initiation of cellular growth. Of note, in different cellular systems it has been shown that

the specific interaction of FGF-2 with its receptor and the subsequent FGFR activation triggers a downstream signal cascade that culminates with the activation and phosphorylation of ERK2 (16). To investigate the role of recombinant exogenous FGF-BP1 on FGF-2-mediated early intracellular biological responses, phosphoprotein analysis was performed in NIH-3T3 murine fibroblasts. Consistent with previous findings that demonstrated the physical interaction of FGF-BP1 with FGF-2 (37), we now provide evidence that FGF-BP1 exerts positive and synergistic modulation of FGF-2-mediated signaling, by enhancing the growth factor-dependent ERK2 phosphorylation. Interestingly, we show that, at concentrations of FGF-2 not sufficient to elicit maximal ERK2 activation, FGF-BP1 significantly amplifies the FGF-2-mediated response in a dose-dependent manner. Sustained activation of ERK2 signal transduction pathway often controls the stimulation of cell proliferation (40,41), and ERK2 activation is required for proliferation of fibroblasts *in vitro* (38). In addition to ERK2, FGF-2-dependent mitogenic signal transduction pathways lead to the activation of PLCγ and p70S6K, respectively. However, it has been observed that PLCγ activation is likely not to be responsible for FGF-2-mediated NIH-3T3 mitogenesis, nor is the signal emanated from p70S6K sufficient to induce cell proliferation (42-44). Indeed, coincident with the ERK2 activation, we found that the interaction of FGF-BP1 with FGF-2 also elicits a dramatic enhancement of FGF-2-mediated NIH-3T3 proliferation. This supports the notion of FGF-BP1 as a chaperone molecule that will serve as a positive modulator of FGF-2-dependent growth controlled by the ERK2 pathway.

FGF-2 is a potent angiogenic molecule, and previous studies have shown that it can induce neovascularization in the chicken embryo CAM assay. Here, we use

this experimental model to address whether recombinant FGF-BP1 can act synergistically with FGF-2 to cause new and directed blood vessel growth. We found that the angiogenic response seen with addition of both recombinant FGF-BP1 and FGF-2 was significantly greater than that seen with FGF-2 alone. Furthermore, FGF-BP1 treatment led to a more rapid establishment of directed blood vessel growth when added to FGF-2 (see e.g. Fig. 5A, left versus right panel). Interestingly, we found that FGF-BP1 added to the CAM, without exogenous FGF-2, was able to induce angiogenesis in a dose-dependent manner on its own; we speculate that this is due to endogenous FGFs present in the CAM. This finding is also consistent with an earlier report in which transfection of FGF-BP1 into a human adrenocortical carcinoma cell line (SW-13) induced the growth of highly-vascularized tumors in athymic nude mice (26), and reduction of FGF-BP1 message in ME-180 cells reduced their angiogenic stimulus during tumor growth (27). In addition, all-*trans*-retinoic acid has been shown to downregulate FGF-BP1 mRNA levels in tumors grown from the ME-180 cells and coincident with that the extent of tumor angiogenesis (45).

Binding proteins have been described for other cytokines, and the most relevant two binding protein families are those for insulin-like growth factor (IGF) and transforming growth factor β (TGFβ). Latent TGFβ binding proteins and IGF binding proteins have been shown to bind and protect their respective ligands from degradation, and can positively or negatively modulate their ligands' functional activities (46,47). Each of these binding proteins represents a family of multiple proteins, with homologous members found in different tissues and species. Similarly, human FGF-BP1 has homologues in chicken, zebrafish, cow, mouse, and rat (2,28,36,37). Recently, we found

a novel FGF-BP, designated FGF-BP2, that is also located on chromosome 4p16, in close proximity to FGF-BP1. The amino acid sequences of these two proteins contain eight cysteine residues that are conserved across different species and between the FGF-BP genes. This suggests identical disulfide bond formation and similar tertiary structure. It will be interesting to see to what extent the different FGF-BPs contribute to the diversity of activities by the more than 20 FGFs (2).

In conclusion, our studies suggest that FGF-BP1 represents an important regulatory factor that positively modulates FGF-2-mediated cellular responses, such as signaling, proliferation and angiogenesis.

Footnotes

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GenBank Accession Numbers. human FGF-BP1, M60047

Abbreviations. BP, binding protein; FGF, fibroblast growth factor; His-BP1, poly-histidine-tagged FGF-BP1; SELDI, surface-enhanced laser desorption/ ionization

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Figures legends

Fig. 1. Protein stain and Western blots of the His-BP1 protein.

(**A**) Silver stain of 30 µl of a pooled, chelate affinity-purified His-BP1 preparation separated by a 4-20% gradient SDS-PAGE. (**B**, **C**) Western blots of 30 µl aliquots from consecutive affinity chromatography fractions loaded onto 4-20% SDS-PAGE. A blot with anti-His tag (**B**) and anti-BP1 (**C**) antibodies is shown.

Fig. 2. Binding of FGF-2 to His-BP1.

(**A**) Binding of different concentrations of ^{125}I-FGF-2 to GST-BP1 (upper panel) and His-BP1 (lower panel) immobilized in 96-well plates. (**B**, **C**, **D**) Competition of FGF-1, FGF-2 and His-BP1 for ^{125}I-FGF-2 binding to immobilized His-BP1. Binding data obtained from wells containing immobilized FGF-BP1 (filled circles) in comparison to blocking solution only (control; open circles) are shown. The data are representative of at least four independent experiments in which each sample was run in triplicate. (**E**) Protein chip analysis using SELDI (surface-enhanced laser desorption/ionization(33)) to characterize FGF-2 binding to immobilized His-BP1. Mass spectrometry analysis of proteins is shown. *Lane 1*, "input" FGF-2 ligand preparation (FGF-2 spiked into cell growth media). *Lane 2* and *3*, proteins present in the "input" preparation that bound to immobilized His-BP1 (lane 2) or background (lane 3) respectively. The arrowheads indicate the peak corresponding to the FGF-2 protein used as a ligand.

Fig. 3. His-BP1 effect on FGF-2-mediated mitogenesis in NIH-3T3 fibroblasts.

Cells were treated for 48 hours with FGF-1 or FGF-2, +/- His-BP1, in the absence (gray bars) or presence (black bars) of anti-FGF-2 antibody and the proliferation rate was measured as described under "Materials and Methods". The data shown are representative of three independent experiments.

Fig. 4. His-BP1 effect on FGF-2-induced ERK2 activation in NIH-3T3 fibroblasts.

Cells were starved overnight and treated for 5 minutes with different concentrations of FGF-2 and/or His-BP1, as indicated. Controls were left untreated. 50 μg of total cell lysates were separated by SDS-PAGE (10%), transferred onto PVDF membranes and immunoblotted with anti-phosphotyrosine monoclonal antibody (pMAPK; upper panel). Detection of endogenous ERK2 in the protein extracts was determined by western blot analysis, using anti panERK monoclonal antibodies (p42/44; lower panel). Quantitation of bands was obtained by densitometry and is expressed relative to control (bar graph).

Fig. 5 His-BP1 effect on FGF-2-induced angiogenesis in the Chorioallantoic Membrane (CAM) assay.

Filter disks with FGF-2 (100 ng) +/- His-BP1 (as indicated) were placed on CAMs of four-day-old chicken embryos. (**A**, **B**) Photograph of angiogenesis on the CAM scored as 1 (**A**) and 3 (**B**) at 24 hours. The bar indicates a 1 mm size. (**C**) Quantitation of the assays; $p = 0.015$ is the overall p-value from ANOVA analysis. * = $p<0.05$, ** = $p<0.01$ shows the comparison relative to the respective control sample.

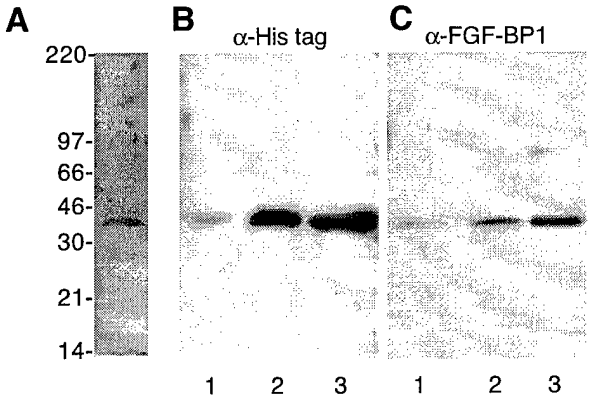


Figure 1 Tassi et al.

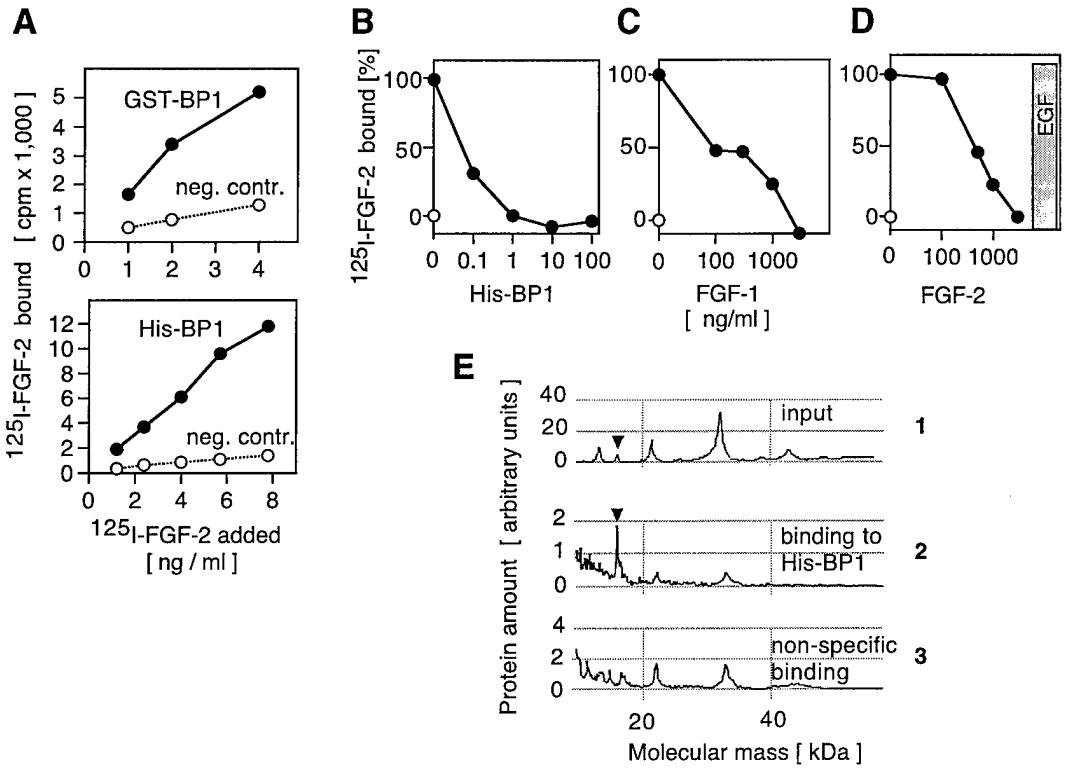
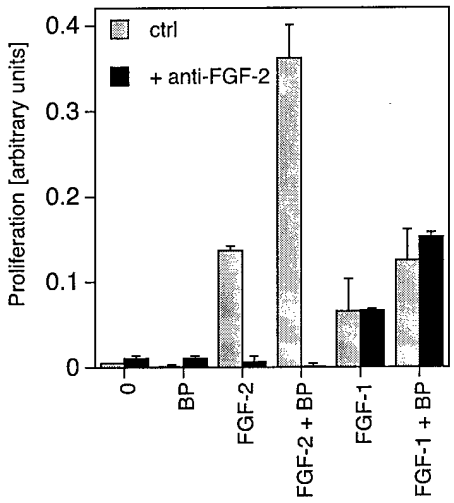


Figure 2 Tassi et al.



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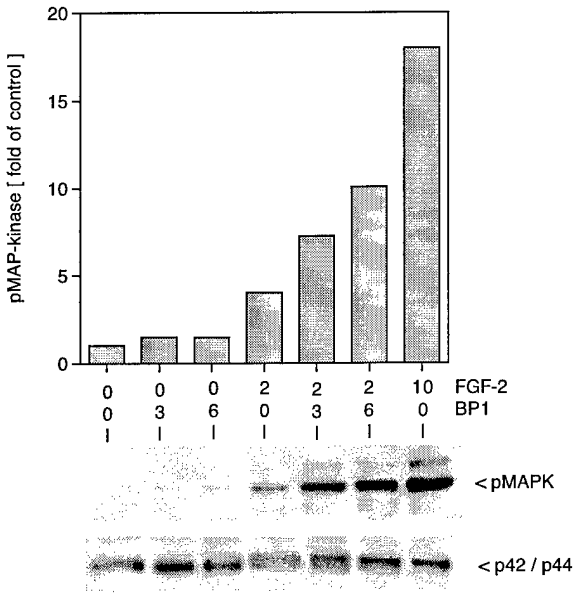

Figure 4 Tassi et al.

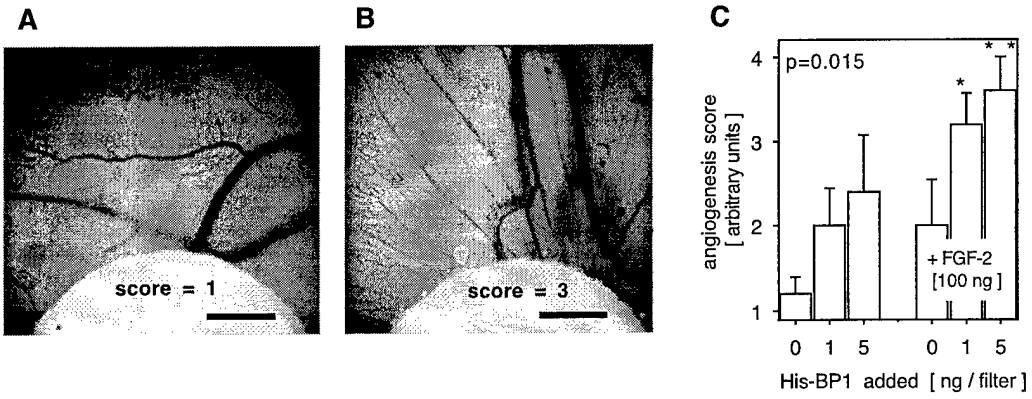

Figure 5 Tassi et al.