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Award Number: DAMD17-99-1-9187

TITLE: Cell Surface Molecules Driving Breast Cancer/Endothelial Interactions

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REPORT DATE: July 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
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**20030130 197**

# REPORT DOCUMENTATION PAGE

Form Approved  
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

<b>1. AGENCY USE ONLY (Leave blank)</b>		<b>2. REPORT DATE</b> July 2002	<b>3. REPORT TYPE AND DATES COVERED</b> Annual Summary (01 Jul 01 - 30 Jun 02)	
<b>4. TITLE AND SUBTITLE</b> Cell Surface Molecules Driving Breast Cancer/Endothelial Interactions			<b>5. FUNDING NUMBERS</b> DAMD17-99-1-9187	
<b>6. AUTHOR(S)</b> Ali Al-Attar				
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> Georgetown University Medical Center Washington, DC 20057  E-Mail: alattara@georgetown.edu			<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			<b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>	
<b>11. SUPPLEMENTARY NOTES</b>				
<b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for Public Release; Distribution Unlimited			<b>12b. DISTRIBUTION CODE</b>	

**13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information)**  
 Fibroblast growth factor-binding protein 1 (FGF-BP1) is a secreted heparin-binding protein that can bind and solubilize members of the fibroblast growth factor (FGF) family. FGF-2 is immobilized on heparan sulfate proteoglycans (HSPGs) in the extracellular matrix and can be released from this storage and activated by degradation of the HSPG or by binding a soluble chaperone molecule that transports it to its cell-surface tyrosine kinase receptor. FGF-BP1 was proposed to serve as such a carrier protein and has been demonstrated to act as an angiogenic switch in models of malignant progression of cancer. Two recombinant FGF-BP1 proteins were produced in prokaryotic and eukaryotic expression systems, and demonstrated to bind several members of the FGF family in a dose-dependent, reversible manner. Furthermore, FGF-2 binding to FGF-BP1 and to heparan sulfate was found to be mutually exclusive. FGF-BP1 did not interfere with FGF-2 binding to its tyrosine kinase receptor (FGFR1) in a cell-free system, and the biologic effects of FGF-2 were enhanced by the addition of exogenous FGF-BP1 in models of growth and angiogenesis. Finally, HIV-1 Tat protein was found to inhibit FGF-2 binding to FGF-BP1 but not to its receptor, suggesting that HIV-1 Tat might function as an FGF binding protein.

<b>14. SUBJECT TERMS</b> breast cancer, fibroblast growth factor, angiogenesis			<b>15. NUMBER OF PAGES</b> 20	
			<b>16. PRICE CODE</b>	
<b>17. SECURITY CLASSIFICATION OF REPORT</b> Unclassified	<b>18. SECURITY CLASSIFICATION OF THIS PAGE</b> Unclassified	<b>19. SECURITY CLASSIFICATION OF ABSTRACT</b> Unclassified	<b>20. LIMITATION OF ABSTRACT</b> Unlimited	

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## Introduction

Fibroblast growth factor-binding protein 1 (FGF-BP1) is a secreted heparin-binding protein that can bind and solubilize members of the fibroblast growth factor (FGF) family (Wu et al. 1991, Czubyko et al. 1994). FGF-2, the prototype member of the FGF family, is typically immobilized on heparan sulfate proteoglycans in the extracellular matrix (Bashkin et al. 1989). FGF-2 can be released from this storage and activated either by degradation of the heparan sulfate proteoglycan, or by binding a soluble chaperone molecule that transports it to its cell-surface tyrosine kinase receptor (Saksela et al. 1988, Bashkin et al. 1989, Ishai-Michaeli et al. 1990, Brunner et al. 1991). FGF-BP1 has been proposed to serve as such a carrier protein (Czubyko et al. 1994). It has been shown to be upregulated in tissue samples from various epithelial cancers (colon, squamous cell, and breast), and has been demonstrated to act as an angiogenic switch in models of malignant progression of these cancers (Kurtz et al. 1997, Czubyko et al. 1997).

Here the mechanism of action of FGF-BP1 was investigated using two recombinant FGF-BP1 proteins, produced in prokaryotic and eukaryotic expression systems. The recombinant FGF-BP1 proteins were purified and demonstrated to bind to FGF-2 in a dose-dependent, reversible manner. FGF-1, 4, 5, 7, and 9 were able to inhibit FGF-2/FGF-BP1 binding, suggesting that these two proteins also serve as ligands for FGF-BP1. Three heparinoids were also able to inhibit FGF-2/FGF-BP1 binding, indicating that FGF-2 binding to FGF-BP1 and to heparan sulfate is mutually exclusive. However, FGF-BP1 was found to not interfere with FGF-2 binding to its tyrosine kinase receptor (FGFR1) in a cell-free system. Furthermore, the biologic effects of FGF-2 were positively modulated by the addition of exogenous recombinant FGF-BP1 protein in several models of growth and angiogenesis, supporting the hypothesis that FGF-BP1 functions as a soluble carrier protein for FGF-2. Finally, studies of HIV-1 Tat protein found that it can also inhibit FGF-2/FGF-BP1 binding, but does not interfere with FGF-2 binding to its receptor, supporting the hypothesis that HIV-1 Tat can also function as an FGF binding protein.

## Body

FGF-2 is the prototype member of the FGF family of proteins, which are involved in multiple biologic processes including tumorigenesis and angiogenesis, organogenesis, and cell differentiation (reviewed in Powers et al. 2000). Following secretion of FGF-2 through an ER/Golgi-independent pathway into the extracellular matrix (ECM), FGF-2 binds to and is immobilized on heparan sulfate (HS) in the matrix (Jackson et al. 1995, Lavallee et al. 1998, Carreira et al. 1998). FGF-2 is therefore sequestered and functionally inactive in the ECM, bound to HS (Bashkin et al. 1989).

Several mechanisms have been suggested for the release of FGF-2 from HS. Both the protein core as well as the glycosaminoglycan side chains of HS molecules have been shown to be selectively degraded with resultant functional activation of FGF-2 (Saksela et al. 1988, Bashkin et al. 1989, Ishai-Michaeli et al. 1990, Brunner et al. 1991). An alternative mechanism has been proposed where a soluble carrier protein, FGF-BP1, can bind FGF-2 and release it from HS in the ECM (Czubayko et al. 1994). FGF-BP1 is a heparin-binding growth factor that has been suggested to reversibly bind FGF-1 and FGF-2 (Wu et al. 1991). Transfection and ribozyme-targeting studies have found that it can act as a rate-limiting step in tumorigenesis and angiogenesis, such that FGF-BP1 might function as an angiogenic switch molecule (Czubayko et al. 1994, Czubayko et al. 1997). Furthermore, expression studies have found that it is upregulated during carcinogenesis in models of phorbol ester-induced squamous cell carcinoma, and that modulation of FGF-BP1 production might be part of the mechanism of action of anti-neoplastic retinoid agents (Liaudet-Coopman et al. 1996, Kurtz et al. 1997, Liaudet-Coopman et al. 1997).

These studies motivated us to investigate FGF-BP1/FGF-2 interaction, and in particular to attempt to define domains that could interfere with FGF-BP1/FGF-2 binding and therefore interrupt FGF-2 functional activation. We initially invested considerable time into using peptide phage display technology in an attempt to identify peptides that selectively bind FGF-2 or FGF-BP1. Our rationale was that some of these identified peptides would bind within the FGF-2/FGF-BP1 interaction domain, or alternatively interact with one of the proteins to induce a conformational change such that FGF-2 and FGF-BP1 no longer bind. In this effort we tested multiple phage display systems, including libraries expressing short random peptides, peptide fragments of FGF-2 and FGF-BP1, as well as protein fragments from cDNA libraries of tumor cell lines. While significant time was invested experimenting with different systems, employing multiple technical parameters, and analyzing plus sequencing the retained clones, this technology consistently did not yield any results that contributed to our investigation. Concurrently, we were attempting to produce recombinant FGF-BP1 proteins, and the eventual successful purification of these proteins allowed us to investigate the protein-protein interaction of FGF-2 and FGF-BP1 through direct binding studies.

The results from much of the work summarized below are found in a publication from our laboratory that is included in the appendix to this Annual Report (Tassi et al. 2001).

Recombinant FGF-BP1 proteins were made in prokaryotic and eukaryotic expression systems. Purified recombinant protein retained the ability to bind radiolabeled FGF-2, suggesting that the proteins re-folded properly following denaturing purification

protocols. Proteins produced in both eukaryotic and prokaryotic expression systems were assayed in initial experiments to determine their binding affinities and document reversible, dose-dependent binding to radiolabeled FGF-2. The recombinant FGF-BP1 produced in the eukaryotic expression system provided a relatively cleaner purification with lower background, and had a much shorter tag (six histidine residues bilaterally, compared with a GST tag in the prokaryotic expression system). Therefore, further experiments were performed using the eukaryotic expression system producing histidine-tagged FGF-BP1.

An *in vitro* binding assay was used to determine the binding characteristics and kinetics of radiolabeled FGF-2 with recombinant FGF-BP1. Reversible, dose-dependent binding was observed with a dissociation constant ( $K_d$ ) of approximately 10 nM, which is weaker than the binding of FGF-2 to its tyrosine kinase receptor, FGFR. This same assay, where recombinant FGF-BP1 is bound to wells and radiolabeled FGF-2 is added in solution, was used to screen for other ligands by determining which ones could interfere with the FGF-2/FGF-BP1 interaction. In addition to non-radiolabeled FGF-2 (a positive control), FGF-1 and FGF-7 were able to fully disrupt radiolabeled FGF-2/FGF-BP1 binding in this assay. FGF-4, -5, and -9 were able to weakly interfere with FGF-2/FGF-BP1 binding, suggesting that they might also serve as ligands for FGF-BP1. In addition, PDGF-BB was able to partially interfere with FGF-2/FGF-BP1 binding, suggesting that it too might be a ligand for FGF-BP1. In contrast, FGF-6, as well as IL-1 $\alpha$  and  $\beta$ , which are structural homologs of FGF-2, were not able to compete with radiolabeled FGF-2 for binding with FGF-BP1. These findings suggest that FGF-BP1 serves as a carrier protein for some, but not all, members of the FGF family, and that the binding exhibits some specificity as it does not extend to FGF-2 structural homologs such as IL-1 $\alpha$  and  $\beta$ .

Further studies evaluated the stoichiometry of the FGF-2/FGF-BP1 interaction. FGFs bind their tyrosine kinase receptors in a 2:2 dimer, and multiple FGFs can simultaneously bind to a single HSPG molecule. In contrast, we performed cross-linking studies using radiolabeled FGF-2 that demonstrated that FGF-2 binds FGF-BP1 with a 1:1 stoichiometry

The next series of experiments examined the interaction of FGF-BP1 with FGF-2 in the presence of HS and heparin-like molecules. Extracellular FGF-2 is usually stored in an inactive form, bound to HS in the ECM. Since FGF-BP1 was hypothesized to be a carrier molecule that lifts FGF-2 from HS, we wanted to investigate the binding of FGF-2 to FGF-BP1 in the presence of HS and heparin-like molecules. We found that increasing concentrations of HS could inhibit binding of FGF-2 to FGF-BP1, as could heparin and, in an especially potent fashion, pentosan polysulfate, a heparinoid in clinical trials for its antineoplastic effects. These findings suggest that FGF-BP1 and HS bind overlapping sites on the FGF-2 molecule, supporting the concept that binding of FGF-2 to FGF-BP1 and to HS is mutually exclusive and therefore FGF-BP1 is capable of releasing FGF-2 from HS.

A series of transfection and ribozyme-targeting experiments previously performed by our laboratory had found that FGF-BP1 expression is critical for tumorigenesis and angiogenesis in several model systems (Czubayko et al. 1994, Czubayko et al. 1997). Since there are several proposed mechanisms for FGF-2 solubilization from HS in the ECM, it is plausible that in different systems, different mechanisms predominate and that only in certain models would the effects of FGF-BP1 become critical. Therefore,

members of our laboratory performed a series of biologic experiments using exogenous addition of recombinant FGF-BP1. We found that in the NIH 3T3 mitogenic assay (an *in vitro* model of tumorigenesis) and the chicken chorioallantoic membrane, or CAM, assay (an *in vivo* model of angiogenesis), exogenous addition of FGF-BP1 resulted in statistically-significant positive modulation of the biologic effects of FGF-2. A similar positive modulation of FGF-2's biologic effects was seen in PC-12 neurite outgrowth experiments, which are a model of neuronal sprouting and differentiation. However, the effects of exogenous FGF-BP1 were not as clearly reproducible in other biologic assays where FGF-2 drives anchorage-independent growth (SW-13 soft agar assay) or angiogenesis (endothelial cell proliferation assay). These findings reflect both the technical complexities of experiments dependent on protein/ECM interactions, as well as the limitations of FGF-BP1's efficacy to those situations where other mechanisms for FGF-2 solubilization are dormant.

As the earlier experiments analyzed the protein/protein interactions of FGF-2 with FGF-BP1, and further studies demonstrated that exogenous FGF-BP1 could positively modulate FGF-2 biologic activity, we wanted to investigate the interaction of FGF-BP1 and FGF-2 with its tyrosine kinase receptor, FGFR. Protein A/G-coated agarose beads were used to precipitate commercially-available FGFR1 $\alpha$ / F<sub>c</sub> fusion protein, along with any bound ligand, in a cell-free system. Radiolabeled FGF-2 was able to specifically bind FGFR1 $\alpha$ / F<sub>c</sub> fusion protein and was precipitated with the protein A/G-coated agarose beads. In order to document that FGF-BP1 could act as a carrier protein without interfering in the FGF/FGFR binding interaction, excess concentrations of recombinant FGF-BP1 were added in this cell-free system and did not have any effect on radiolabeled FGF-2 binding to FGFR1 $\alpha$ / F<sub>c</sub> fusion protein. This finding suggests that FGF-BP1 neither interferes with nor enhances FGF/FGFR binding interactions. We were unsuccessful in determining using immunodetection techniques whether FGF-BP1 was bound to the FGF-2/FGFR1 $\alpha$  complex.

A final component of this study examined the possibility that other angiogenic proteins might function as FGF binding proteins, in a manner analogous to FGF-BP1. Tat protein is an HIV-1 early gene product that was initially described for its function as a transactivator of transcription (reviewed in Jeang et al. 1999). Later studies found that Tat has multiple biologic effects, among which it can function as a heparin-binding angiogenic growth factor (Albini et al. 1995, Albini et al. 1996). Implicated in the pathogenesis of the aggressive variant of Kaposi's sarcoma seen in AIDS patients, Tat has been described to modulate FGF-2 biologic activities through interference with FGF-2/HS binding (Ensoli et al. 1990, Ensoli et al. 1994, Albini et al. 1995, Albini et al. 1996, Barillari et al. 1999). Tat's function as a heparin-binding growth factor and positive modulation of FGF-2 biologic effects are strikingly reminiscent of FGF-BP1. In order to investigate whether Tat could be functioning as an FGF binding protein, we tested the ability of Tat to interfere with the binding of FGF-2 to FGF-BP1 or to FGFR1 $\alpha$  in cell-free systems. We found that Tat protein could successfully compete with FGF-BP1 for binding to radiolabeled FGF-2, suggesting that Tat and FGF-BP1 bind overlapping sites on FGF-2. This experiment was the first to document that Tat binds FGF-2. We next found that, like FGF-BP1, Tat does not interfere with FGF-2 binding to its tyrosine kinase receptor FGFR1 $\alpha$ , consistent with a function as a carrier protein for FGF-2.

It is therefore plausible that Tat is functioning as an FGF binding protein analogous to FGF-BP1.

This series of experiments was initially aimed at interrupting FGF-2/FGF-BP1 binding interactions by using phage display technology to identify novel ligands. Significant effort was placed into developing this approach, but continued technical difficulties encouraged investigation through parallel avenues. The purification of two recombinant FGF-BP1 proteins that were functionally active allowed the investigation of FGF-2/FGF-BP1 binding interaction through a series of *in vitro* and *in vivo* studies. Significant understanding of the characteristics of the FGF/FGF-BP1 interaction was achieved, with additional insight into the role of FGF-BP1 in FGF-2's relationship with HS as well as with its tyrosine kinase receptor, FGFR. Finally, similarities between HIV-1 Tat protein and FGF-BP1 in their binding characteristics with FGF-2 suggest that Tat might function as an FGF binding protein, with significant implications for its role in the pathogenesis of the variant of Kaposi's sarcoma seen in AIDS patients.

## Key Research Accomplishments

- Produced and purified two recombinant FGF-BP1 proteins, in prokaryotic and eukaryotic systems;
- Determined the binding characteristics and kinetics of FGF-BP1 binding to FGF-2 ( $K_d=10$  nM), as well as to other members of the FGF family (FGF-1, -4, -5, -6, -7, -9), structural analogs of FGF-2 (IL-1 $\alpha$  and  $\beta$ ), and PDGF-BB;
- Demonstrated that binding of FGF-2 to FGF-BP1 and to heparan sulfate is mutually-exclusive, and that heparin and the heparinoid pentosan polysulfate can interact with FGF-2 in a manner similar to heparan sulfate;
- Analyzed the stoichiometric ratio of FGF-2/FGF-BP1 interaction and determined it to be 1:1;
- Demonstrated that FGF-2 can bind its cell-surface tyrosine kinase receptor FGFR1 in the presence of FGF-BP1, in a cell-free system;
- Documented that FGF-BP1 can positively modulate FGF-2 biologic activity in *in vitro* and *in vivo* direct add-on experiments;
- Found that HIV-1 Tat protein binds FGF-2 at an overlapping site with that bound by FGF-BP1, and that FGF-2 bound to Tat can still bind FGFR, supporting the possibility that Tat can function as an FGF binding protein.

## Reportable Outcomes

Tassi, E., Al-Attar, A., Aigner, A., Swift, M.R., McDonnell, K., Karavanov, A., Wellstein, A.(2001) Enhancement of fibroblast growth factor (FGF) activity by an FGF-binding protein. *J. Biol. Chem.* **276**: 40247-40253.

## Conclusions

- (1) FGF-BP1 binds to different members of the FGF family with different affinities. FGF-BP1 binds FGF-1, 2, and 7 with high affinity, and FGF-4, 5, and 9 with low affinity.
- (2) FGF-2 binding to FGF-BP1 and to heparinoids is mutually exclusive.
- (3) FGF-BP1 enhances FGF efficacy in different biological systems.
- (4) FGF-BP1 does not inhibit FGF-2 receptor binding.
- (5) HIV-1 Tat shares FGF-2 binding characteristics with FGF-BP1.

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## Enhancement of Fibroblast Growth Factor (FGF) Activity by an FGF-binding Protein\*

Received for publication, May 30, 2001, and in revised form, July 29, 2001  
Published, JBC Papers in Press, August 16, 2001, DOI 10.1074/jbc.M104933200

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Fibroblast growth factor-binding protein (FGF-BP) 1 is a secreted protein that can bind fibroblast growth factors (FGFs) 1 and 2. These FGFs are typically stored on heparan sulfate 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 up-regulated 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-1 and -2 and investigated interactions with recombinant human FGF-BP1 protein as well as effects on signal transduction, cell proliferation, and angiogenesis. We show that recombinant FGF-BP1 specifically binds FGF-2 and that this binding is inhibited by FGF-1, heparan sulfate, and heparinoids. Furthermore, FGF-BP1 enhances FGF-1- and FGF-2-dependent proliferation of NIH-3T3 fibroblasts and FGF-2-induced extracellular signal-regulated kinase 2 phosphorylation. Finally, in the chicken chorioallantoic membrane angiogenesis assay, FGF-BP1 synergizes with exogenously added FGF-2. We conclude that FGF-BP1 binds directly to FGF-1 and FGF-2 and positively modulates the biological activities of these growth factors.

Fibroblast growth factors (FGFs)<sup>1</sup> represent a family of over 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 16–18-kDa protein, is one of the best-studied members 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 Refs. 1 and 2).

FGF-2 interacts with low affinity cell surface and extracellular matrix heparan sulfate proteoglycans, 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 heparan sulfate proteoglycans can modulate the action of FGF-2 by increasing its affinity for FGFRs (7). Moreover, heparan sulfate proteoglycans seem to protect FGF-2 from degradation by proteases in the extracellular environment (8, 9) and 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 phospholipase C $\gamma$  (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/MAPK 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, MAPK/extracellular signal-regulated kinase kinase, and p42/44<sup>MAPK</sup>, also known as extracellular signal-regulated kinase (ERK) 1 and 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 endoplasmic reticulum- 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 more easily. Two distinct mechanisms by which locally stored FGF-2 can be released from the ECM have been described. One mechanism involves digestion of the sugar backbone in heparan sulfate proteoglycans by heparinases or other glycosaminoglycan-de-

\* Supported by American Cancer Society Grant CB-202 (to A. W.), National Institutes of Health Grant CA71508 from the National Cancer Institute, Bethesda, Maryland (to A. W.), a grant from the US Army Medical Research Materiel Command Breast Cancer Program (to A. W. and A. A.-A.), and a grant from the Studienstiftung des Deutschen Volkes (to A. A.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) M60047.

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<sup>1</sup>The abbreviations used are: FGF, fibroblast growth factor; FGFR, FGF receptor; FGF-BP, FGF-binding protein; ERK, extracellular signal-regulated kinase; CAM, chorioallantoic membrane; MAPK, mitogen-activated protein kinase; ECM, extracellular matrix; TBS, Tris-buffered saline (50 mM Tris-HCl and 150 mM NaCl, pH 7.5); GST, glutathione *S*-transferase; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

grading enzymes (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 have previously shown that the binding of FGF-2 to a secreted binding protein (FGF-BP) might represent such a mechanism (26, 27). Wu *et al.* (28) initially described FGF-BP as a low affinity heparin-binding protein isolated from human epidermoid carcinoma A431 cells. FGF-BP has been shown to bind to FGF-1 and -2 in a noncovalent, reversible manner. Moreover, FGF-BP protects and presents FGF-2 to its high affinity cell surface receptor (26, 27, 29), and a recent study (30) demonstrates the interaction of FGF-BP with perlecan, a heparan sulfate proteoglycan in the basement membrane. This most likely represents a local reservoir for FGF-BP. A related protein designated FGF-BP2 has recently been identified by our laboratory (2), and we will thus refer to the original protein as FGF-BP1.

FGF-BP1 is expressed below the level of detection by Northern blotting in normal adult human tissues, whereas its expression is significantly elevated in various tumors, including head and neck, skin, cervical, and lung squamous cell carcinomas (26, 30). In addition, FGF-BP1 is up-regulated in some colon cancers and breast adenocarcinomas (27). Furthermore, we have recently shown that phorbol esters as well as epidermal growth factor can up-regulate FGF-BP1 gene transcription (31–33). We reported previously (26) that expression of human FGF-BP1 cDNA in the FGF-2-positive SW-13 cells led these cells to grow anchorage independently. 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 previously that the depletion of FGF-BP1 from squamous cell carcinoma 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 support a role for FGF-BP1 as a proangiogenic molecule in human tumors (27).

In the present study, we used recombinant FGF-BP1 protein to directly evaluate its binding to FGF-1 and -2 *in vitro* and study the functions of the protein. We found that FGF-BP1 was able to bind  $^{125}\text{I}$ -FGF-2 in a dose-dependent and specific manner and can be competed by FGF-1 and FGF-2 as well as by different heparinoids. Furthermore, we studied the role of FGF-BP1 on the activation of the Ras/MAPK 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 the 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.) 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<sub>2</sub>.

**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.). The baculovirus construct contains 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, which was then transformed into bac-

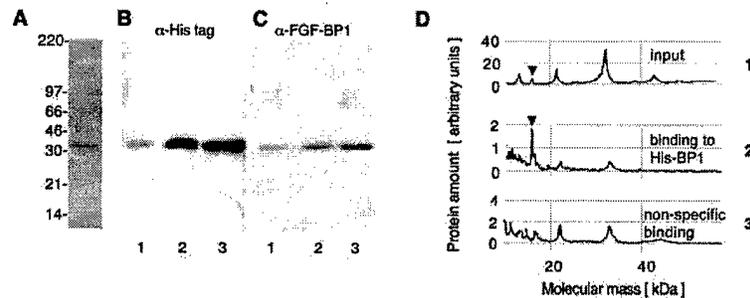
mid-containing DH10BAC competent cells. LacZ-negative clones containing the recombinant bacmid were identified. The bacmid DNA was isolated and then transfected into Sf-9 cells to generate baculovirus. Infected Sf-9 cells were grown for 5 days, pelleted, and lysed in a buffer containing 6 M guanidine-HCl, 0.01 M Tris-HCl, and 0.1 M sodium phosphate, pH 8.0. Cell lysates were homogenized and then incubated for 1 h on ice. Cellular debris was removed by centrifugation at 10,000 × *g* for 15 min. 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 5× Laemmli's buffer, boiled at 95 °C for 5 min, 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 (Bio-Rad). The His-tagged protein was detected with a rabbit polyclonal anti-FGF-BP1 (27) or a mouse monoclonal anti-His antibody (Invitrogen Corp., Carlsbad, CA) that was then visualized by enhanced chemiluminescence detection using horseradish peroxidase-linked donkey anti-rabbit or anti-mouse immunoglobulin G as the secondary antibodies, respectively (Amersham Pharmacia Biotech).

**Phosphorylation Studies**—50% confluent NIH-3T3 cells were serum-depleted overnight and treated for 5 min with 2 and 10 ng/ml FGF-2 (Invitrogen Corp.) and recombinant FGF-BP1. Controls were left untreated. Cells were then washed with cold phosphate-buffered saline, 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, and 100 μg/ml pefabloc. Cellular debris was removed by centrifugation at 14,000 rpm for 15 min. Phosphorylated proteins were immunoprecipitated from the cleared lysates by incubation with agarose-conjugated anti-phosphotyrosine (4G10; Upstate Biotechnology, Lake Placid, NY) monoclonal antibody for 2 h at 4 °C. Immunocomplexes were recovered by centrifugation and washed five times with cold lysis buffer. Samples were then resuspended with 15 μl of 2× Laemmli's buffer and boiled for 5 min at 95 °C. Alternatively, 10–50 μg of total protein cell extracts were resuspended with 5× Laemmli's buffer and heated for 5 min at 95 °C. Both immunoprecipitates and total cell lysates were separated on 10% SDS-polyacrylamide gels, transferred onto polyvinylidene difluoride membranes, and analyzed by immunoblot analysis. Tyrosine-phosphorylated proteins and ERK2 were detected with the corresponding mouse monoclonal antibody and then visualized by enhanced chemiluminescence detection using horseradish peroxidase-linked goat anti-mouse antibody (Amersham Pharmacia Biotech), respectively. Monoclonal anti-pan ERK antibody was purchased from BD Transduction Laboratories (Lexington, KY).

**FGF/FGF-BP1 Binding Assays**—100 ng/ml His-BP1 (a bilaterally hexahistidine-tagged FGF-BP1 protein) diluted in Tris-buffered saline (TBS) was incubated overnight in 96-well plates (EIA/RIA Strip Plate; Corning Inc., Corning, NY) at 4 °C with constant rocking. Excess unbound His-BP1 was removed by washing the wells twice with TBS. Nonspecific binding was blocked by the addition of 300 μl of LB medium (Bio 101, Carlsbad, CA) to the wells for 1 h at room temperature. Wells were then washed five times with TBS.  $^{125}\text{I}$ -FGF-2 (1–20 ng/ml) was added to the wells and incubated for 2 h at room temperature with constant rocking. Unbound  $^{125}\text{I}$ -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, His-BP1, pentosanpolysulfate (bene Chemie, Munich, Germany), and heparin or heparan sulfate (Sigma) were simultaneously added with  $^{125}\text{I}$ -FGF-2. Binding of radiolabeled FGF-2 to His-BP1 was measured by counting the radioactive emission from the individual wells.  $^{125}\text{I}$ -FGF-2 was purchased from Amersham Pharmacia Biotech. Human recombinant FGF-1 and FGF-2 were purchased from Life Technologies, Inc.

**Protein-protein Interaction Studies on Protein Arrays (Protein Chip Assay)**—The analysis was performed with a surface-enhanced laser desorption/ionization (34) (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 α-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



**FIG. 1. Silver stain, Western blots, and binding of FGF-2 to the recombinant His-BP1 protein.** *A*, silver stain of 30  $\mu$ l of a pooled, chelate affinity-purified His-BP1 preparation separated by a 4–20% gradient SDS-PAGE. *B* and *C*, Western blots of 30- $\mu$ l 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. *D*, protein chip analysis using surface-enhanced laser desorption/ionization to assess FGF-2 binding to immobilized His-BP1. Mass spectrometry analysis of proteins is shown. 1, input FGF-2 ligand preparation (FGF-2 spiked into cell growth media with 10% fetal calf serum). 2 and 3, proteins present in the input preparation that bound to immobilized His-BP1 (2) or to background (3), respectively. The arrowheads indicate the peak corresponding to the FGF-2 protein used as a ligand.

FGF-2, 3  $\mu$ l of a 240 mg/ml solution of FGF-BP1 in phosphate-buffered saline 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. For further details, see Ref. 35.

**Proliferation Assay**— $5 \times 10^3$  NIH-3T3 fibroblasts were seeded in three replicates in 96-well plates for 8 h. Cells were serum-deprived for 16 h and then treated with human recombinant FGF-1 (10 ng/ml), FGF-2 (5 ng/ml), anti-FGF-2 (15  $\mu$ g/ml), and His-BP1 (6 ng/ml), unless indicated otherwise. The proliferation rate was evaluated after 48 h by the addition of 10  $\mu$ l/well WST-1 reagent, as suggested by the manufacturer (Roche Molecular Biochemicals). Rabbit polyclonal anti-FGF-2 was purchased from R&D Systems (Minneapolis, MN).

**CAM Assay**—The CAM assay was carried out as described previously (36). In brief, 2-day-old fertilized chicken eggs were broken open into 35  $\times$  10-mm Petri dishes and incubated at 37 °C for 48 h. Sterile Whatmann filter disks (8 mm in diameter) were prewetted 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. CAMs were photographed using a digital camera at 0, 12, 24, and 36 h after 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 toward the disk). Scoring was carried out blinded, and the results were averaged. Baseline (no treatment) was subtracted from the average score as indicated.

**Statistics and Data Analysis**—Graphpad/Prism Software (San Diego, CA) was used for statistical analysis as well as curve fitting to obtain half-maximal inhibitory concentrations ( $IC_{50}$ ) from binding studies. From this,  $K_d$  values for FGF-2 were derived by using the Cheng-Prusoff equation ( $K_d = IC_{50}/(1 + L/K_d)$ ), (37) where  $L$  is the concentration of the FGF-2 radioligand, and  $IC_{50}$  is the half-maximal inhibitory concentration calculated from the competition isotherm of the unlabeled FGF-2.

## RESULTS

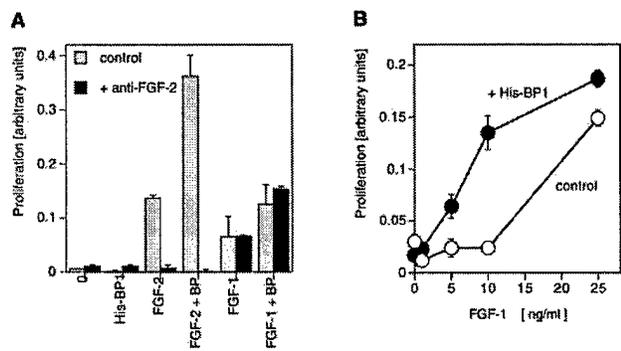
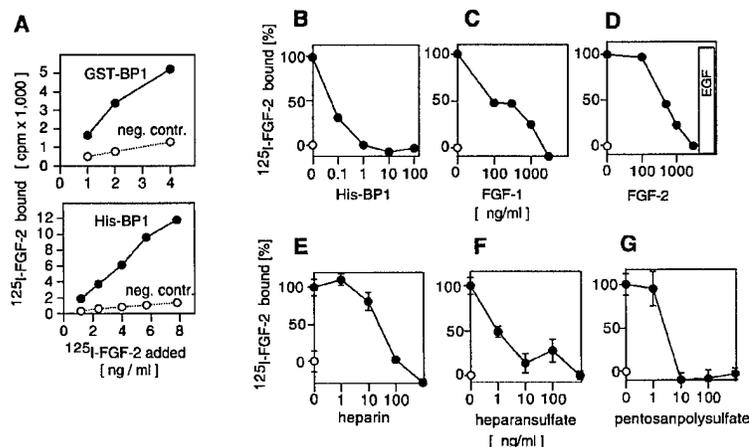
**Generation of Recombinant FGF-BP1 Protein**—In previous studies, we investigated the effects of FGF-BP1 by overexpression experiments in FGF-BP1-negative cells or by depletion of endogenous FGF-BP1 from cells using ribozyme targeting (26, 27, 29). Because FGF-BP1 is secreted from cells and acts as 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 nucleotides 197–799 of human FGF-BP1 cDNA (GenBank™ accession number 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 designated GST-BP1 was generated in BL21 bacteria using a pGEX-2TK construct that contains the same nucleotides (data

not shown). This protein was used to generate antibodies and used in some of the initial protein-protein interaction studies (see below). To determine whether the protein generated in the Sf-9 cells did indeed represent FGF-BP1, serial elutions obtained from the final affinity chromatography were separated by SDS-PAGE, and a single protein was detected by silver staining and Western blotting with anti-BP1 as well as anti-histidine tag antibodies (Fig. 1, A–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 His-BP1 protein is slower than that predicted by its molecular mass (26.9 kDa), most likely due to its basic nature, a feature also reported for the bovine BP1 protein (see Ref. 38).

**FGF Binding to FGF-BP1 in Cell-free Assays**—We first asked whether the recombinant His-BP1 will specifically recognize FGF-2 when the FGF-2 ligand is present at low abundance in a diverse mixture of molecules comprising fetal calf serum. To address this question, we used surface-enhanced laser desorption/ionization protein chip technology coupled with mass spectrometry (34) (see Fig. 1D). This approach was previously applied by us to characterize ligand-receptor interaction when studying pleiotrophin and its receptor, anaplastic lymphoma kinase (35). In the present experimental series, the His-BP1 protein was immobilized on a protein chip and incubated with FGF-2 that had been mixed with growth media containing 10% fetal calf serum. FGF-2 comprised only a very small portion of the overall preparation used as the input (arrowhead in Fig. 1D, 1, input). However, FGF-2 was specifically recognized by the immobilized His-BP1 in this mixture (arrowhead in Fig. 1D, 2), and only nonspecific binding was observed without the immobilized His-BP1 (Fig. 1D, 3). From this, we conclude that FGF-2 does indeed bind specifically to His-BP1, even when the growth factor is only present at very low abundance in a complex mixture of proteins and other molecules, and that no other ligand for His-BP1 is present in fetal calf serum.

We next sought to quantitate FGF binding to the His-BP1 recombinant protein. For this, a cell-free 96-well binding assay was established using immobilized His-BP1 as a bait and  $^{125}$ I-FGF-2 as a ligand. As shown in Fig. 2A, His-BP1 (bottom panel) and GST-BP1 (top panel) bound to the radiolabeled FGF-2 in a dose-dependent manner. The bacterial GST-BP1 displayed less specific and more nonspecific binding to  $^{125}$ I-FGF-2 per unit of protein in comparison with the eukaryotic His-BP1, and we thus decided to use the latter as the major tool for our additional studies. To support the specificity of His-BP1 binding to the FGF-2 radioligand, competition assays were performed with excess cold FGF-2, FGF-1, or His-BP1 as well as epider-

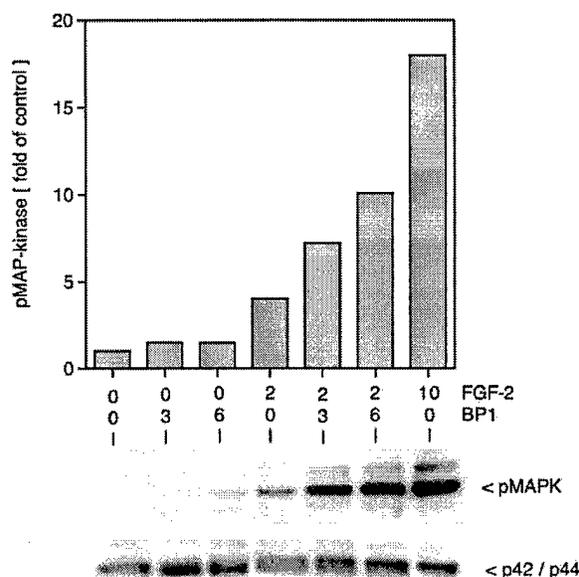
**FIG. 2. Binding of FGF-2 to His-BP1 and competition by different agents.** A, binding of different concentrations of  $^{125}\text{I}$ -FGF-2 to GST-BP1 (top panel) and His-BP1 (bottom panel) immobilized in 96-well plates. B–G, competition of His-BP1, FGF-1, FGF-2, heparin, heparan sulfate, and pentosanpolysulfate for  $^{125}\text{I}$ -FGF-2 binding to immobilized His-BP1.  $^{125}\text{I}$ -FGF-2 binding obtained from wells containing immobilized His-BP1 (●; 100%) in comparison to blocking solution only (○; 0%) is shown. The data are representative of at least four independent experiments, in which each sample was run in triplicate.



**FIG. 3. Effect of His-BP1 on FGF-1- and FGF-2-mediated mitogenesis in NIH-3T3 fibroblasts.** A, cells were treated for 48 h with FGF-1 (10 ng/ml) or FGF-2 (5 ng/ml)  $\pm$  His-BP1 (6 ng/ml) in the absence (□) or presence (■) of anti-FGF-2 antibody. B, concentration-response curve of FGF-1 in the absence (○) or presence of His-BP1 (●). The proliferation rate was measured as described under "Materials and Methods," and the data shown are representative of three independent experiments.

mal growth factor as a nonspecific growth factor control. As shown in Fig. 2B, increasing concentrations of His-BP1 were able to inhibit the binding of  $^{125}\text{I}$ -FGF-2 to the immobilized His-BP1, and an  $\sim 10$ -fold excess of the His-BP1 in solution completely inhibited FGF-2 binding. FGF-2 also competed for FGF-2 radioligand binding (Fig. 2D). From a series of such competition assays, we calculated an apparent dissociation constant ( $K_d$ ) value of  $\sim 10$  nM for FGF-2 binding to His-BP1 (for details, see "Materials and Methods"). Furthermore, in support of the original report on FGF-BP1 by Wu *et al.* (28), we found that FGF-1 also competed with FGF-2 for its binding to His-BP1 (Fig. 2C). Epidermal growth factor was used as a negative control and did not inhibit FGF-2 binding to the immobilized His-BP1 even at 10  $\mu\text{g}/\text{ml}$  (bar in Fig. 2D), supporting a specific interaction of FGF-BP1 and FGF-2.

Earlier studies from our laboratory have shown that FGF-BP1 lowers the affinity of heparin for FGF-2 (29) in support of the notion that FGF-BP can release FGF-2 from its local storage on glycosaminoglycans in the extracellular matrix (27). Conversely, based on this mechanism of action of FGF-BP1, we speculated that glycosaminoglycans in solutions should be able to disrupt binding of FGF-2 to FGF-BP. We thus studied the effects of heparin, heparan sulfate, and pentosanpolysulfate on the binding of FGF-2 to His-BP1 (Fig. 2, E–G). The extracellular matrix glycosaminoglycan heparan sulfate inhibited binding of FGF-2 to the immobilized His-BP1 at very low concentrations ( $\text{IC}_{50} = 1$  ng/ml), and the semisynthetic heparinoid

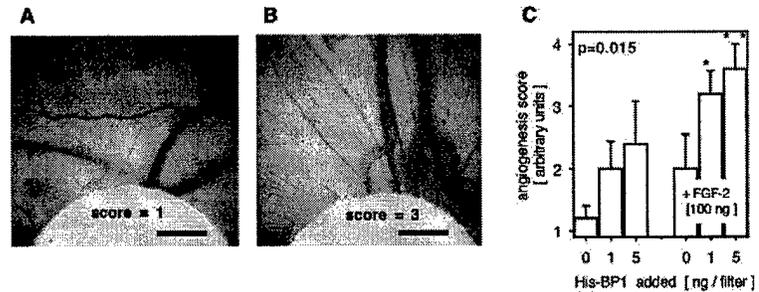


**FIG. 4. Effect of His-BP1 on FGF-2-induced ERK2 activation in NIH-3T3 fibroblasts.** Cells were starved overnight and treated for 5 min with different concentrations of FGF-2 and/or His-BP1, as indicated (ng/ml). Controls were left untreated. 50  $\mu\text{g}$  of total cell lysates were separated by 10% SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and immunoblotted with anti-phosphotyrosine monoclonal antibody (pMAPK). Detection of endogenous ERK2 in the protein extracts was determined by Western blot analysis, using anti-pan ERK monoclonal antibodies (p42/44). Quantitation of bands was obtained by densitometry and is expressed relative to control (bar graph).

pentosanpolysulfate (39–41) and the anticoagulant heparin were somewhat less potent ( $\text{IC}_{50} = 3$  and 30 ng/ml). All of these heparinoids completely inhibited binding of FGF-2 to the immobilized His-BP1, and we propose from these data that the heparin-binding domain in FGF-2 overlaps with the binding domain that interacts with FGF-BP. Also, this mutually exclusive binding of FGF-2 to FGF-BP or to a glycosaminoglycan supports the notion of FGF-BP as an extracellular chaperone molecule that releases locally stored FGFs (26, 27, 30, 42).

**FGF-BP1 Enhances FGF-induced Proliferation in NIH-3T3 Fibroblasts**—After we demonstrated the ability of the recombinant human FGF-BP1 to specifically bind FGF-1 and -2 in a cell-free system, we sought to determine the effect of this interaction on biological activity *in vitro*. FGF-1 and -2 are potent mitogens for a number of cell lines, including fibroblasts and

**Fig. 5. Effect of His-BP1 on FGF-2-induced angiogenesis in the CAM assay.** Filter disks with FGF-2 (100 ng)  $\pm$  His-BP1 (as indicated) were placed on CAMs of 4-day-old chicken embryos. A and B, photograph of angiogenesis on the CAMs scored as 1 (A) and 3 (B) at 24 h. Bar, 1 mm. C, quantitation of the assays;  $p = 0.015$  is the overall  $p$  value from analysis of variance. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  (comparison relative to the respective control sample).



endothelial cells (17), and we used NIH-3T3 fibroblasts as an experimental model system to study the effect of His-BP1 on FGF-induced proliferation (Fig. 3). 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 h 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 submaximally effective concentration of FGF-2 (5 ng/ml) and added His-BP1 (6 ng/ml) (Fig. 3A). Whereas no proliferation was detected when cells were grown in the presence of His-BP1 alone, the addition of 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 as well as the synergistic effects between His-BP1 and FGF-1. As for FGF-2, a concentration-response curve for FGF-1 showed that His-BP1 enhances the effects of low concentrations of FGF-1 ( $p < 0.01$ ; analysis of variance) and does not increase the maximal effect of the growth factor (Fig. 3B). 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 presence of 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 (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, 43). 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, as determined by immunoblot analysis with an anti-phosphotyrosine antibody, was significantly enhanced in NIH-3T3 fibroblasts when cells were co-stimulated for 5 min with different concentrations of FGF-2 and His-BP1. In particular, 3 and 6 ng/ml His-BP added to FGF-2 (2 ng/ml) exhibited an enhancement of phosphorylation of 1.8- and 2.5-fold, respectively, when compared with the levels obtained with FGF-2 alone (Fig. 4, bar graph). The same results were obtained in immunoprecipitation studies (data not shown). In addition, as shown in the bottom panel of Fig. 4, the levels of expression of ERK2 were not affected by these treatments and, consistent with the phospho-MAPK 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, 44). As an experimental approach to investigate the effects of FGF-BP1 on FGF-2-mediated angiogenesis *in vivo*, we used the chick embryo CAM assay. As shown in Fig. 5, FGF-2 and 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, 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 that binds FGF-2 and is hypothesized to mobilize FGF-2 from its storage in the ECM (28). Previous studies have found that endogenous FGF-BP1 is overexpressed in several cancers (26, 27, 30). 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 in athymic nude mice (27). Because endogenous FGF-BP1 plays a critical role in tumor growth and angiogenesis, we set out to explore the effects of exogenously added FGF-BP1 protein on FGF-dependent cellular responses.

For our experiments, we produced and purified human recombinant, polyhistidine-tagged FGF-BP1. This His-BP1 protein gave a single band after SDS-PAGE by silver staining as well as by immunoblotting, and recombinant His-BP1 binds radiolabeled FGF-2 *in vitro*. This binding is specific because it is competed by excess concentrations of cold FGF-1 and -2 or recombinant FGF-BP1 and not by an unrelated growth factor, epidermal growth factor. Heparinoids inhibit binding between FGF-2 and FGF-BP1 at low concentrations (nanograms/milliliter), and we conclude from this that binding of FGF-2 to heparinoids and to FGF-BP1 is mutually exclusive. This lends additional support to the role of FGF-BP1 as a chaperone that can shuttle FGFs from their glycosaminoglycan storage. The His-BP1 purified as a monomeric protein and chemical cross-linking studies of radiolabeled FGF-2 with His-BP1 suggested that these proteins bind at a 1:1 ratio.<sup>2</sup>

There have been conflicting reports with regard to whether FGF-BP1 binds FGF-1. In the original description of FGF-BP1, Wu *et al.* (28) found that FGF-BP1 purified from the media of A431 human epidermoid carcinoma cell cultures was also able to bind FGF-1. In contrast, Lametsch *et al.* (38) reported that FGF-BP1 purified from bovine parturition mammary secretion binds FGF-2, but not FGF-1. 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. Furthermore, FGF-BP1 synergized with FGF-1 as well as with FGF-2 in the proliferation assays with

<sup>2</sup> A. Al-Attar and A. Wellstein, unpublished data.

NIH-3T3 cells (Fig. 3), and the effect of FGF-BP1 on both of these FGFs was indistinguishable.

The MAPK pathway has been studied extensively 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 trigger 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 (28), 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 the ERK2 signal transduction pathway often controls the stimulation of cell proliferation (45, 46), and ERK2 activation is required for proliferation of fibroblasts *in vitro* (43). In addition to ERK2, FGF-2-dependent mitogenic signal transduction pathways lead to the activation of phospholipase C $\gamma$  and p70S6K, respectively. However, it has been observed that phospholipase C $\gamma$  activation is likely not to be responsible for FGF-2-mediated NIH-3T3 mitogenesis, nor is the signal that emanates from p70S6K sufficient to induce cell proliferation (47–49). 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 the addition of both recombinant FGF-BP1 and FGF-2 was significantly greater than that seen with the addition of FGF-2 alone. Furthermore, FGF-BP1 treatment led to a more rapid establishment of directed blood vessel growth when added to FGF-2 (see 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 down-regulate FGF-BP1 mRNA levels in tumors grown from the ME-180 cells and, coincident with that, the extent of tumor angiogenesis (50).

Binding proteins have been described for other cytokines, and the most relevant two binding protein families are those for insulin-like growth factor and transforming growth factor  $\beta$ . Latent transforming growth factor  $\beta$ -binding proteins and insulin-like growth factor-binding proteins have been shown to bind and protect their respective ligands from degradation and can positively or negatively modulate their ligands' functional

activities (51, 52). 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, 29, 38). 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-mediated cellular responses, such as signaling, proliferation, and angiogenesis.

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