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

A secreted carrier protein has been described which is able to bind to FGF-1 and FGF-2 in a non-covalent, reversible manner. FGF-2 bound to this protein was not subject to degradation and retained its mitogenic activity. This **FGF-binding protein (FGF-BP)** has been studied extensively by our laboratory. FGF-BP is highly expressed in squamous cell carcinomas (SCC) and EGF is able to increase the expression of FGF-BP in SCC derived cell lines through PKC, MEK/ERK, and p38 MAPK signaling. We have found FGF-BP mRNA to be expressed in two breast cancer cell lines (MDA-MB-468, MCF-7/ADR), by Northern Analysis/Ribonuclease Protection. EGF treatment of MDA-MB-468 cells resulted in an increase in FGF-BP mRNA expression in a time-dependent manner. EGF signaling occurs primarily through the PKC, and p38 MAPK pathways. Finally, EGF induction of the FGF-BP promoter is mediated through CCAAT/enhancer binding protein and AP-1 transcription factor binding sites on the promoter.

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Annual report for Grant Number DAMD17-00-1-0265
August 1, 2000 to July 31, 2001

P.I.: Benjamin L. Kagan

Title: Growth Factor Regulation of an Angiogenic Factor, the Fibroblast Growth Factor-Binding Protein (FGF-BP), in Breast Cancer

I. Introduction

Paracrine and autocrine growth factors have many functions, including a crucial role in inducing the formation of new blood vessels in a healing wound, as well as in a growing tumor. Many studies have demonstrated that a solid tumor mass cannot grow beyond a few millimeters in size without a sufficient supply of blood to the tumor. Tumor blood vessels provide a pathway for tumor cells to metastasize to distal sites, as well as a source of nourishment [1-4]. The most important and best-studied angiogenesis factors belong to the family of fibroblast growth factors (FGFs) [5, 6]. FGF-1 and FGF-2 (aFGF and bFGF, respectively) are unique in that their biological activities can be quenched by binding tightly to heparansulfate proteoglycan molecules in the extracellular matrix [7-10]. Two alternate mechanisms of FGF-1 and FGF-2 activation have been theorized as a result of a multitude of studies over the last decade. One mechanism involves the solubilization of FGF-2 from its storage site by heparanase digestion of the glycosaminoglycan portion of the cell attachment [11-14]. The second mechanism involves the binding of FGF to a secreted carrier protein delivering the activated FGF to its target receptor. A secreted carrier protein has been described which is able to bind to FGF-1 and FGF-2 in a non-covalent, reversible manner [15]. FGF-2 bound to this protein was not subject to degradation and retained its mitogenic activity [15]. This **FGF-binding protein (FGF-BP)** has been studied extensively by our laboratory.

Expression of FGF-BP in cell lines that express FGF-2 results in these cells having a tumorigenic and angiogenic phenotype [16]. FGF-BP transfected cells have been shown to release the protein into their media along with FGF-2 in a non-covalently bound form; the released FGF-2 is now biologically active [17]. FGF-BP mRNA is expressed in SCC, colon, and breast tumor cell lines and primary tumor tissue [16]. The role of FGF-BP during tumor progression has been studied by our laboratory using skin carcinogenesis as a model for epithelial cancers. We have shown that FGF-BP mRNA is upregulated in the skin during mouse development, but drops to low levels in adult mouse skin. In both mouse and human skin, FGF-BP mRNA and protein levels increase at least 3-fold upon treatment with PKC-activating TPA (12-*O*-tetradecanoylphorbol-13-acetate), and increase further in DMBA/TPA induced papillomas and carcinomas [18].

II. Body

The human FGF-BP promoter was recently isolated and cloned revealing positive and negative regulatory elements within a 118 base pair region just upstream of the FGF-BP transcription start site (**Figure 1**). The phorbol ester TPA was then shown to upregulate FGF-BP transcription in ME180 squamous cell carcinoma cells. This transcription was mediated through the activation of protein kinase C, and the Sp1, AP-1, and C/EBP positive regulatory elements in the FGF-BP promoter [19]. Treatment of ME180 SCC cells resulted in the upregulation of FGF-BP mRNA. Signal transduction was mediated through the EGFR, PKC, MEK/ERK, and p38 pathways, while transcription was mediated through the AP-1 and C/EBP regulatory elements in the promoter [20]. Finally, we have also shown that serum upregulates FGF-BP expression in ME180 cells, predominantly through PKC and p38 signaling, while only through the C/EBP site on the FGF-BP promoter [21]. **In my accepted proposal, I hypothesized that an angiogenic "funneling" effect exists in which intracellular signals intiated by EGF and related ligands result in the activation of FGF through the**

modulation of the FGF-BP gene. I planned to consider the relevance of this "funneling" effect with respect to the breast cancer system.

We have found FGF-BP mRNA to be expressed in two breast cancer cell lines, and 4 out of 6 clinical samples of human breast cancers, by Northern Analysis/Ribonuclease Protection, and RT-PCR, respectively. We have also detected FGF-BP mRNA in the human and mouse mammary gland. **This report summarizes the findings by Benjamin Kagan as PI of the funded research, testing the role of FGF-BP in human breast cancer cell progression and its regulation by the epidermal growth factor.**

Aim 1: To study the regulation of the FGF-BP mRNA, by growth factors, in breast cancer.

Detection of endogenous FGF-BP mRNA in MCF-7/ADR and MDA-MB-468 human breast cancer cell lines. Previously, we were able to show that FGF-BP mRNA was expressed in 9 out of 15 breast cancer cell lines, by RT-PCR. To study the regulation of FGF-BP expression in breast cancer cell lines, we wanted to use a quantitative method for detection of FGF-BP mRNA. A ribonuclease protection assay specific for human FGF-BP was developed using a riboprobe derived from a pRC/CMV vector plasmid containing the FGF-BP open reading frame [16]. We were able to detect FGF-BP mRNA only in the MCF-7/ADR cell line, an adriamycin resistant clone of the MCF-7 cell line, as well as the ME180 SCC cell line, which was used as a positive control. Northern analysis was also used, screening a wider array of breast cancer cell lines. **We were able to detect expression of FGF-BP mRNA in both MCF-7/ADR and the MDA-MB-468 cell lines. Expression of FGF-BP mRNA, as determined by RNase protection and Northern analysis, is summarized in Table 1.**

EGF regulation of endogenous FGF-BP in MDA-MB-468 cells. Studies have shown that the MDA-MB-468 cell line overexpresses the EGFR as compared to MCF-7 breast cancer cells [22-24]. Biscardi et al. [24] measure levels of EGFR to be 35 fold that of MCF-7 cells. Because the MDA-MB-468 cell line, like the ME180 cell line, express high levels of the EGFR [22], we decided to test whether FGF-BP mRNA expressed in these cells can be regulated by EGF and/or TPA. MDA-MB-468 cells were grown to 80% confluency, serum starved for 24 hours, and treated with EGF for 1, 3, 6, or 24 hours. FGF-BP mRNA levels were analyzed by Northern analysis, and we were able to observe that EGF induced FGF-BP upregulation at about 3-fold above control, peaking at 6 hours of EGF treatment (**Figure 2**). The time-course of EGF induction of FGF-BP mRNA in MDA-MB-468 cells was similar to that observed in the ME180 SCC cell line, suggesting similar mechanisms of regulation [20]. **These data demonstrate that EGF can regulate FGF-BP in MDA-MB-468 cells, in a similar manner to ME180 SCC cells**

EGF induction of FGF-BP in MDA-MB-468 cells is mediated through PKC and p38 MAPK signaling. EGF regulation of FGF-BP mRNA in ME-180 cells occurs through PKC, and the MEK/ERK and p38 MAPK signaling pathways [20]. Serum, in contrast, mediates FGF-BP transcription through PKC and p38 MAPK signaling, but not MEK/ERK [21]. To discern between the possible signaling pathways involved in EGF induction of FGF-BP in MDA-MB-468 cells, we tested pharmacological inhibitors of signal transduction at various concentrations for their affect on FGF-BP regulation. We found that treatment with the EGFR tyrosine kinase inhibitor PD153035 resulted in a significant concentration dependent inhibition of EGF induction of FGF-BP mRNA (**Figure 3**). Therefore, as expected, EGFR tyrosine kinase activity is essential for the EGF effect. To establish whether PKC activation was also required for the EGF effect on FGF-BP, we treated MDA-MB-468 cells with the bisindoylmaleimide PKC inhibitor Ro 31-8220 [25]. At concentrations of 1 μM and 10 μM, Ro 31-8220 was able to signficantly inhibit the EGF induction of FGF-BP (**Figure 3**). At these concentrations Ro 31-8220 is also able to inhibit other kinases including the mitogen- and stress-activated protein kinase-1 (MSK1) [26], therefore we tested whether the PKC-specific inhibitor calphostin C [27] could also inhibit the EGF effect. Treatment with 100 nM calphostin C significantly reduced EGF-induced FGF-BP mRNA expression by 50% (**Figure 3**). Taken together, these data suggest a role for PKC in the EGF induction of FGF-BP in MDA-MB-468 cells.

To determine whether different MAP kinase pathways were also involved in the EGF effect on FGF-BP, we used the MEK1/2 specific inhibitor U0126 and the p38 MAPK specific inhibitor SB202190 [28, 29]. Treatment with 1 μM and 10 μM U0126 did not significantly inhibit EGF induction (**Figure 3**). Although 20 μM U0126 significantly inhibited EGF induction of FGF-BP, the overall inhibition was only around 30% as compared to the ability of U0126 to inhibit the EGF induction of FGF-BP in ME-180 cells by 70% [20]. This suggests a lesser role for the MEK/ERK pathway in the EGF effect in MDA-MB-468 cells. In contrast, as seen in the ME-180 cells, treatment with increasing concentrations of the p38 MAPK inhibitor SB202190, resulted in a concentration-dependent inhibition of EGF-induced FGF-BP mRNA expression ranging from 55% inhibition at 5 μM to 80% inhibition at 20 μM. Furthermore, as described above, the bisindoylmaleimide Ro 31-8220 was able to significantly inhibit EGF-induced FGF-BP mRNA expression at concentrations specific for PKC and other kinases such as MSK1. MSK1 has been shown to be activated by p38 MAPK phosporylation [26, 30]. Taken together, these data suggest that p38 MAPK plays a dominant role in the induction of FGF-BP by EGF in MDA-MB-468 cells.

Other intracellular targets for EGF receptor-induced intracellular signaling include members of the c-Src protein tyrosine kinase family. c-Src family members interact with the EGFR at tyrosine residues via SH2 domains [31]. MDA-MB-468 cells have been shown to express moderate levels of c-Src protein as compared normal breast epithelium [24]. Therefore, we used the c-Src family specific inhibitor PP1 [32]. Treatment with PP1 resulted in a maximal inhibition of EGF induction of FGF-BP of 20% only at the highest concentration, 10 μM (**Figure 3**). Concentrations of 1 μM and 0.1 μM, also shown to inhibit s-Src family members [32], had no effect. This suggests that c-Src family members do not play a role in the EGF effect.

<u>Aim 2: To study the regulation of the human FGF-BP promoter in breast cancer cells.</u>

EGF regulation of the FGF-BP promoter in MDA-MB-468 cells. As described above, EGF induces the upregulation of FGF-BP in MDA-MB-468 breast cancer cells. To determine if this regulation occurred at the transcriptional level, we tested whether EGF regulated the activity of FGF-BP promoter in MDA-MB-468 cells. As described above, various portions of the human FGF-BP promoter, full-length, mutated, or deleted, have been cloned upstream of a luciferase reporter gene. These constructs have been used successfully to assess the activity of the FGF-BP promoter in ME180 cells [19, 20, 33]. We were able to show that in MDA-MB-468 cells, treatment with EGF was able to induce the activity of the –1060/+62 and –118/+62 promoter constructs 4- to 5-fold above basal (**Figure 4**). Deletion of either the AP-1 or the C/EBP, and not the Sp1(b) site, reduced the induction by EGF of the promoter constructs, suggesting the AP-1 and the C/EBP sites were necessary for EGF induced FGF-BP transcription in this cell line. This observation is similar to what was observed in the ME180 cells [20]. Upon further investigation, cell-type specific differences were observed. Deletion of the AP-1 site resulted in a statistically significant decrease in promoter basal activity, suggesting the AP-1 site is necessary for basal activity. Deletion of the C/EBP site revealed a statistically significant increase in promoter basal activity, suggesting differences in C/EBP binding to the site affecting both basal and EGF induced activity of the FGF-BP promoter. **These data show that EGF is able to induce the activity of the FGF-BP promoter in MDA-MB-468 cells, through the AP-1 and C/EBP sites, as seen in ME180 cells. In addition, C/EBP binding to the FGF-BP promoter may repress basal activity while enhancing promoter activity after EGF treatment.**

III. Key Research Accomplishments

- Expression of FGF-BP mRNA was detected in both MCF-7/ADR and the MDA-MB-468 cell lines by Northern analysis and RNase protection.
- EGF upregulates FGF-BP expression in MDA-MB-468 cells, in a similar manner to ME180 SCC cells. This occurs predominantly through the PKC and p38 MAPK signaling pathways.

- EGF is able to induce the activity of the FGF-BP promoter in MDA-MB-468 cells, through the AP-1 and C/EBP sites, as seen in ME180 cells. Deletion of the C/EBP site on the FGF-BP promoter results in a significant increase in basal promoter activity.

IV. Reportable Outcomes

Manuscripts, abstracts, and publications produced as a result of this funded research:

Kagan BL, Harris VK, Coticchia CM, Ray R, Wellstein A and Riegel AT, Transcriptional regulation of a binding protein for FGF (FGF-BP) through p38/SAPK2 signaling. In: *Proceedings of the American Association for Cancer Research, New Orleans, LA,* March 24-March 28, *2001.*

Kagan BL, Cabal-Manzano R, Stoica GE, Nguyen Q, Wellstein A, and Riegel AT, EGF-induced fibroblast growth factor–binding protein (FGF-BP) expression in breast cancer is mediated through C/EBPβ-regulated transcription and p38 MAPK signaling. *2001* (manuscript in preparation)

V. Conclusions

We were able to observe FGF-BP expression in the MDA-MB-468 cell line. In this model we demonstrated that EGF was able to upregulate FGF-BP transcription. This is important in the context of breast cancer because expression of the EGFR has been inversely correlated with ER expression, and along with expression of the EGFR family member HER2, the EGFR has been correlated with a poor prognosis for breast cancer. FGF-BP expression, and its regulation by EGF in the MDA-MB-468 breast cancer cell line, may suggest that FGF-BP plays a role in the expression of a more angiogenic phenotype in breast cancer.

As described above, deletion of the C/EBP site on the FGF-BP promoter resulted in a significant increase in promoter basal activity. This suggests that a C/EBP factor binding to this site acts as a repressor. Recently, a variant of C/EBPβ, the liver enriched inhibitory protein (LIP), translated from the same mRNA as the full length protein (also called liver enriched activating protein or LAP), has been described [34, 35]. LIP is similar to LAP, except that it does not contain a transcactivating domain. The LIP-LAP dimer is able to bind to its normal consensus site on a promoter, with greater affinity than LAP-LAP dimers, but is not able to promote transcription, therefore acting as a dominant negative [35]. LIP has also been found to be expressed in human breast cancer samples that are both ER and PR negative [36]. **These data suggest the possibility that LIP may be present on the FGF-BP promoter in the MDA-MB-468 cells under basal conditions acting as a repressor of FGF-BP basal activity. When stimulated with EGF, the C/EBP dimer might change to a LAP/LAP dimer and therefore enhance FGF-BP promoter activity.** These hypotheses are currently under investigation.

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Appendix:

Figure 1. Regulatory region of the FGF-BP promoter

Table 1. Levels of FGF-BP, ER, and EGFR in human breast cancer cell lines. (adapted from Biscardi et al., *Mol Carcinog* 21:261-272, 1998)

Cell Line	EGFR	ER	FGF-BP
MCF-7	+	++	-
MCF-7/ADR	+++	-	++
BT20	++	-	-
BT474	-	+	-
T47D	-	+	-
T47Dco	-	-	-
MDA-MB 231	++	-	+/-
MDA-MB 468	++++	-	+++
BT549	++	-	+/-
Hs 578T	++	-	+/-

Figure 2. EGF induction of FGF-BP mRNA in MDA-MB-468 cells.

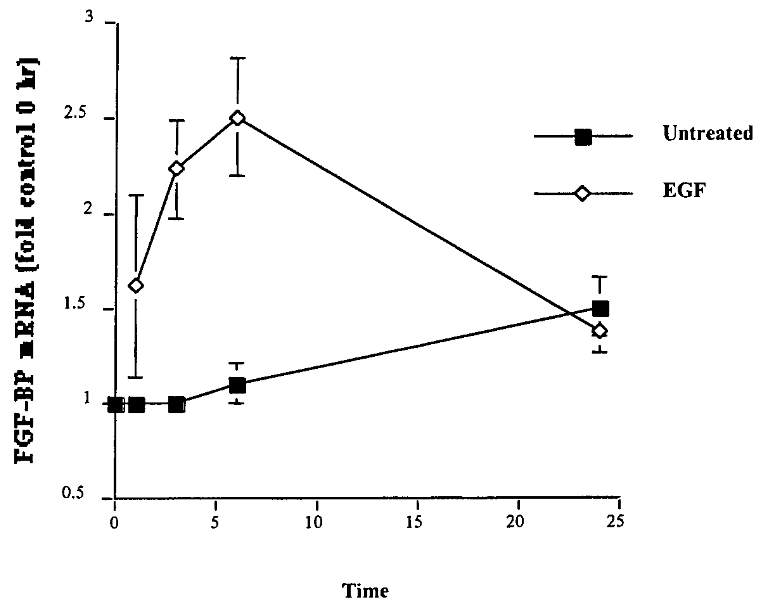

Figure 3. Effect of signal transduction inhibitors on the EGF induction of FGF-BP in MDA-MB-468 cells. The following inhibitors were used: Calphostin C (PKC), PD153035 (EGFR), U0126 (MEK1/2), PP1 (c-Src), Ro 31-8220 (PKC, MSK1), and SB202190 (p38/MAPK).