Extracellular Matrix Fibronectin Increases Prostaglandin E₂ Receptor Subtype EP4 in Lung Carcinoma Cells through Multiple Signaling Pathways

THE ROLE OF AP-2

We have previously demonstrated that fibronectin (Fn) stimulates the proliferation of non-small cell lung carcinoma (NSCLC) cell growth through the induction of cyclooxygenase-2 (COX-2) and prostaglandin E₂ secretion. Here, we demonstrate that NSCLC cells express mRNA and protein for the prostaglandin E₂ receptor EP4 and that Fn enhances its stimulatory effect by inducing the expression of EP4, but not of EP1, EP2, and EP3 receptor subtypes. The effect of Fn on EP4 was inhibited by an antibody against α5β1 integrin and by inhibitors of phosphoinositide 3-kinase (wortmannin) and extracellular signal-regulated kinase (ERK) (5, 10). PGE₂, acting via EP4, contributes to tumor growth and progression of gallbladder and colorectal carcinoma (7, 8). Taken together, our results indicate that manipulation of prostaglandin E₂ receptors, designated EP4, and AP-2α may be the basis for new approaches for the prevention of lung carcinoma.

At present, the mechanisms that link Fn and EP4 gene expression and how they might relate to lung carcinoma are unknown. Herein, we explore the relationship between these molecules and their role in lung carcinoma cell growth. Our results show that Fn stimulates lung carcinoma cell growth and that this inductive effect is partly dependent upon stimulation of PGE₂ production and induced PGE₂ receptor subtype EP4 gene expression, which is mediated through integrin-dependent signals, including the activation of phosphoinositide 3-kinase (PI3K) and extracellular signal-regulated kinase (ERK) signaling pathways.
Fibronectin Stimulates EP4 in Lung Cancer

EXPERIMENTAL PROCEDURES

Culture and Chemicals—NSCLC cell lines H1838 and H2106 were obtained from the American Type Culture Collection (Manassas, VA), and were grown in RPMI 1640 medium (H1838) supplemented with 10% heat-inactivated fetal bovine serum, HEPES buffer, 50 IU/ml penicillin/streptomycin, and 1 μg of amphotericin (complete medium) or in Dulbecco’s modified Eagle’s medium/F-12 medium (H2106) supplemented with 10% heat-inactivated fetal bovine serum, 0.005 mg/ml insulin, 0.01 mg/ml transferrin, 30 nm sodium selenite, 10 nm hydrocortisone, 10 nm β-estradiol, 10 mm HEPES, as described previously (4). Afterward, cells were harvested and replaced in serum-free medium on Fn- or collagen type 1-coated culture plates for all experiments described later (the plates were coated with the matrix components diluted in buffer containing bovine serum albumin overnight at 4 °C). Afterward, the supernatants were removed, and the dishes were washed with phosphate-buffered saline three times before experiments were initiated. Mouse anti-human integrin α5β1 (MAB1969) and anti-integrin αβ1 antibodies (MAB1967) were purchased from Chemicon International Inc. (Temecula, CA), 16,16-dimethylprostaglandin E2 (dmPGE2), Polyclonal antibodies against Akt, ERK1, ERK2, and their phosphorylated isoforms (AktS473) (ERK Thr421/Ser422) from Cell Signaling Inc. The ERK1/2 inhibitor PD98095, the protein kinase C (PKC) inhibitor H89, the mammalian target of rapamycin (mTOR) inhibitor, rapamycin, and control nonspecific siRNA oligonucleotides with scrambled sequences. They were synthesized by Sigma (The Woodlands, TX) according to published data (13, 14). Final results, which were expressed as n-fold differences in EP4 gene expression relative to the GAPDH gene, were calculated using a formula based on a doubling of the product after each cycle (12). The procedures for treatment and total RNA preparation were identical to those described for RT-PCR. All DNA Master SYBR Green 1 kits and CellTiter-Glo luminescent cell viability assay were purchased from Roche biochem. The CellTiter-Glo (PKA) inhibitor wortmannin, and polyethylene glycol (PEG)-ester, CO), as described previously (15). For the transfection of ERK kinases, a mixture of respectively antisense or sense ODN or siRNA was added. After incubation for 15 min at room temperature, the mixture was diluted with medium and added to each well. The final concentration of siRNAs in each well was 100 nM. After culturing for 30 h, cells were washed and resuspended in new culture medium in the presence of 7 min at 72 °C. Analysis of amplicons was accomplished on 1% agarose gel containing 0.2 μg/ml ethidium bromide and visualized under UV transilluminator. The densitometric analysis of PCR products was performed by computer software (Bio-Rad Quantity One) and a GS-800 Imaging Densitometer (Bio-Rad) and standardized to the GAPDH product. EP4/GAPDH density bands in control groups were considered as 100%. Values of treatment group EP4/GAPDH ratios are given as percentage of controls. A 100-base pair ladder (Invitrogen) was used as a size standard.

Real Time RT-PCR—This procedure, which is based on the time point during cycling when amplification of the PCR product is first detected, rather than on the amount of PCR product accumulated after a fixed number of cycles, was described previously (12). Final results, which were expressed as n-fold differences in EP4 gene expression relative to the GAPDH gene, were calculated using a formula based on a doubling of the product after each cycle (12). The procedures for treatment and total RNA preparation were identical to those described for RT-PCR. All DNA Master SYBR Green 1 kits and CellTiter-Glo luminescent cell viability assay were purchased from Roche biochem. The CellTiter-Glo (PKA) inhibitor wortmannin, and polyethylene glycol (PEG)-ester, CO), as described previously (15). For the transfection of ERK kinases, a mixture of respectively antisense or sense ODN or siRNA was added. After incubation for 15 min at room temperature, the mixture was diluted with medium and added to each well. The final concentration of siRNAs in each well was 100 nM. After culturing for 30 h, cells were washed and resuspended in new culture medium in the presence of 7 min at 72 °C. Analysis of amplicons was accomplished on 1% agarose gel containing 0.2 μg/ml ethidium bromide and visualized under UV transilluminator. The densitometric analysis of PCR products was performed by computer software (Bio-Rad Quantity One) and a GS-800 Imaging Densitometer (Bio-Rad) and standardized to the GAPDH product. EP4/GAPDH density bands in control groups were considered as 100%. Values of treatment group EP4/GAPDH ratios are given as percentage of controls. A 100-base pair ladder (Invitrogen) was used as a size standard.

Reverse Transcriptase PCR—Total RNA was prepared from human lung carcinoma cells using TRIZol reagent (Invitrogen) according to the manufacturer’s instructions. To amplify 465-bp EP4 and 200-bp GAPDH cDNA fragments, the sequences of PCR primers (Sigma) were 5’-TCGGCGAGAAGCTACTGCTG-3’ (for EP4 sense), 5’-GACGGTGGCAGAATGGAGAAAGGA-3’ (for EP4 antisense), 5’-CCATTGGAGACTGCGG-3’ (for GAPDH sense), and 5’-CCATTGGGATCGAATGAC-3’ (for GAPDH antisense) according to published data (11, 12). The RT-PCR was carried out as previously described (12). The samples were first denatured at 95 °C for 30 s, followed by 32 PCR cycles, each with temperature variations as follows: 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. The last cycle was followed by an additional extension incubation of 7 min at 72 °C. Analysis of amplicons was accomplished on 1% agarose gel containing 0.2 μg/ml ethidium bromide and visualized under UV transilluminator. The densitometric analysis of PCR products was performed by computer software (Bio-Rad Quantity One) and a GS-800 Imaging Densitometer (Bio-Rad) and standardized to the GAPDH product. EP4/GAPDH density bands in control groups were considered as 100%. Values of treatment group EP4/GAPDH ratios are given as percentage of controls. A 100-base pair ladder (Invitrogen) was used as a size standard.

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or absence of Fn for an additional 24 h for Western blot analysis, cell growth, and gel mobility shift assays.

**Western Blot Analysis**—The procedure was performed as previously described (16). Protein concentrations were determined by the Bio-Rad protein assay. Equal amounts of protein from whole cell lysates were solubilized in 2× SDS-sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 5–10% 2-mercaptoethanol, and 0.004% bromophenol blue) and separated on SDS-8–10% polyacrylamide gels. The separated proteins were transferred onto nitrocellulose using a Bio-Rad Trans Blot semidyed transfer apparatus for 1 h at 25 V, blocked with Blotto (1× TBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl)) with or without 5% bovine serum albumin, 5% nonfat dry milk, and 0.1% Tween 20 overnight at 4 °C, and washed twice for 5 min with wash buffer (1× TBS and 0.1% Tween 20). Blots were incubated with polyclonal antibodies against COX-2, EP1, EP2, EP3, EP4, Akt, ERK1, ERK2, and their phosphorylated forms and for AP-2, AP-2α, AP-2β, and AP-2δ (1:1000) overnight at 37 °C, washed three times for 5 min with wash buffer, and incubated with a secondary antibody raised against rabbit IgG conjugated to horseradish peroxidase (1:1000; Sigma) for 1 h at room temperature. The blots were washed four times in wash buffer, transferred to freshly made ECL solution (Amersham Biosciences) and exposed to x-ray film. Protein bands were quantified by densitometric scanning using a Bio-Rad GS-800 calibrated densitometer. In controls, antibody was omitted or replaced by serum IgG.

**Cell Viability Assay**—NSCLC cells (10 × 10^5 cells/well) were transfected with EP4 siRNA for 30 h and Fn (20 μg/ml)-coated culture plates for an additional 48 h in 96-well plates. The cells in culture were detached with 0.1% trichloroacetic acid at 4 °C for 20 min and washed with 1× PBS. Afterward, the attached cells were treated with ice-cold 6% trichloroacetic acid at 4 °C for 20 min and washed once with 6% trichloroacetic acid. Cells were solubilized with 0.1 N NaOH and counted in a liquid scintillation counter in 4 ml of scintillation fluid.

**Siteld-directed Mutagenesis**—To prepare site-directed mutants of the promoter, the following oligonucleotides were synthesized: mutated AP-2 (−1529 bp), 5′-GGTTTTTAATGGCTGTTTCCGATC; mutated AP-2 (−1133 bp), 5′-GTCGGTCTTCCCTGCTTTCCTTG; mutated AP-2 (−1000 bp), 5′-GCTCCTGCCAAGTCTTACCAGGAGCTCG; the lowercase letters indicate mutation, and the underlined letters indicate the AP-2 binding site. The EP4 plasmid constructs containing site-directed mutations of AP-2 cis-acting elements were generated by oligonucleotide-directed mutagenesis using the GeneEditor in vitro site-directed mutagenesis system according to recommendations by the manufacturer (Promega). Briefly, double-stranded EP4 promoter plasmid was alkaline-denatured, precipitated, washed, and resuspended in Tris-EDTA buffer. Mutated AP-2 oligonucleotides and selection oligonucleotides were annealed; mutant strands were synthesized, ligated, and transformed into BMH 71-18 mutS competent cells. The mutated AP-2 EP4 plasmid was isolated and transformed into JM109 competent cells. Colonies (10–15) were selected and screened for mutants by sequencing using Applied Biosystems ABI Prism 377 DNA sequencer.

**Transient Transfection Assay**—The human EP4 wild-type and deletion promoter constructs (pGleP4-1 to -5) ligated to the luciferase reporter gene have been reported previously (18). The EP4 promoter construct contains ~4200 bp of the 5′-flanking region of the mouse EP4 receptor gene connected to the pGL3 basic luciferase reporter vector (Promega). NSCLC cells were seeded at a density of 5 × 10^5 cells/well in 6-well dishes and grown to 50–60% confluence. For each well, 2 μg of the above plasmid DNA with or without 0.2 μg of the internal control phRL-SV40 was cotransfected with TLRG E6 lipofection reagent (Promega). The reporter constructs and measurement were determined using the dual luciferase gene expression assay by the manufacturer (Promega). Nuclear proteins (5 g/10^5 cells) were prepared sequentially and were used in the luciferase activity measurements. Firefly luciferase activity was normalized with changes in the Renilla luciferase activity within the same sample.

**Electrophoretic Mobility Shift Assay**—Electrophoretic mobility shift assays were performed as described before (12). The 5′-flanking oligonucleotides used as probes were as follows: wild type Sp1 (5′-CTCCCCGGCCAAGCTGG-3′), mutant Sp1 (5′-CTCCCCGCACAAGCTGG-3′), wild type C/EBP (5′-GATAATTTAACATGAT-3′), mutant C/EBP (5′-GACTTGAATTTAACATGAT-3′), wild type AP-2 (5′-CTTCCGGCAGGGATCTGGCCTG-3′), and mutant AP-2 (5′-CTTCCGGCAGGGATCTGGCCTG-3′), which is based on the EP4 promoter sequences (18) and consensus AP-2 binding motif (5′-GATCGAAGTACCGCCGGCCGGCGCGG-3′). The complementary oligonucleotides were annealed and purified following the manufacturer’s protocol. The Sp1, C/EBP, and AP-2 oligonucleotides were end-labeled with [γ-32P]ATP using T4 polynucleotide kinase as recommended by the manufacturer. Nuclear proteins (5 μg) were first incubated under binding conditions (10 mM HEPES, 10 mM Tris-HCl (pH 7.9), 50 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 12% (v/v) glycerol, and 2 μg of poly(dI-dC)) for 10 min, followed by the addition of [γ-32P]ATP probe for another 20 min at room temperature in a final volume of 20 μl in the presence or absence of AP-2 antibodies (2 μg/ml). For cold competition, a 100-fold excess of the respective unlabeled consensus oligonucleotides was incubated for 15 min before adding the
of EP4 could influence the effects of Fn on cell growth. We first depleted EP4 from cells in culture using siRNA approaches. Treatment of H1838 cells with EP4 siRNA blocked EP4 production. Levels of EP4 were unchanged in cells transfected with control siRNA oligonucleotides (Fig. 1B). To determine if EP4 siRNA can block Fn-induced lung carcinoma cell growth in our system, H1838 cells were transfected with EP4 siRNA duplexes. Afterward, the cells were plated onto Fn-coated culture plates for an additional 24 h. As shown in Fig. 1C, the EP4 siRNA duplexes inhibited Fn-induced H1838 cell proliferation, whereas the control siRNA had no effects as determined by the [3H]thymidine incorporation assay. Similar results were also found by cell viability assays (Fig. 1D).

Since EP4 has been shown to be involved in human lung carcinoma biology, we tested if Fn can affect its expression. H1838 cells exposed to Fn showed increased EP4 protein levels in a time- and dose-dependent manner with maximal increases noted in 24 h at concentrations of 20 μg/ml (Fig. 2, A and C). Similar results were also observed in an additional NSCLC cell line (H2106) (Fig. 2, B and D), which significantly stimulated EP4 mRNA levels in a dose-dependent manner, with maximal increases noted at concentrations of 20 μg/ml Fn as determined by the CellTiter-Glo Luminescent cell viability assay (Fig. 2F). This result was consistent with our previous studies (Fig. 2G). Of note, cells cultured on Fn-coated plates adhered well to the plates. In order to determine whether Fn-induced EP4 expression was dependent on other EP receptors, we examined other EP receptors. We found that treatment of H1838 cells with or without anti-integrin α5β1 antibodies (MAB1969) or anti-integrin α2β1 antibodies (MAB1967; 25 μg/ml each) for 2 h before exposing the cells to Fn-coated plates with Fn. We found that Fn-induced EP4 protein was eliminated in the presence of α5β1 antibodies, whereas the anti-α2β1 antibodies had no effect (Fig. 3A). This suggests that α5β1 integrin mediates Fn-induced EP4 expression.

Fn has been shown to affect kinase signaling pathways in several studies (21–23). We previously demonstrated that Fn activated ERK and PI3K/Akt signaling pathways in NSCLC cells (4, 23, 24). Here, we examined if inhibition of these kinase signal pathways diminished or abrogated the effects of Fn on EP4. The specific inhibitors of ERK (PD98095 (25 μM)) and of PI3K (wortmannin (100 nM)) significantly blocked Fn-induced EP4 protein levels in H1838 cells (Fig. 3B). We also demonstrated that mTOR signals were mediating some of the effects of Fn on NSCLC cell growth (23). However, we found that rapamycin, an inhibitor of mTOR, had no effect on inhibition of Fn-induced expression of EP4 protein (Fig. 3C). Also, the inhibitor of PKA (H89 (10 μM)) or of PKC (calphostin C (Cal; 0.5 μM)) had no effects (Fig. 3, D and E). In addition, we showed probe. The same amount of probe as the probe was used for hybridization. 

Statistical Analysis—All experiments were performed at least three times. All data from gel shift assays, luciferase activity assays, RT-PCR, and Western blot analysis were expressed as mean ± S.D. The data presented in some figures are from representative experiment, which was qualitatively similar in the replicate experiments. Statistical significance was determined with Student’s t test (two-tailed) comparison between two groups of data sets. The asterisks shown in the figures indicate significant differences of experimental groups in comparison with the corresponding control condition (p < 0.05; see figure legends).

RESULTS

Effect of Fn on EP4 Gene Expression in Human Lung Carcinoma Cells—We previously showed that mRNAs encoding for the four PGE2 receptor subtypes are present in human NSCLC cells (17). Consistent with this, we showed that the PGE2 receptor subtype EP4 protein is expressed in the two NSCLC cell lines studied (Fig. 1A). We also demonstrated that Fn stimulated NSCLC cell growth (4). Here, we examined whether blockade
Fibronectin Stimulates EP4 in Lung Cancer

FIGURE 2. The effect of Fn on EP4 gene expression in human lung cancer cells. A and B, dose-dependent effect of Fn on EP4 expression. Cellular proteins were isolated from H1838 (A) and H2106 (B) cells cultured on plates coated with Fn (20 μg/ml) for the indicated time period. Afterward, Western blot was performed to detect EP4 protein levels. C and D, time-dependent effect of Fn on EP4 expression. Cellular proteins were isolated from H1838 (C) and H2106 (D) cells cultured on plates coated with increased concentrations of Fn or collagen and then subjected to Western blot analysis for EP4 expression as determined by real time RT-PCR (B and D). E and F, dose-dependent effect of Fn and collagen on EP4 expression. Total RNA was isolated from H1838 cells cultured on plates coated with increased concentrations of Fn or collagen and then subjected to RT-PCR analysis. GAPDH served as internal control for normalization purposes. *, significant differences from control (p < 0.05). Con, untreated control; Fn, fibronectin; Collagen, type 1 (20 μg/ml). G, dose-dependent effect of Fn on EP4 expression. H1838 cells treated with Fn for 24 h showed a significant increase in EP4 protein levels. Actin served as internal control for normalization purposes.

that the inhibitor of PI3K, wortmannin, blocked ERK activation in H1838 cells (not shown).

Fn Increased EP4 Promoter Activity—We next examined whether the effects of Fn on EP4 expression occur at the transcriptional level. The EP4 promoter contains multiple transcription factor binding sites, including NF-κB, NF-IL6 (C/EBP), Sp1, and AP-2, among others (Fig. 4A). These sites have been shown to be differentially responsive to various stimuli (15, 18, 25, 26). We found that H1838 cells, transfected with the full-length wild-type EP4 promoter (−4200/−116 bp) luciferase reporter construct, exposed to Fn showed increased promoter activity. Collagen type 1 had no effect on the wild-type promoter (Fig. 4B). The Fn-induced EP4 promoter activity was slightly reduced in one EP4 deletion reporter construct (−1555/−116 bp). There was no response to Fn with another EP4 deletion reporter construct (−992/−116 bp) (Fig. 4B), indicating that the region between −1555 and −992 bp in the EP4 promoter played an important role in stimulation of EP4 gene expression in response to Fn. We also tested whether the effect of Fn on EP4 promoter activity was mediated by Sp1, C/EBP, and AP-2 binding activities (Fig. 4B). These sites have been shown to be differentially responsive to various stimuli (15, 18, 25, 26). We found that H1838 cells, transfected with the full-length wild-type EP4 promoter (−4200/−116 bp) luciferase reporter construct, exposed to Fn showed increased promoter activity. Collagen type 1 had no effect on the wild-type promoter (Fig. 4B). The Fn-induced EP4 promoter activity was slightly reduced in one EP4 deletion reporter construct (−1555/−116 bp). There was no response to Fn with another EP4 deletion reporter construct (−992/−116 bp) (Fig. 4B), indicating that the region between −1555 and −992 bp in the EP4 promoter played an important role in stimulation of EP4 gene expression in response to Fn. We also tested whether the effect of Fn on EP4 promoter activity was mediated by Sp1, C/EBP, and AP-2 binding activities (Fig. 4B). These sites have been shown to be differentially responsive to various stimuli (15, 18, 25, 26). We found that H1838 cells, transfected with the full-length wild-type EP4 promoter (−4200/−116 bp) luciferase reporter construct, exposed to Fn showed increased promoter activity. Collagen type 1 had no effect on the wild-type promoter (Fig. 4B). The Fn-induced EP4 promoter activity was slightly reduced in one EP4 deletion reporter construct (−1555/−116 bp). There was no response to Fn with another EP4 deletion reporter construct (−992/−116 bp) (Fig. 4B), indicating that the region between −1555 and −992 bp in the EP4 promoter played an important role in stimulation of EP4 gene expression in response to Fn. We also tested whether the effect of Fn on EP4 promoter activity was mediated by Sp1, C/EBP, and AP-2 binding activities (Fig. 4B). These sites have been shown to be differentially responsive to various stimuli (15, 18, 25, 26). We found that H1838 cells, transfected with the full-length wild-type EP4 promoter (−4200/−116 bp) luciferase reporter construct, exposed to Fn showed increased promoter activity. Collagen type 1 had no effect on the wild-type promoter (Fig. 4B). The Fn-induced EP4 promoter activity was slightly reduced in one EP4 deletion reporter construct (−1555/−116 bp). There was no response to Fn with another EP4 deletion reporter construct (−992/−116 bp) (Fig. 4B), indicating that the region between −1555 and −992 bp in the EP4 promoter played an important role in stimulation of EP4 gene expression in response to Fn. We also tested whether the effect of Fn on EP4 promoter activity was mediated by Sp1, C/EBP, and AP-2 binding activities (Fig. 4B). These sites have been shown to be differentially responsive to various stimuli (15, 18, 25, 26). We found that H1838 cells, transfected with the full-length wild-type EP4 promoter (−4200/−116 bp) luciferase reporter construct, exposed to Fn showed increased promoter activity. Collagen type 1 had no effect on the wild-type promoter (Fig. 4B). The Fn-induced EP4 promoter activity was slightly reduced in one EP4 deletion reporter construct (−1555/−116 bp). There was no response to Fn with another EP4 deletion reporter construct (−992/−116 bp) (Fig. 4B), indicating that the region between −1555 and −992 bp in the EP4 promoter played an important role in stimulation of EP4 gene expression in response to Fn. We also tested whether
Fibronectin Stimulates EP4 in Lung Cancer

FIGURE 3. Involvement of α5β1 integrin, PI3K, ERK, and mTOR pathways in the induction of EP4 by Fn. A, effect of anti-α5β1 antibodies on Fn-induced EP4 protein levels. Cellular protein was isolated from H1838 cells cultured for up to 2 h in the presence or absence of anti-α5β1 and anti-α2β1 antibodies (25 μg/ml each) before exposing the cells to culture plates coated with Fn for an additional 24 h and then subjected to Western blot analysis for EP4. B, effect of inhibitors of ERK1/2 and PI3K on Fn-induced EP4 protein levels. Cellular protein was isolated from H1838 cells cultured for up to 2 h in the presence or absence of PD98095 (25 μM) or wortmannin (100 nM) before exposing the cells to Fn-coated culture plates for an additional 24 h and then subjected to Western blot analysis for EP4. C, effect of mTOR inhibitor on Fn-induced EP4 protein levels. Cellular protein was isolated from H1838 cells cultured for up to 4 h in the presence or absence of rapamycin (10 μM) before exposing the cells to Fn-coated culture plates for an additional 24 h and then subjected to Western blot analysis for EP4. D, effect of PKA inhibitors on Fn-induced EP4 protein levels. Cellular protein was isolated from H1838 cells cultured for up to 2 h in the presence or absence of H89 (10 μM) before exposing the cells to Fn-coated culture plates for an additional 24 h and then subjected to Western blot analysis. E, effect of PKC inhibitors on Fn-induced EP4 protein levels. Cellular protein was isolated from H1838 cells cultured for up to 2 h in the presence or absence of calphostin C (0.5 μM) before exposing the cells to Fn-coated culture plates for an additional 24 h, then subjected to Western blot analysis. F, effect of P38K inhibitor on ERK1/2. Cellular protein was isolated from H1838 cells cultured for 1 h in the presence or absence of wortmannin (100 nM) before exposing the cells to Fn (20 μg/ml) coated onto the culture plates for an additional 1 h. Afterward, Western blot was performed to detect phosphorylated ERK1/2 and total ERK1 and ERK2 proteins. G, effect of ERK inhibitor on Akt. Cellular protein was isolated from H1838 cells cultured for 1 h in the presence or absence of PD98095 (25 μM) before exposing the cells to Fn (20 μg/ml) for an additional 1 h. Afterward, Western blot was performed to detect phosphorylated Akt and total Akt protein. Actin served as internal control for normalization purposes. Con, untreated control cells.

induced AP-2 binding activity (Fig. 5E). The addition of an AP-2α antibody induced a super-shift band, whereas AP-2β and AP-2γ antibodies had no effects (Fig. 5F). The specific bands for AP-2, Sp1, and C/EBP were attenuated by a 100-fold molar excess of unlabeled wild-type oligonucleotides but were not inhibited by the mutated unlabeled oligonucleotides (Fig. 5, Mut). Oligonucleotides containing a mutated AP-2 (Mut AP-2), Sp1 (Mut Sp1), or C/EBP (Mut C/EBP) site were end-labeled with [γ-32P]ATP and used as another control to confirm the binding specificity. Similar results were obtained with H2106 cells (not shown).

The Role of Transcription Factor AP-2 in Fn Induction of EP4 and Cell Growth—We further tested the role of AP-2 in mediating Fn-induced EP4 expression in human lung carcinoma cells by using the antisense approach. We showed that a specific AP-2 antisense ODN completely blocked the production of EP4 by Fn, whereas this antisense ODN had no effect on the AP-2 expression (Fig. 6A). Consistent with these findings, we found that cells transfected with AP-2 antisense ODN resulted in inhibition of Fn-induced cell growth as determined by [methyl-3H]thymidine incorporation assay (Fig. 6B) and inhibition of Fn-stimulated effect on EP4 promoter activities (Fig. 6C). The control sense ODN had no effect. By using site-directed mutated EP4 promoter constructs in which each of three AP-2 binding sites were mutated (−1529, −1133, and −1000 bp), we found that the stimulatory effect of Fn on EP4 promoter activity was lost with EP4 promoter constructs in which one AP-2 site was mutated (−1000 bp) (Fig. 6D).

P38K, ERK, and α5β1 Integrin Signaling Are Involved in Fn-induced AP-2 Protein Expression—We also examined the effects of Fn on AP-2 protein expression, and, consistent with the gel shift experiment results,
we found that Fn induced COX-2 and AP-2α protein production; no changes were noted in AP-2α protein expression. This was the stimulatory effect of Fn. Similar results were obtained with H2106 cells (not shown).

**DISCUSSION**

Fn is a heterodimeric extracellular matrix glycoprotein implicated in a number of physiological events during embryogenesis, angiogenesis, thrombosis, and inflammation (27–29). Fn expression is increased in lung carcinomas, particularly in non-small cell lung carcinoma (3, 29–31). Also, the adhesion of lung carcinoma cells to Fn enhances tumorigenicity and confers resistance to apoptosis induced by standard chemotherapeutic agents (32). Previously, we found that Fn stimulates human lung carcinoma cell growth in vitro by increasing expression of COX-2 and PGE2 biosynthesis (4). We also demonstrated that all four PGE2 receptors are expressed in NSCLC cells studied (17). Based on these data, we predicted that Fn stimulates NSCLC cell proliferation through one or more of these four EP receptors capable of recognizing PGE2. The development of aberrant crypt foci and putative preneoplastic lesions in the colon was decreased in the EP4 knock-out mice (7). Blockade of EP4 production also mediated inhibition of NSCLC cell invasion by stimulating EP4 expression. Therefore, we tested whether Fn on cell growth shown in Fig. 1, C and D, the stimulatory effect of Fn on cell proliferation was diminished in the presence of EP4 siRNA, whereas the control siRNA had no effect as determined by the [3H]thymidine incorporation assay (Fig. 8F) and by cell viability assays (Fig. 8F). Similar results were obtained with H2106 cells (not shown).
Fibronectin Stimulates EP4 in Lung Cancer

Here, we reported that Fn only induced the expression of EP4, whereas it had no effect on other EP receptors. A similar lack of changes in the other EP receptors was observed when EP4 was knocked down by EP4 siRNA. This finding indicates that Fn selectively targets EP4 receptor subtypes in NSCLC cells. In view of the above, we focused on EP4 and explored the mechanisms involved.

First, we demonstrated that EP4 siRNA antagonized Fn-induced lung carcinoma cell growth, suggesting a direct role for EP4 in mediating this process in our system. This is consistent with data from others who reported that EP4 antisense oligonucleotides diminished EP4 protein expression and abolished the PGE2-stimulated production of cAMP and blocked the ability of PGE2 to augment release of immunoreactive substance P and calcitonin gene-related peptide in sensory neurons (33). The EP4 antagonist, ONO-AE3-208, and the EP4 siRNA have been shown to inhibit extracellular matrix-induced metalloproteinase-9 expression in macrophages (34). More recently, EP4 antagonists inhibited breast cancer cell growth and reduced breast, lung, and colon cancer metastasis, suggesting that blockade of EP4 may be an alternative approach to the use of COX-2 inhibitors to prevent tumor metastasis (35, 36).

In this study, we confirm that Fn increased EP4 gene expression in NSCLC cells, whereas collagen type 1 had no effect. This suggested that Fn may induce NSCLC growth not only by stimulation of PGE2 but may also enhance this process by inducing the expression of EP4. The connection of Fn and EP4 expression has never been reported in lung cancer cells, although an antagonist of the PGE2 receptor EP1 has been shown to decrease Fn synthesis in a rat diabetic nephropathy model (37). Also, PGE2-accelerated ProNectin F(TM) (a proteolytic fragment of Fn)-dependent adhesion was mediated through coop-

FIGURE 5. Electrophoretic mobility shift assay to determine DNA binding of AP-2, Sp1, and C/EBP protein in the EP4 promoter in response to Fn.
A–C, effect of Fn on C/EBP, Sp1, and AP-2 binding activities. Oligonucleotides containing the C/EBP (A), Sp1 (B), and AP-2 (C) sites were end-labeled with [γ-32P]ATP and incubated with nuclear extracts (5 μg each) from H1838 cells treated with Fn or collagen type 1 (20 μg/ml each) for an additional 24 h. D, ERK antisense blocks Fn-induced AP-2 binding activities. Top, cellular protein was isolated from H1838 cells transfected with control oligonucleotide or ERK antisense oligonucleotide (1 μM each) for 24 h before exposing the cells to Fn (20 μg/ml)-coated culture plates for an additional 24 h. E, the inhibitors of ERK1/2 and PI3K abrogated the Fn-induced AP-2 binding activities. Oligonucleotides containing AP-2 sites were end-labeled with [γ-32P]ATP and incubated with nuclear extracts (5 μg) from H1838 cells treated with PD98059 (25 μM) and wortmannin (100 nM) for 1 h before exposing the cells to culture plates coated with Fn (20 μg/ml) for an additional 24 h. F, anti-AP-2 antibody supershift. Oligonucleotides containing AP-2 sites were end-labeled with [γ-32P]ATP and incubated with nuclear extracts (5 μg) and AP-2α, -β, and -γ antibodies (2 μg/l each) for 24 h. For competition assays, a molar excess (100 ×) of consensus Sp1 (Cold Sp1) or C/EBP (Cold C/EBP) or AP-2 (Cold AP-2) oligonucleotide was added to the binding reaction. Oligonucleotides containing a mutated Sp1 (Mut Sp1) or C/EBP (Mut C/EBP) or AP-2 (Mut AP-2) site that were end-labeled with [γ-32P]ATP were used to confirm the binding specificity. Con, untreated control cells.

RETRACTED
Fibronectin Stimulates EP4 in Lung Cancer

We previously demonstrated that Fn up-regulated COX-2 expression through activation of the ERK signal pathway and that blockade of ERK completely abrogated Fn-induced COX-2 expression (4). These findings altogether suggested a strong connection between COX-2, EP4, and ERK signals. However, ERK played no role in the increase of EP4 expression induced by peroxisome proliferator-activated receptor β/δ activation (15), suggesting the existence of independent pathways that differ according to the stimulus. Fn activates MMP-9 via the ERK and PI3K/Akt signaling pathways in NSCLC and ovarian cancer cells (24, 42, 43). The inhibitor of PI3K, wortmannin, blocked the effect of Fn on stimulation of ERK phosphorylation, indicating cross-talk between the PI3K and ERK1/2 pathways in NSCLC cells. The cross-talk between these kinases has been reported in other cell systems as well (44, 45). In contrast, our data indicated that mTOR, PKC, and PKA signaling pathways are not involved in the up-regulation of the EP4 gene induced by Fn, although the latter two kinases were involved in prostaglandin E receptor expression in other studies (46, 47). This might suggest distinct effects, depending on the stimulant and cells studied.

EP4 has been shown to be regulated at the level of gene transcription in different cell types (21, 47). We found that Fn, not collagen type I, increased EP4 promoter activity. Furthermore, the region between −1555 and −992 was demonstrated to play a critical role in AP-2 binding. Our previous study demonstrated that Fn stimulated lung carcinoma cell growth through its receptor α5β1, since anti-α5β1 antibodies eliminated the mitogenic response (4). Here, the Fn effect on EP4 protein levels was blocked by anti-α5β1 antibodies but not by anti-α2β1 antibodies, indicating that the integrin receptor α5β1 mediated this regulation. In order to elucidate the mechanism(s) involved in Fn induction of EP4, we attempted to delineate the signaling pathways involved in induction of EP4 expression in lung carcinoma cells in response to Fn treatment. Data from our laboratory (4, 21, 24, 39, 40) and data of others (3, 41) have demonstrated that adhesion to Fn treatment. Data from our laboratory (4, 21, 24, 39, 40) and data of others (3, 41) have demonstrated that adhesion to Fn activates several kinase signaling pathways, including ERK, PI3K, PKC, and PKA. We found that inhibitors of the PI3K kinase and ERK prevented Fn-induced EP4 protein expression, suggesting that the activation of these dual kinase signaling pathways is required for Fn-induced EP4 protein expression. We have previously demonstrated that Fn up-regulated COX-2 expression through activation of the ERK signal pathway and that blockade of ERK completely abrogated Fn-induced COX-2 expression (4). These findings altogether suggested a strong connection between COX-2, EP4, and ERK signals. However, ERK played no role in the increase of EP4 expression induced by peroxisome proliferator-activated receptor β/δ activation (15), suggesting the existence of independent pathways that differ according to the stimulus. Fn activates MMP-9 via the ERK and PI3K/Akt signaling pathways in NSCLC and ovarian cancer cells (24, 42, 43). The inhibitor of PI3K, wortmannin, blocked the effect of Fn on stimulation of ERK phosphorylation, indicating cross-talk between the PI3K and ERK1/2 pathways in NSCLC cells. The cross-talk between these kinases has been reported in other cell systems as well (44, 45). In contrast, our data indicated that mTOR, PKC, and PKA signaling pathways are not involved in the up-regulation of the EP4 gene induced by Fn, although the latter two kinases were involved in prostaglandin E receptor expression in other studies (46, 47). This might suggest distinct effects, depending on the stimulant and cells studied.

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Fibronectin Stimulates EP4 in Lung Cancer

FIGURE 8. The role of COX-2 sigaling in mediating and inducing lung carcinoma cell growth and proliferation. Cellular protein was isolated from H1838 cells treated with or without dmPGE2 (0.1 μM) for 4 h before exposing the cells to culture plates coated with Fn (20 μg/ml) for an additional 48 h. Afterward, viable cell numbers were determined by the CellTiter-Glo luminescent cell viability assay. All data are presented as means ± S.D. * indicates significant difference from control (p < 0.05). Con, untreated control cells.

a major role. The results showing that both PD98095 and wortmannin partially prevented Fn-stimulated EP4 promoter activity further suggested a role for PI3K and ERK kinase signaling pathways in mediating Fn up-regulation of EP4 gene expression. Several transcription factor binding sites within regions of the EP4 promoter have been characterized, including regulatory elements for AP-2, C/EBP, Sp1, and others (15, 18, 25). We showed that treatment of H1838 cells with Fn significantly increased protein binding activities of AP-2 in the EP4 promoter, whereas it had little effect on C/EBP and Sp1. This, together with the supershift assay results, indicated that AP-2 binding to the AP-2 site was necessary for the up-regulation of EP4 gene transcription in response to Fn. There are three AP-2 binding sites in this region. By mutations of each of these sites, we found that only one site (~1000 bp) was involved in the Fn-induced EP4 promoter activity. To our knowledge, a role for the AP-2 site in regulation of EP4 expression has never been reported. The transcription factor AP-2 regulates genes involved in a spectrum of important biological functions. Data obtained from different experimental models in vitro and in vivo indicate that AP-2 proteins function as important regulators of c-Myc targets in cell cycle progression and apoptosis (48). AP-2α overexpression leads to anchorage-independent growth and malignant transformation in vitro (50). It has therefore been suggested that AP-2 is involved in malignant transformation of breast cancer (49). Immunohistochemical analysis of AP-2α and AP-2γ-specific antibodies confirmed up-regulation of these proteins in human breast tumors (50). Other data indicate that AP-2α might be involved in proliferation by inducing terminal differentiation, apoptosis, and growth retardation (51). We are a few studies that explore the transcriptional regulation of EP4 and transcription factor interactions in its promoter region. Studies show that a GC-rich/Sp1 binding site located within the first 80 bases of the transcription start site in the EP4 promoter region is important in transcription initiation of the EP4 gene (24), and several negative, positive, and lipopolysaccharide/serum-responsive regions are located at different areas in the mouse EP4 promoter (16). We confirmed that AP-2 sites were involved in Fn-induced EP4 gene expression using mutated EP4 constructs.

Fn increased nuclear AP-2α protein levels, and inhibition of ERK signals prevented Fn-induced AP-2 expression. The connection between the AP-2 and ERK signal has been reported in other studies. For example, the ability of estradiol to increase AP-2 protein expression and AP-2 DNA binding activity was reversed by PD98059 (52). ERK activation is necessary for induction of the binding activities of AP-2 in T cells (53). Also, increased ERK signal has been reported in the absence of AP-2α in mouse epidermis (54). In addition, we found that blockade of AP-2 by AP-2 antisense approaches had no effect on ERK activ-
Fibronectin Stimulates EP4 in Lung Cancer

higher doses of exogenous PGE2 have been shown to suppress dmPGE2 up-regulated expression of the receptor subtype EP4, reduced cell growth, our data suggest that induction of AP-2 showing that AP-2 antisense oligonucleotides abolished Fn-induced AP-2 signaling (55, 56). PGE2 not only stimulated the baseline but literature contains data that link expression of COX-2 and AP-2 production, the effect of Fn on EP4 could be solely mediated by binding to its α5β1 integrin receptor, Fn stimulates PI3K/Akt, followed by the induction of MEK-1/ERK and COX-2 signal pathways. These events stimulate the production of PGE2 and expression of the PGE2 subtype EP4 receptor gene through activation of the transcription factor AP-2a. These two events, induction of PGE2 and EP4, serve to amplify the mitogenic effects of Fn on lung carcinoma cells. We previously demonstrated that Fn increased COX-2 expression and stimulated the production of PGE2 in lung carcinoma cells. Apoptotic cell death is at least partly responsible for the mitogenic effects of Fn on lung carcinoma cells. By binding to its α5β1 integrin receptor, Fn stimulates PI3K/Akt, followed by the induction of MEK-1/ERK and COX-2 signal pathways. These events stimulate the production of PGE2 and expression of the PGE2 subtype EP4 receptor gene through activation of the transcription factor AP-2a. These two events, induction of PGE2 and EP4, serve to amplify the mitogenic effects of Fn on lung carcinoma cells.

FIGURE 9. Schematic representation of signal pathways triggered in NSCLC in response to Fn. By binding to its α5β1 integrin receptor, Fn stimulates PI3K/Akt, followed by the induction of MEK-1/ERK and COX-2 signal pathways. These events stimulate the production of PGE2 and expression of the PGE2 subtype EP4 receptor gene through activation of the transcription factor AP-2a. These two events, induction of PGE2 and EP4, serve to amplify the mitogenic effects of Fn on lung carcinoma cells.

In summary, our studies show that Fn stimulates human lung carcinoma cell proliferation through the PGE2 receptor subtype EP4. This effect is enhanced by Fn-induced EP4 expression. Control of EP4 gene expression by Fn is dependent on α5β1 integrin-mediated signals that include activation of PI3K/Akt, ERK, and COX-2. These signals stimulate PGE2 production, which induces the transcription factor AP-2a and stimulates AP-2a interactions with critical DNA regions within the EP4 gene promoter (−1555 to −992 bp). Thus, Fn, by increased PGE2 production and EP4 gene expression, induces mitogenic signals in NSCLC cells (Fig. 9). This study reveals a novel molecular mechanism for Fn regulation of human lung carcinoma cell growth and provides further evidence for the role of E prostanooid receptors in lung carcinoma biology. 

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MARCH 16, 2007•VOLUME 282•NUMBER 11

JOURNAL OF BIOLOGICAL CHEMISTRY 7971

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Fibronectin Stimulates EP4 in Lung Cancer

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