Complex Regulation of the Fibroblast Growth Factor-binding Protein in MDA-MB-468 Breast Cancer Cells by CCAAT/Enhancer-binding Protein β1

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ABSTRACT

The fibroblast growth factor-binding protein (FGF-BP) binds and activates fibroblast growth factors in the extracellular matrix, and can have a rate-limiting role in tumor angiogenesis. Here we demonstrate high levels of FGF-BP expression in invasive human breast cancer, relative to normal breast and in situ carcinoma, and in MDA-MB-468 human breast cancer cells. In these cells, FGF-BP was up-regulated by treatment with epidermal growth factor (EGF), dependent on protein kinase C and p38 mitogen-activated protein kinase signaling. Mutational analysis revealed that the activator protein 1 and CCAAT/ enhancer binding protein (C/EBP) sites on the FGF-BP gene promoter were required for the EGF effect, whereas deletion of the C/EBP site resulted in a significant increase in promoter basal activity indicating a basal repressive control mechanism. These data suggest that the C/EBP site is a central regulatory element for the regulation of FGF-BP promoter activity in MDA-MB-468 cells. We found that MDA-MB-468 cells express high endogenous levels of both the activating (LAP) and repressive (LIP) isoforms of C/EBPβ. Overexpression of C/EBPβ-LAP in MDA-MB-468 cells resulted in a large 80-fold increase in FGF-BP promoter basal activity, which was reversed by coexpression of LIP. Gel-shift analysis revealed that four LIP- and LAP-containing complexes (α-d) bind to the C/EBP site. DNA binding of the LIP and LAP-containing complex and the b complex in the presence of EGF was modulated by inhibition of p38 mitogen-activated protein kinase, suggesting a role for these complexes in the EGF induction of the FGF-BP promoter. This study suggests that along with its well-defined role in mammary gland development, C/EBPβ may well play a role in the pathology of breast cancer, in particular in the control of angiogenesis in the invasive phenotype.

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 angiogenic factors belong to the family of FGFs (5, 6). FGF-1 and FGF-2 are unique in that their biological activities can be quenched by binding tightly to heparan sulfate proteoglycan molecules in the extracellular matrix (7–10). A mechanism by which FGF can be activated involves the binding of FGF to a secreted carrier protein delivering the activated FGF to its target receptor. This secreted carrier protein, called the FGF-BP, is able to bind to FGF-1 and FGF-2 in a noncovalent, reversible manner (11). FGF-2 bound to FGF-BP protein is not subject to degradation (12) and can enhance the mitogenic activity of FGF-2 in mouse fibroblasts (13).

Expression of FGF-BP in cell lines that express EGF-2 results in these cells having a tumorigenic and angiogenic phenotype (12). FGF-BP-transfected cells are able to release the protein into their medium along with FGF-2 in a noncovalently bound form; the released FGF-2 is then biologically active (14). FGF-BP mRNA is expressed in SCC and colorectal carcinoma tumor tissue (12). FGF-BP mRNA is up-regulated in the skin of mice during embryonic development but drops to low levels in adult skin. In both mouse and human skin, FGF-BP mRNA and protein levels increase at least 3-fold on treatment with the PKC-activating phorbol ester TPA, and increase additionally in 7,12-dimethylbenz(a)anthracene/TPA-induced papillomas and carcinomas (15). We have also determined that FGF-BP can be up-regulated in SCC by EGF (16), and both TPA and EGF regulation of FGF-BP are dependent on an AP-1 and a C/EBP site in the FGF-BP promoter (16, 17).

C/EBPβ is a member of the C/EBP family of transcription factors (C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, and C/EBPε) that bind to consensus DNA sequences as homo- and heterodimers, and affect the transcription of various genes involved in proliferation and differentiation, especially in the liver and the immune system (18, 19). A variant of C/EBPβ, the C/EBPβ-LIP, translated from the same mRNA as the full-length protein (also called C/EBPβ-LAP), has been described (20, 21). The LAP variant is similar to LAP, except that it does not contain a transactivating domain. The C/EBPβ-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 factor (20). C/EBPβ, especially LAP, may have a central role in mouse mammary gland differentiation and proliferation (22, 23), and C/EBPβ-LIP is expressed in human breast cancer samples that are both estrogen receptor- and progesterone receptor-negative (24).

Like many other growth factors, EGF plays a role in the tumorigenesis of many different types of cancers such as SCCs (reviewed in Refs. 25–27) and in the tumorigenesis of breast cancer (28). Expression of the EGF receptor and other members of the EGF receptor family, especially HER2, has been associated with poor prognosis in breast cancer (28, 29). In SCC, FGF-BP is an EGF target gene, whereas in colon cancer cells we do not observe induction of FGF-BP by EGF (4). Therefore, it was of interest to determine the level of

Cancer Tissue Resource; CMV, cytomegalovirus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FBS, fetal bovine serum; MAPK, mitogen-activated protein kinase; EGFR, epidermal growth factor receptor; MEK, mitogen-activated protein kinase.

4 R. Ray and A. T. Riegel, unpublished observations.
expression of FGF-BP in human breast cancer, and to determine the EGF and basal regulation of this gene in this setting.

MATERIALS AND METHODS

Cell Culture and Reagents. The MDA-MB-468 human breast cancer cell line and the ME-180 human cervical SCC cell line were obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in IMEM with 10% FBS (Invitrogen Inc., Carlsbad, CA). Human recombinant EGF was purchased from Collaborative Biochemical Products (Bedford, MA). Tyrophostin AG1517 (PD153035), Ro 31–8220 (bisindolylmaleimide IX) and PPI were purchased from Alexis Corp. SB202190 was purchased from Calbiochem (San Diego, CA). U0126 was purchased from Promega. Calphostin C was purchased Sigma-RBI (Natick, MA). Wortmannin was purchased from Biomol (Plymouth Meeting, PA). All of the compounds were dissolved in Me2SO.

Case Samples. A total of 205 paraffin-embedded tissue blocks from patients with breast cancer who had undergone surgery were obtained from the Lombardi Cancer Center/Georgetown University Medical Center. Pathology reports and clinical histories at the time of admission were reviewed to determine the nature of the lesion and tumor staging. The samples were classified as follows: 143 in situ, 34 invasive, and 28 normal, noncancerous breast tissue. Serial recuts of 4-μm paraffin-embedded tissue sections were used for detecting FGF-BP mRNA expression by ISH. In addition, TAMs from the National Cancer Institute CBCTR were used. This TMA contained samples from invasive cancer, as well as in situ carcinoma and control breast tissues from reduction mammoplasty.

ISH. The expression of FGF-BP mRNA in human breast tissue samples was assessed by ISH. The FGF-BP riboprobe consisted of a 668-bp internal sequence of FGF-BP cDNA (11), subcloned into the pRc/CMV vector (5.5 kb; Invitrogen). Digoxigenin-labeled antisense and sense riboprobe were made using the DIG RNA labeling kit (Roche) according to protocol. Tissue sections were cut (4-μm) and mounted on (+)-charged glass slides (Fisher Scientific, Pittsburgh, PA) using standard histology technique.

The protocol used for ISH has been described previously (30–32). Briefly, tissues were microwaved (high) for 3 min, and incubated at 56°C overnight and at 65°C 1 h before deparaffinization. Paraffin was removed by two 5-min immersions in xylene followed by two 5-min washes in ethanol, and one wash in diethylpyrocarbonate-treated water. After deparaffinization, samples were digested in a 1 × PBS/10 μg/ml proteinase K solution at 37°C for 10 min. The slides were washed once in diethyl pyrocarbonate water and once in 2 × SSC [0.9 mM NaCl and 0.09 m sodium citrate (pH 7.0)]. For tissue deproteinization, Serial recuts of 4-μm paraffin-embedded tissue sections were used for detecting FGF-BP mRNA expression by ISH. In addition, TAMs from the National Cancer Institute CBCTR were used. This TMA contained samples from invasive cancer, as well as in situ carcinoma and control breast tissues from reduction mammoplasty.

Northern analysis was carried out as described previously (17) using 20 μg of total RNA. Hybridization probes were prepared by random-primed DNA labeling (Amersham Biosciences, Piscataway, NJ) of purified insert fragments from human FGF-BP (12) and human GAPDH (Clontech, Palo Alto, CA). Quantification of mRNA levels was performed using a PhosphorImager (Amersham Biosciences).

Plasmids. Human FGF-BP promoter fragments were cloned into the pXP1 promoterless luciferase reporter vector and have been described previously (17). The MEK2 (K101A) dominant-negative construct was provided by Dr. J. Holt (Vanderbilt University, Nashville, TN). The expression plasmids containing the type pGAPDH-LAP (pCDNA3-Flag-pGAPDH-LAP) and constitutively active MKK6 (pCDNA3-Flag-MKK6(Glu)) were provided by Dr. R. Davis (University of Massachusetts, Boston, MA). The expression vectors for human CEBPβ-LAP and CEBPβ-LIP (CMV-LAP and CMV-LIP, respectively) were gifts from Dr. U. Schibler (University of Geneva, Geneva, Switzerland) courtesy of Dr. J. Schwartz (University of Michigan, Ann Arbor, MI). Wild-type CEBPβ mRNA contains three in-frame AUG translation start sites, from which LAP and LIP are translated from the second and third sites, respectively (20). The second in-frame AUG is flanked by an imperfect Kozak’s sequence, GAC-CATGG (21), compared with the Kozak’s consensus sequence of CCA/GCCAUGG (33, 34), whereas the third in-frame AUG is flanked by a perfect Kozak’s sequence (20), resulting in translation of both LAP and LIP. The CMV-LAP construct contains only the second and third translation start sites, but both are flanked by perfectly matched Kozak’s sequences resulting in the more efficient translation of LAP alone (20). The effects of dominant negatives or activated constructs were compared with their empty vector control or with the empty vector pCDNA3 (Invitrogen).

Transient Transfections and Reporter Gene Assays. Twenty-four h before transfection, MDA-MB-468 cells were plated at a density of 3 × 10^6 cells in 10-cm dishes. pRL-CMV Renilla luciferase reporter vector (Promega, Madison, WI) was included as a control for transfection efficiency. MDA-MB-468 cells were transfected by electroporation as described by Raja et al. (35). Briefly, cells were trypsinized and washed twice by centrifugation in IMEM containing 10% FBS. The cells from each plate were then resuspended
in 400 μl of IMEM containing 20% FBS. A total of 30 μg of plasmid DNA (29 μg of FGF-BP promoter construct and 3.0 ng of pRL-CMV) was added to the cell suspension 5 min before electroporation. For cotransfection, 24 μg of −118/+62Luc FGF-BP promoter construct, 5 μg or indicated amounts of expression vector, and 3.0 ng of pRL-CMV were added to cells. Electroporation of the entire cell sample was carried out in a cuvette with an electrode gap of 0.4 cm at 350 V and 500 μF, using a Bio-Rad GenePulser II (Bio-Rad, Hercules, CA). The electroporated cells were then distributed equally to a six-well plate, each well having been prefilled with 3 ml of IMEM with 10% FBS. Cells were allowed to recover and attach for 16 h before treatment. Transfected cells were washed twice with serum-free IMEM, treated with or without EGF (10 ng/ml) in serum-free IMEM for 16 h, and then lysed in 150 μl of passive lysis buffer (Promega). Twenty μl of extract was assayed for both firefly and Renilla luciferase activity using the Dual-Luciferase reporter assay system (Promega). To correct for transfection efficiency and a small background induction (1.5–2.0-fold) of the pRL-CMV plasmid by EGF (16), Renilla luciferase values were corrected for protein content, and these numbers were then used to normalize firefly luciferase values. Protein content of cell extracts was determined by Bradford assay (Bio-Rad).

**Gel-Shift Assays.** MDA-MB-468 cells were grown to 80% confluence on 150-mm dishes, serum starved for 16 h, and treated with or without 10 ng/ml EGF for 1 h. As a control, ME-180 SCC cells were treated with 5 ng/ml EGF for 1 h. For gel-shift assays using transiently transfected cells, MDA-MB-468 cells were plated at a density of 6 × 10^5 cells in 150-mm dishes and transfected with 10 μg of expression vector or empty vector by electroporation as described above. Cells were then either untreated or treated with 10 ng/ml EGF for 1 h. To study the effects of inhibition of p38 MAPK, cells were pretreated for 1 h with 10 μM SB202190, then treated with or without 10 ng/ml EGF for 1 h. Nuclear extracts were prepared as described previously (17). Binding reactions with the −70/-51 and −55/-30 probe was carried out as described previously (17) with 6 μg of MDA-MB-468 nuclear extracts, binding buffer [20 mM Tris (pH 7.5), 60 mM KCl, 5% glycerol, 0.5 mM DTT, and 2.0 mM EDTA], and 500 ng of poly(dIdC).

Supershift antibodies (2 μg) or cold consensus oligonucleotide (20- and 50-fold molar excess) were added to the binding reaction for 10 min on ice before adding 20 fmol of labeled probe. Reactions were carried out for 20 min at room temperature and analyzed by 6% PAGE. Fox-specific antibodies c-Fos (K-25), c-Fos (4), Fos B (102), Fra-1 (R-20), and Fra-2 (Q-20); Jun-specific antibodies c-Jun/AP-1 (D), c-Jun/AP-1 (N), JunB (N-17), and JunD (329); and C/EBP-specific antibodies C/EBPα (C-19) and C/EBPβ (Δ198) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The C/EBP consensus oligonucleotide (Santa Cruz Biotechnology) had a sequence of 5′-TGCAAGTGGCAGAATCTGCA-3′.

**Western Analysis.** Forty μg of crude nuclear extracts from untreated or EGF-treated MDA-MB-468 and ME-180 cells, or 20 μg of lysates from MDA-pc468 transiently transfected cells were electrophoresed on Novex precast 4–20% Tris-glycine polyacrylamide gels (Invitrogen) at 150 V for 60 min. The protein was then transferred to polyvinylidene difluoride membranes (Millipore) for 2 h at 200 mA. Blots were blocked for 1 h in PBST (1× PBS and 0.5% Tween 20) containing 4% BSA (Sigma, St. Louis, MO), and then incubated for 1 h in 1× PBST/0.4% BSA, containing antibodies (1 μg/ml) for C/EBPα (C-19; Santa Cruz). Blots were washed with PBST (without BSA) four times for 5 min each, with agitation. Blots were then incubated for 1 h in antibody solution containing a 1:5000 dilution of horseradish peroxidase-labeled donkey antirabbit immunoglobulin (Amersham) and washed as before. Lastly, blots were assayed for enhanced chemiluminescence using SuperSignal West Pico Chemiluminescent Substrate (Pierce) and enhanced chemiluminescence film (Hyperfilm ECL; Amersham). Signal intensities of the M, 46,000, 36,000 (C/EBPα-LAP), and 20,000 (C/EBPβ-LIP) bands were measured by densitometry using multiple exposures to Hyperfilm ECL to assess the linear range. Expression of LAP and LIP were corrected for loading differences by comparing to the band intensity of the M, 46,000 band, as described by Zahnow et al. (23).

**Statistics.** The GraphPad Prism software package was used for graphics and data evaluation. Student t test was applied for continuous variables and χ² (Fisher’s exact test) for discontinuous variables. Values of P < 0.05 were considered significant.

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**RESULTS**

Expression of FGF-BP Correlates with Invasive Breast Cancer. In this study, we assessed the expression of FGF-BP in 205 (cancer and noncancer) paraffin-embedded tissue samples from breast surgical patients. IISH was performed using an FGF-BP-specific digoxigenin-labeled antisense riboprobe, and case samples were categorized by status (in situ or invasive) to correlate FGF-BP with cancer grade. Fig. 1A shows a representative example of parallel staining for FGF-BP mRNA expression in normal breast (negative) as well as breast cancer (one positive and one negative). FGF-BP mRNA expression was detected in 47% (67 of 143) of invasive cases, 23% (8 of 34) of in situ cases, and 14% (4 of 28) of normal and noncancerous breast (Table 1). These data show that expression of FGF-BP is associated with malignant transformation (P = 0.0002 for trend normal versus in situ versus invasive) and with invasiveness of human breast cancer (P = 0.014 in situ versus invasive; Table 1).

EGF Treatment Increases FGF-BP mRNA in MDA-MB-468 Human Breast Cancer Cells through PKC and p38 MAPK Signaling. To find a tissue culture model that recapitulated the high FGF-BP expression that we observed in invasive breast cancer, we screened a number of cell lines for FGF-BP mRNA expression. In this study and as reported previously (16, 36, 37), we have assayed FGF-BP mRNA expression, rather than protein expression, because FGF-BP is a secreted protein (12), and therefore it is difficult to measure using whole cell extracts. Although it has been shown previously that most breast cancer cell lines are negative for FGF-BP expression (12), we have found that MDA-MB-468 cells have high FGF-BP expression (Fig. 1). Interestingly, MDA-MB-468 is a breast cancer cell line that can be invasive and metastatic in vivo when injected into mice along with angiogenesis-stimulating Matrigel (38). In addition, MDA-MB-468 cells have been characterized as showing high expression of the EGFR receptor (39) and are EGF-responsive for invasion (40). Overexpression of the EGFR receptor is a poor prognostic indicator in breast cancer (28) presumably through EGF stimulation of a more invasive phenotype, although the precise target genes are not yet defined. Treatment of MDA-MB-468 cells with 10 ng/ml EGF resulted in a rapid increase in the steady-state levels of FGF-BP mRNA (Fig. 2A). Induction of FGF-BP mRNA was observed after 1 h of treatment and was maximal after 6 h with an average increase of 2.5–3-fold (Fig. 2B). 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 molecules at various concentrations for their effect on EGF-BP regulation. Treatment with the EGFR tyrosine kinase inhibitor PD153035 resulted in a significant concentration dependent inhibition of EGF induction of FGF-BP mRNA (Fig. 2C). In addition to EGFR tyrosine kinase activity, PKC is also involved in the EGF effect, because the bisindoylmaleimide PKC inhibitors Ro 31–8220 (41), at concentrations of 1 μM and 10 μM, and calphostin C (42) were both able to significantly inhibit the EGF induction of FGF-BP (Fig. 2C).

To determine the role of the additional downstream signaling pathways on EGF induction of FGF-BP, we used MEK1/2 and p38 MAPK inhibitors (43, 44). The MEK1/2-specific inhibitor U0126 was
only effective at higher concentrations of 10 μM and 20 μM, and not at the concentration of 1 μM (Fig. 2C); 1 μM of U0126 is usually used to specifically inhibit MEK-induced signaling (45). In contrast, treatment with increasing concentrations of the p38 MAPK inhibitor SB202190 resulted in a concentration-dependent inhibition of EGF-induced FGF-BP mRNA (Fig. 2C). 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 also potentially include members of the c-Src protein tyrosine kinase family. However, the c-Src-family-specific inhibitor PP1 (46) resulted only in a maximal 10% reduction in EGF-induced FGF-BP mRNA levels, only at the highest concentration used (10 μM; Fig. 2C). These data indicate only a minimal role for Src in EGF control of FGF-BP expression. The phosphatidylinositol 3'-kinase pathway also is not involved because treatment of MDA-MB-468 cells with 1 μM wortmannin did not have any significant effect on EGF induction of FGF-BP (data not shown). Overall, our pharmacological inhibitor data indicate a major role for PKC, and more significantly, p38 MAPK in EGF effects on FGF-BP in breast cancer cells.

**EGF Regulation of the FGF-BP Gene Promoter in MDA-MB-468 Cells.** The promoter of the human FGF-BP gene was cloned previously (17), and functional analysis of the promoter in ME-180 SCC cells revealed that activity is mediated through the first 118 bp of the proximal promoter sequence (16, 17). In contrast, in colon cancer cells the first 1060 bp of the promoter is involved in basal and regulated expression of the FGF-BP promoter. The important regulatory sites within this region are shown in Fig. 3, center panel, and include two Sp1 binding sites [Sp1(a) and Sp1(b)], AP-1, E-box, and C/EBP binding sites. We examined the promoter elements involved in the basal activity and EGF regulation of the FGF-BP promoter in MDA-MB-468 cells using a series of promoter mutant or deletion constructs linked to a luciferase reporter gene. After transfection into MDA-MB-468 cells, the fold activity or induction of each construct in the presence or absence of EGF treatment was determined. As shown in Fig. 3, left histogram, basal activity of the promoter was not significantly affected when deleted from −1060 to −118. In contrast, the −118 construct harboring a deletion of the AP-1 site (ΔAP-1) resulted in a significant decrease in basal promoter activity. Surprisingly, deletion of the C/EBP site resulted in a significant increase in basal activity of ∼3.5-fold equivalent to that seen with EGF treatment of the promoter. This suggested that the C/EBP site acts as a repressor element for FGF-BP promoter basal activity in breast cancer cells. This effect of deletion of the C/EBP site on the FGF-BP promoter was not seen in ME-180 cells (16, 17), suggesting a tissue-specific mechanism by which FGF-BP basal promoter activity is regulated.

Consistent with the response of the endogenous FGF-BP gene to EGF, the FGF-BP promoter was induced 4-fold by EGF in MDA-MB-468 cells, and this effect was still evident within the first 118 bp upstream of the transcription start site (Fig. 3, right histogram). Deletion of the AP-1 or C/EBP site on the FGF-BP promoter resulted in a 50% decrease in EGF induction (Fig. 3, right histogram), suggesting that in combination these sites constitute the majority of the transcriptional effects of EGF on the FGF-BP gene promoter in MDA-MB-468 cells. Consistent with this conclusion, deletion of the Sp1(b) site did not affect the ability of EGF to induce the FGF-BP promoter in MDA-MB-468 cells (Fig. 3, right histogram), nor did mutation of the E-box site centered at −58. This mutation has resulted previously in superinduction of promoter activity in both TPA- and EGF-treated SCC cells (47). Interestingly, this mutation did increase basal promoter activity to 2-fold over control (Fig. 3, right histogram). The necessity of the C/EBP site on the FGF-BP promoter for both basal and EGF-induced activity in MDA-MB-468 cells may represent a novel phenomenon, specific to the transcriptional regulation of FGF-BP in breast cancer cells.

**Contribution of the p38 Pathway to FGF-BP Promoter Activity.** Inhibition of p38 MAPK with pharmacological agents demonstrated the importance of this kinase in mediating the induction of FGF-BP by EGF only at the concentration of 1 μM (Fig. 2C); 1 μM of U0126 is usually used to specifically inhibit MEK-induced signaling (45). In contrast, treatment with increasing concentrations of the p38 MAPK inhibitor SB202190 resulted in a concentration-dependent inhibition of EGF-induced FGF-BP mRNA (Fig. 2C). 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 also potentially include members of the c-Src protein tyrosine kinase family. However, the c-Src-family-specific inhibitor PP1 (46) resulted only in a maximal 10% reduction in EGF-induced FGF-BP mRNA levels, only at the highest concentration used (10 μM; Fig. 2C). These data indicate only a minimal role for Src in EGF control of FGF-BP expression. The phosphatidylinositol 3'-kinase pathway also is not involved because treatment of MDA-MB-468 cells with 1 μM wortmannin did not have any significant effect on EGF induction of FGF-BP (data not shown). Overall, our pharmacological inhibitor data indicate a major role for PKC, and more significantly, p38 MAPK in EGF effects on FGF-BP in breast cancer cells.
EGF in MDA-MB-468 cells. To confirm this result, we demonstrated that expression vectors for p38 MAPK or a constitutively active mutant of MKK6 [MKK6(Glu)], a MEK that specifically phosphorylates p38 MAPK (48), along with the FGF-BP promoter luciferase construct and a CMV driven Renilla luciferase reporter vector for transfection efficiency, and were untreated or treated with 10 ng/ml of EGF for 18 h. Promoter constructs are described under “Experimental Procedures” and by Harris et al. (17). Values represent the mean from at least three separate experiments, each done in triplicate wells, bars, ±SE. Statistically significant differences relative to the −118/+62 promoter construct are indicated (*, P < 0.05, t test).

AP-1 and C/EBP Factors Bind to the FGF-BP Promoter in MDA-MB-468 Cells. To determine which AP-1 factors bind to the promoter in EGF-treated MDA-MB-468 cells, gel-shift analysis was carried out using labeled promoter sequence fragment −70 to −51 (Fig. 5A), and nuclear extracts from untreated or EGF-treated MDA-MB-468 cells. Protein binding in the uppermost complex, shown previously to represent AP-1 (16, 17, 37), is induced by EGF in MDA-MB-468 cells (Fig. 5B, Lanes 1 and 2, arrow). Incubation with an antibody for Fos family members resulted in a supershifted complex, and incubation with antibodies specific for c-Fos and FosB inhibited the formation of the AP-1 complex, suggesting that c-Fos and FosB bind to the AP-1 site on the FGF-BP promoter in MDA-MB-468 cells. The AP-1 complex was also blocked when EGF-treated MDA-MB-468 nuclear extracts were incubated with antibodies specific for Jun family members and JunB, suggesting that JunB also binds to the AP-1 site on the FGF-BP promoter (Fig. 5B, Lanes 8 and 10).

To demonstrate C/EBP binding to the FGF-BP promoter in MDA-MB-468 cells, we carried out gel-shift analysis using a labeled promoter sequence fragment from −55 to −30 (Fig. 5A), which was incubated in the presence of nuclear extracts from untreated or EGF-treated MDA-MB-468 cells. As shown in Fig. 5C (Lanes 1 and 2) and Fig. 8 (Lanes 1–3 and 12–14), we observed four specific complexes in nuclear extracts from MDA-MB-468 cells. However, no qualitative differences in any of these C/EBP complexes were observed between untreated and EGF-treated cells (Fig. 5C, Lanes 1, 2, 5, and 8). Complexes a-d, formed in MDA-MB-468 cells, were confirmed to bind C/EBPβ by supershift analysis using antibodies C/EBPβ (Δ198) and C/EBPβ (C-19), as well as by competition with a cold excess C/EBP consensus oligonucleotide (Fig. 5C, Lanes 6, 7, 9, and 10). Incubation with the Δ198 antibody, which recognizes all of the C/EBP family members, resulted in one supershifted band and the disappearance of band a when extracts from EGF-treated MDA-MB-468 cells were used (Fig. 5C, Lane 3). The C-19 antibody, which is specific for only C/EBPβ, resulted in a decrease in binding of complexes a and b.
pared the levels of C/EBP could account for the tissue-specific variations observed, we con-
considered that these differences in LAP:LIP ratios might explain the differences in basal activity seen with the ΔC/EBP promoter construct (Ref. 16; Fig. 3).

Effect of C/EBPβ Isoforms on the Activity of the FGF-BP Promoter. C/EBPβ-LIP has been shown to have a higher DNA-binding affinity than LAP, and that small increases in the levels of LAP present, relative to levels of LAP, confer a significant increase in the ability of transcriptional repression to occur (20). These results are consistent with the notion that the ratio of LAP:LIP present in a cell is an important factor that determines the transactivating ability of a C/EBPβ complex. To determine whether changes in C/EBPβ isomeric levels impacted on the activity of the FGF-BP promoter, we transiently cotransfected expression vectors for either LAP alone or LAP and LIP together (CMV-LAP and CMV-LIP, respectively) along with FGF-BP promoter constructs in MDA-MB-468 cells. Overexpression of LAP alone resulted in a significant increase of 80-fold in the basal activity of the −118/+62 FGF-BP promoter construct (Fig. 7A). In contrast, overexpression of LAP in ME-180 cells resulted in a much lesser 8-fold increase in basal activity of the −118/+62 construct.

The expression vector CMV-LIP was then introduced into MDA-MB-468 cells in increasing concentrations (2 μg and 4 μg), along with constant amounts of CMV-LAP (5 μg) and FGF-BP promoter constructs, to ascertain the effects of decreasing the ratio of LAP:LIP on the FGF-BP promoter. Cotransfection of 2 μg of LIP along with LAP, conferred a LAP:LIP ratio of ~2 (Fig. 7B), and this change in LIP:LAP ratio reduced the superinduction of the −118/+62 construct by 50% (Fig. 7C). Increasing LIP to 4 μg conferring a LAP:LIP ratio of 1 (Fig. 7B), and this significantly decreased the superinductive effect of LAP to a level 70% below the level of induction seen with LAP alone (Fig. 7C). The effects observed because of the changes in LAP:LIP ratio were primarily limited to the C/EBP site, because transfection with the ΔC/EBP construct in a constant activity level (Fig. 5C, Lane 4). These data suggest that C/EBPβ binding to the FGF-BP promoter in MDA-MB-468 cells is complex, but specific, and that the degree of binding is independent of EGF treatment.

C/EBPβ-LAP and C/EBPβ-LIP Are Expressed in MDA-MB-468. As shown in Fig. 3, deletion of the C/EBP site on the FGF-BP promoter results in a significant increase in basal promoter activity in MDA-MB-468 cells. This suggests that a transcription factor complex that binds to this site might act as a repressor, reducing the basal activity of the FGF-BP promoter, which is a unique aspect of regulation of FGF-BP promoter activity not observed previously. A variant of C/EBPβ, the C/EBPβ-LIP, translated from the same mRNA as the full-length protein but at a downstream start codon (also called C/EBPβ-LAP), has been described (20, 21). The LIP variant is similar to LAP, except that it does not contain a transactivating domain. The C/EBPβ-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 (20).

To determine whether differences in C/EBPβ isomeric expression could account for the tissue-specific variations observed, we compared the levels of C/EBPβ isoform expression in MDA-MB-468 (where the C/EBP site is repressive) and ME-180 cells (where the C/EBP site has no impact on basal activity). Immunoblot analysis using an antibody specific for the COOH terminus of C/EBPβ re-
vealed that MDA-MB-468 cells express higher levels of LIP relative to LAP (Fig. 6A, M, 20,000 band) as compared with ME-180 cells. In fact, as shown in Fig. 6B, ME-180 cells have a 3–4-fold higher LAP:LIP ratio than MDA-MB-468 cells, and we conjectured that these differences in LAP:LIP ratios might explain the differences in basal activity seen with the ΔC/EBP promoter construct (Ref. 16; Fig. 3).

Fig. 5. Transcription factor binding to FGF-BP promoter elements in MDA-MB-468 cells. A, double-stranded oligonucleotide sequences of promoter elements used for gel-shift analysis. B, supershift analysis of transcription factor binding to the AP-1, and C, C/EBP sites of the FGF-BP promoter. Labeled FGF-BP promoter sequences as indicated were incubated with nuclear extracts from untreated or EGF-treated MDA-MB-468 cells. Binding reactions were performed in the presence of “supershifting” antibodies or cold excess consensus oligonucleotide as indicated. An arrow or bar to the left of each panel indicates specific binding of AP-1 and C/EBP. * indicate supershifted complexes.

Fig. 6. Expression of C/EBPβ in ME-180 and MDA-MB-468 cells. A, a representative Western blot analysis of C/EBPβ protein levels using 40 μg of nuclear extracts from untreated and EGF-treated MDA-MB-468 and ME-180 cells. C/EBPβ was specifically recognized using a polyclonal antibody specific for the COOH terminus of the protein (C/EBPβ-C19). The positions of LAP (35 kDa) and LIP (20 kDa) isoforms are indicated by arrows. B, ratios of LAP to LIP in MDA-MB-468 and ME-180 cells. Levels of LAP and LIP were quantified from multiple exposures of Western blot analyses using densitometry and corrected for levels of the M, 46,000 nonspecific band. Values are expressed as actual ratios of LAP to LIP for each cell line.
EGF and C/EBPβ Regulation of FGF-BP in Breast Cancer

MB-468 with the radiolabeled −55/−30 oligonucleotide probe resulted in the formation of four complexes a-d. However, it should be noted that frequently in different preparations of nuclear extracts, one or more of the complexes was indistinguishable, and EGF treatment of cells resulted in no consistent differences in the mobility or the amount of any of the complexes (Fig. 8, Lanes 1 and 2 versus 9 and 10). To improve the resolution and to help identify components of these complexes, we examined binding to the FGF-BP promoter C/EBP element in extracts from cells in which levels of LIP or LAP had been increased by transient overexpression. Increasing levels of LIP, which functionally results in FGF-BP promoter repression (Fig. 7C), enhanced both the c and d complexes in the untreated and EGF-treated extracts (Fig. 8, Lanes 3 and 11). In contrast, the overexpression of LAP significantly increased the b complex and also slightly increased the c complex (Fig. 8, Lanes 6 and 14). We also determined that an antibody to C/EBP supershifted the b, c, and d complexes produced after overexpression of LIP or LAP (Fig. 8, Lanes 5, 8, 13 and 16).

The next question was then to determine how EGF and p38 MAPK signaling affected the binding of these complexes. Under conditions of basal levels of LIP and LAP, inhibition of p38 MAPK produced no consistent change in complex binding either in the presence or absence of EGF, although again in many of the extracts the levels of binding to the complexes were low and somewhat variable (Fig. 8, Lanes 2 and 10). Furthermore, under conditions of LIP overexpression, inhibition of p38 MAPK slightly decreased binding on the d complex (Fig. 8, Lanes 4 and 12). In contrast, inhibition of p38 MAPK with SB202190 decreased the LAP-induced binding of the c complex in basal and slightly increased the binding in EGF-treated conditions (Fig. 8, Lanes 7 versus 15). These data suggest that binding

Fig. 7. Effects of LAP and LIP overexpression on the FGF-BP promoter activity. A, MDA-MB-468 cells and ME-180 were cotransfected with the indicated FGF-BP promoter constructs and 5 μg CMV-LAP. Relative luciferase activity of the cells transfected with ΔC/EBP (■) or −118/+62 (□) constructs are expressed as fold over control (empty vector transfected cell lines). B, shown is a representative Western blot of C/EBPβ protein levels in MDA-MB-468 cells transiently transfected with CMV-LIP and CMV-LAP. The levels of LAP and LIP were quantified from multiple exposures of Western blot analyses using densitometry, and corrected for levels of the M, 46,000 band. Values are expressed as relative ratios of LAP to LIP for each transfection condition. C, MDA-MB-468 cells were cotransfected with the −118/+62 or ΔC/EBP (C, inset) FGF-BP promoter constructs, 5 μg CMV-LAP, and the indicated amounts of CMV-LIP or empty vector (pcDNA3). MDA-MB-468 cells were treated with vehicle alone (Me2SO) or 10 μM SB202190 for 1 h and then treated with or without 10 ng/ml EGF for 1 h. Binding reactions were incubated with a C/EBP supershift antibody where indicated. Specific binding is indicated by bars (a–d) to either side of each panel, nonspecific binding is indicated (NS), and supershifted complexes are indicated by *.

Fig. 8. Binding of C/EBPβ-LAP and -LIP to the C/EBP Site on the FGF-BP Promoter. To additionally investigate the interactions of C/EBPβ-LIP and -LAP with the C/EBP site we performed gel mobility shift analysis. As seen in Fig. 5C and Fig. 8 (Lanes 1 and 9), incubation of nuclear extracts from untreated and EGF-treated MDA-

80% below the level seen with the −118/+62 construct (Fig. 7C, inset). Interestingly, there is an apparent increase in the expression of LAP, concurrent with the increase in LIP, although the amount of transfected LAP DNA remains constant (Fig. 7B). This phenomenon may be accounted for by differences in transfection efficiencies between LAP-LIP-transfected cells or by the possible ability of LAP protein to be stabilized by increases in LIP expression. These scenarios remain to be studied.

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of the c complex can be changed by activation of the p38 MAPK signaling cascade. Interestingly, the binding of the b complex is differentially regulated under basal versus EGF-induced conditions. If EGF is present, then inhibition of the p38 MAPK increases binding of complex b (Fig. 8, Lanes 14 versus 15), whereas in the absence of EGF, inhibition of p38 MAPK decreases binding of b (Fig. 8, Lane 6 versus 7). This suggests that other EGF-induced signaling pathways can alter the susceptibility of the b complex to regulation by p38 MAPK. In summary it seems that basal repression of the promoter by LIP is well correlated with the formation of the d complex. Whereas inhibition of p38 MAPK had some effect on the binding of the d complex, the effect of p38 MAPK is mainly on the b and c complexes, the DNA binding of which can be modulated by p38 MAPK signaling especially by ongoing EGF stimulation.

**DISCUSSION**

In this study, we show that FGF-BP is highly expressed in invasive breast carcinoma, relative to normal breast tissues and *in situ* carcinoma, and that this pattern of expression is recapitulated in the MDA-MB-468 human breast cancer cell line. Previous studies from our laboratory have demonstrated that FGF-BP is also highly expressed in SCC and colon cancer (12, 15). However, in both of these tumor types we have observed that FGF-BP gene expression is turned on at early dysplastic stages of disease (15),6 whereas in breast cancer high FGF-BP gene expression is confined to invasive disease. This suggests that angiogenesis in late-stage breast disease may be controlled by a different set of growth factors than early breast cancer. This switching of rate-limiting angiogenic factors has precedent, for instance during colon cancer progression FGF-BP plays a role in early angiogenesis in the polyp and early dysplastic lesions, but vascular endothelial growth factor is a predominant factor during later stages (49, 50).

A unifying factor in terms of FGF-BP regulation in both breast and SCC is its dependence on high EGFR signaling for high levels of FGF-BP expression. In breast cancer, 30% of tumors have increased EGFR and HER2 signaling, and this is associated with a poor prognosis (28, 51–54). FGF-BP may well be a critical gene target for the EGFR/HER2 signaling in breast cancer, enhancing the angiogenic phenotype in late-stage disease. The involvement of the p38 MAPK signaling pathway, as a predominant mechanism for activating FGF-BP gene expression, is also common to SCC and breast cancer cells. Interestingly, activation of PKC is also a requirement for activation of FGF-BP gene expression in these two cell types, suggesting its involvement as another dominant regulator of FGF-BP transcription. PKC has been shown to be associated with the activation of p38 MAPK signaling (55, 56). Therefore, EGF-induced signaling through PKC may activate p38 MAPK-mediated FGF-BP transcription. A number of genes have been reported that can be selectively activated by EGF induction of p38 MAPK (57–59), and along with FGF-BP, these genes may represent a subset that are activated to initiate a particular EGF-induced phenotype. The selective use of p38 MAPK signaling for expression of an angiogenic molecule in invasive disease indicates that it may be possible to selectively target signaling pathways to abrogate the tumorigenic phenotype without unnecessarily dampening all of the proliferative signaling.

The data presented here suggest that the AP-1 and C/EBP sites in the FGF-BP promoter can be regulated by p38 MAPK. The factors binding to the AP-1 site are c-fos, Fos-B, and JunB, all of which can be phosphorylated and activated by p38 MAPK signaling (60). The pattern of AP-1 activation and binding to the FGF-BP promoter is similar to that observed in SCC and represents a conserved transcriptional target for p38 MAPK induction of FGF-BP in different tumor types. In contrast, the targeting of p38 MAPK signaling to the C/EBP site in the FGF-BP promoter, specifically to C/EBPβ, is less well defined. However, at this detection level, a major problem we found in this analysis was that the C/EBP-binding site in MDA-MB-468 extracts were frequently faint and indistinct, thereby making clear conclusions about the changes in factor binding after p38 MAPK activation difficult.

The overexpression of C/EBPβ/LIP and -LAP helped to determine whether these isoforms were part of complexes a-d. From gel-shift analysis we can conclude that complex b contains LAP, complex c contains LIP and LAP, and complex d contains LIP. Given the relative mobility of the LIP and LAP homo- and heterodimers reported previously (61), it could be postulated that complex b represents LAP homodimers, complex c represents LAP/LIP heterodimers, and the d complex is a LAP homodimer. However, because C/EBP family members can heterodimerize with other C/EBP family members (62) and with other leucine zipper family members such as Fos and Jun (62), we cannot rule out that the a-d complexes represent heterodimers with these proteins. Nevertheless, the b and c complexes are clearly regulated by p38 MAPK signaling. The c complex has the DNA binding activated by p38 MAPK, which suggests that if the c complex is responsible for EGF-induced gene activation, it is likely not a LAP/LIP heterodimer, because this is a repressive transactivation complex (20). The DNA binding of the b complex is increased by p38 MAPK in the presence of EGF-induced signaling but not when EGF signaling is absent. If the b complex is indeed the LAP/LIP homodimer, it can activate transcription from a C/EBPβ site (21), but our data suggest that p38 MAPK could be involved only when other signaling pathways in the cell are induced. In this regard, it has been reported that p38 MAPK can phosphorylate LAP (63), and this might be one possible mechanism of activation of the LAP homodimer. However, signaling through p38 MAPK can result in the phosphorylation of a number of other leucine zipper transcription factors, including ATF-2 (64, 65), Elk-1 (48), c-Jun (64), and CHOP (66). Therefore, we cannot rule out the possibility that heterodimeric complexes of these factors with LAP are playing a role in the EGF and p38 MAPK induction of the FGF-BP promoter at the C/EBP element. Overall, the regulation of FGF-BP transcription by EGF at the FGF-BP promoter C/EBP element appears to be complex, potentially dependent not only on the relative amounts of the C/EBP homodimers and their heterodimerization partners, but also on the activity of p38 MAPK and other signaling pathways in the cell.

Perhaps the most significant observation in our study was the difference seen in the regulation of the basal activity of the FGF-BP promoter in MDA-MB-468 cells on deletion of the C/EBP site, which resulted in a significant increase in basal promoter activity (80-fold). This is in contrast to ME-180 SCC cells where deletion of the same site did not result in any change in basal activity (16). These data suggest that although the C/EBP site is a necessary element for EGF-induced FGF-BP promoter activity in both SCC and breast cancer cells, this site has a unique dual role in the regulation of promoter activity specific to breast cancer cells. This also suggests that a cell type-specific repressive mechanism is present that can silence this promoter until an inductive signal, such as signaling through p38 MAPK, is received. Because LIP levels are high in the MDA-MB-468 cells and the LIP/LIP homodimer is a high affinity repressor (20), this complex could be responsible for this basal repressive activity. The d complex is a good candidate for the LAP/LIP homodimer because it has fast mobility (61) and is selectively increased by LIP overexpression. In addition, the d complex is unaffected by inhibition of the p38 MAPK pathway, which suggests that

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6 R. Cabal-Manzano, unpublished observations.
simple removal of the repressor may not explain the activation by EGFR, but that it must be replaced by an active transcription complex in order for FGF-BP gene expression to be induced. The repressive mechanism may not operate in SCC cells because the LIP levels are not high enough.

In conclusion, this study characterizes the signal transduction and transcriptional mechanisms that are important in the basal control and EGFR induction of FGF-BP expression in breast cancer cells. Understanding the regulatory pathways involved in the expression of FGF-BP is important, especially because deregulated FGF-BP expression is rate-limiting in tumor formation and tumor angiogenesis (12, 14). We have now observed a link between EGFR signaling and FGF-BP gene induction in both SCC (16) and breast cancer cells involving p38 MAPK signaling via AP-1 and C/EBPβ induced transcription. Although the phosphorylation site of C/EBPβ in response to p38 MAPK has not been defined in vivo, it is tempting to speculate that p38 MAPK can directly phosphorylate C/EBPβ and, thus, activate this molecule. We have also demonstrated the ability for the differential binding of C/EBP factors, specifically C/EBPβ-LAP and LIP, to regulate both unstimulated and EGFR-stimulated promoter activity of the human FGF-BP gene in human breast cancer cells. Whereas FGF-BP expression is associated with the malignant progression to invasive breast cancer, it is absent from most breast cancer cell lines assayed. This phenomenon may be explained by increased methylation of the FGF-BP gene in breast cancer cell lines versus tissues or the in vitro growth conditions in contrast to intact tissues. In addition, as demonstrated by this study, differences in LAP/LIP ratios differentially modulate FGF-BP promoter activity. The MDA-MB-468 cell line, which expresses FGF-BP, has a LAP:LIP ratio of 5:1, whereas the other cell lines that do not express FGF-BP may have LAP:LIP ratios that do not favor expression. These differences would be important to investigate to additionally understand the role of FGF-BP in human breast cancer.

Although many studies demonstrate that p38 MAPK has a role in angiogenesis (67) and the regulation of expression of angiogenic factors such as vascular endothelial growth factor (68), a role for C/EBP factors in the process of angiogenesis is not well characterized. This study suggests that along with its well-defined role in mammary gland development, C/EBPβ may well play a role in the pathology of breast cancer, particularly through the control of angiogenesis in the invasive phenotype.

ACKNOWLEDGMENTS

We thank Drs. Graciela Piwen-Pilipuk and Jessica Schwartz (University of Michigan at Ann Arbor, Ann Arbor, MI) for kindly providing expression plasmids for C/EBPβ-LAP and -LIP, Drs. Andrew Paterson and Jeffrey Kudlow (University of Alabama at Birmingham, Birmingham, AL) for kindly providing suggestions on the transfection of MDA-MB-468 cells; Dr. Violaine Harris for kindly providing suggestions and FGF-BP promoter luciferase reporter constructs; Dr. Ranjan Ray for sharing unpublished data; and the Riegel and Wellstein laboratories for providing helpful discussions.

REFERENCES


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Complex Regulation of the Fibroblast Growth Factor-binding Protein in MDA-MB-468 Breast Cancer Cells by CCAAT/Enhancer-binding Protein β
