A Novel Binding Factor of 14-3-3β Functions as a Transcriptional Repressor and Promotes Anchorage-independent Growth, Tumorigenicity, and Metastasis*

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Received for publication, April 1, 2008 Published, JBC Papers in Press, May 5, 2008, DOI 10.1074/jbc.M802530200

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The 14–3–3 proteins form a highly conserved family of dimeric proteins that interact with various signal transduction proteins and regulate cell cycle, apoptosis, stress response, and malignant transformation. We previously demonstrated that the β isofrom of 14–3–3 proteins promotes tumorigenicity and angiogenesis of rat hepatoma K2 cells. In this study, to analyze the mechanism of 14–3–3β-induced malignant transformation, yeast two-hybrid screening was performed, and a novel 14–3–3β-binding factor, FBI1 (fourteen-three-three beta interactant 1), was identified. In vitro binding and co-immunoprecipitation analyses verified specific interaction of 14–3–3β with FBI1. The strong expression of FBI1 was observed in several tumor cell lines but not in non-tumor cell lines. Forced expression of antisense FBI1 in K2 cells inhibited anchorage-independent growth but had no significant effect on cell proliferation in monolayer culture. Down-regulation of FBI1 also inhibited tumorigenicity and metastasis accompanying a decrease in MMP-9 (matrix metalloproteinase-9) expression. In addition, the duration of ERK1/2 activation was curtailed in antisense FBI1-expressing K2 cells. A luciferase reporter assay revealed that the FBI1-14–3–3β complex could act as a transcriptional silencer, and MKP-1 (MAPK phosphatase-1) was one of the target genes of the FBI1-14–3–3β complex. Moreover, chromatin immunoprecipitation analysis demonstrated that FBI1 and 14–3–3β were present on the MKP-1 promoter. These results indicate that FBI1 promotes sustained ERK1/2 activation through repression of MKP-1 transcription, resulting in promotion of tumorigenicity and metastasis.

The 14–3–3 family is a highly conserved, ubiquitously expressed protein family (1–3). In mammals, there are at least seven isoforms: β, γ, ε, σ, ζ, τ, and η. The 14–3–3 proteins form homo- and heterodimers that can interact with a variety of cellular proteins. The 14–3–3 proteins are phosphoserine/threonine-binding proteins. These interactions control the enzyme activities, subcellular localization, and protein–protein interactions of target proteins. 14–3–3 proteins regulate many cellular processes, including the cell cycle, metabolism, signal transduction, malignant transformation, and apoptosis. More than 100 binding partners have been identified. Although target proteins show a distinct preference for particular isoforms of 14–3–3 (4), the functional specificity of 14–3–3 isoforms is still not completely understood. In contrast to 14–3–3σ, which is known as a tumor suppressor gene (5–7), we previously reported that 14–3–3β is implicated in the positive regulation of cell cycle progression and tumorigenesis (8). 14–3–3β was overexpressed in various cancer cell lines, including aflatoxin B1-induced rat hepatocellular carcinoma K1 and K2 cells (9, 10). Enforced expression of antisense 14–3–3β inhibited cell proliferation, colony formation in soft agar, tumorigenicity, and angiogenesis of K2 cells. Furthermore, Takihara et al. (11) also reported that overexpression of 14–3–3β in NIH3T3 cells confers tumorigenicity in nude mice via the activation of the mitogen-activated protein kinase (MAPK)2 cascade. Thus, these data support the idea that 14–3–3β could function as an oncogene and has a unique function among the 14–3–3 families; however, the molecular mechanisms of 14–3–3β-induced carcinogenesis are poorly understood.

The MAPK pathway plays an important role in diverse cellular functions, including cell proliferation, differentiation, migration, metastasis, and survival (reviewed in Refs. 12–14). In response to various extracellular stimuli, extracellular signal-regulated kinases (ERKs) are activated by sequential phosphorylation. Activated ERKs phosphorylate and activate downstream kinases and transcriptional regulators. Activated ERKs induce rapid transcriptional activation of immediate early genes and control the cell cycle and cell survival. Recent studies have demonstrated that differences in the duration, strength, and subcellular localization of ERK activities determined signal specificity (12, 13). In addition, many studies have identified factors that may regulate the duration, strength, and subcellular

* This work was partially supported by the “Academic Frontier” project for private universities and matching fund subsidy from MEXT (Ministry of Education, Culture, Sports, Science, and Technology) Grant 2006-2010 (to F.T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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localization of ERK activities. These factors contain the cell surface receptor density, the expression of scaffold proteins, and the interplay between kinases and phosphatases. Mutations in key components that result in sustained ERK activation correlate with carcinogenesis (14). For example, human tumors frequently express Ras proteins that have been activated by point mutation (15). Thus, abnormal activation of ERKs is implicated in the malignant transformation.

In this study, to further analyze the oncogenic function of 14-3-3β, we screened K2 cell and rat brain cDNA libraries using the yeast two-hybrid system with 14-3-3β as a bait. One of the novel cDNA clones we isolated was designated FBI1 (fourteen-three-three beta interactant 1). We established that K2 cells down-regulated the FBI1 transcript by the introduction of an antisense FBI1 cDNA expression vector. Tumors that were formed in the flanks of nude mice by these transfectants were much smaller, and lung metastasis was robustly reduced compared with those of the parental cells. In antisense FBI1 K2 transfectants, the expression level of MMP-9 (matrix metalloproteinase-9) mRNA was reduced, whereas the expression of MKP-1 (MAPK phosphatase) was substantially up-regulated by proteinase-9) mRNA was reduced, whereas the expression of

RNA Isolation and Northern Blot Analysis—Total RNAs were prepared from various cell lines and Fisher 344 rat tissues by the acidic guanidine thiocyanate/phenol/chloroform method. Northern blotting was performed as described previously (16).

Stable Transfections—Antisense FBI1 expression vector was constructed by the insertion of an FBI1 cDNA fragment into the pcDNA3 expression vector (pcDNA3-AS-FBI1). K2 cells were transfected with antisense FBI1 expression vector or empty vector using DOTAP transfection reagent (Roche Applied Science) according to the manufacturer’s instruction. After 2 weeks of selection with 1 mg/ml G418 (Wako, Tokyo, Japan), resistant clones were expanded and analyzed for the expression level of antisense FBI1 RNA by Northern blotting.

Growth and Colonization in Vitro—Cells (4 × 10⁴) were plated in 24-well plates containing Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum (FCS) and cultivated for various times. The cell number was counted using a TATAI hematocytometer. Soft agar assay was performed as described previously (8).

Tumorigenicity and Lung Metastasis—Cells (1 × 10⁷/200 μl of phosphate-buffered saline/flank) were inoculated subcutaneously into 5-week-old athymic mice (BALB/c Jcl nu/nu; Clea Japan, Tokyo, Japan). After 24 days, tumor volumes were calculated as (short axis² × long axis)/2. Thirty-one days after the injection, the lungs were removed to investigate metastatic foci. The metastatic ability was estimated by counting foci in whole mount samples. For the assay of lung metastasis, cells (1 × 10⁶ cells/200 μl of serum-free medium) were injected into the tail vein of nude mice. After 7 weeks, metastatic foci formed in the lungs were investigated by measuring the lung weight. Mouse care and handling conformed to the National Institutes of Health guidelines for animal research. The experimental protocols were approved by the Institutional Animal Care and Use Committee.

Reverse Transcription-PCR—Total RNAs were prepared from K2 and its antisense FBI1 transfectants as described above. Reverse transcription (RT) reactions were performed using total RNAs and Moloney murine leukemia virus reverse transcriptase with 84HF primer.
transcriptase, according to the manufacturer’s instructions (Invitrogen). The RT reaction mixtures were subjected to PCR amplification using the specific primers as follows: rat mmp-9, 5'-TGG CTC TAG GCT ACA GCT TTG CTG C-3' (5'-primer) and 5'-CGA AGG AGT CAT CGA TCA CGT CTC G-3' (3'-primer) (GenBank accession number NM_031055); rat mmp-1, 5'-CCA TGG TGA TGG AGG TGG GCA TCC T-3' (5'-primer) and 5'-CTT TCA GCA GCT CGG AGA AGT GTG TGT G-3' (3'-primer) (GenBank accession number NM_053769); rat cyclin D1, 5'-GAT GGC ATG GAC TGT-3' (5'-primer) and 5'-CCT CAG G-3' (3'-primer) (GenBank accession number NM_031055); rat mRNA expression vector (pcDNA3-AS-14-3-3β, GenBank accession number AF357203). Transfection and detection of luciferase activity were performed as described above.

**ChIP Analysis**—K2 cells were transfected with FLAG-tagged FBI1 or enhanced green fluorescent protein-fused FBI1 expression vector. After 36 h, cells were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100, blocked with 10% goat serum in phosphate-buffered saline, and stained with anti-FLAG (1:500; Sigma) or anti-14-3-3β (1:200; Santa Cruz Biotechnology) antibodies and visualized with Cy3-conjugated goat anti-mouse IgG antibody (1:200; Sigma). The cells were counterstained with Hoechst 33258 to visualize the nuclei.

**Luciferase Reporter Assay**—A luciferase reporter construct (pGAL4-TK-Luc) and GAL4DBD expression vector (pcDNA3GAL4DBD) were constructed as described previously (16). The GAL4DBD-fused FBI1 expression vector was constructed by the insertion of the FBI1 open reading frame in-frame downstream of the GAL4DBD-coding sequence in pcDNA3GAL4DBD and was designated as pGAL4DBD-FBI1. Antisense 14-3-3β RNA expression vector (pcDNA3-AS-14-3-3β) was constructed as described previously (8). For each transfection, 3 × 10⁵ cells/35-mm dish were transfected with 0.25 μg of pGAL4-TK-Luc, 0.25 μg of β-galactosidase expression vector pDNA3.1/Myc-His/lacZ (Invitrogen), and a total of 1.75 μg of pcDNA3GAL4DBD and pGAL4DBD-FBI1 in various combinations. pcDNA3-AS-14-3-3β was also coexpressed in various combinations with pGAL4DBD-FBI1. Thirty-six hours after transfection, cells were lysed with the lysis buffer of the luciferase assay kit (Promega), and luciferase activities were determined according to the manufacturer’s instructions using a Luminous CT9000D fluorescent photometer (Diatron, Tokyo, Japan). Reporter gene activities were normalized using β-galactosidase activity as an internal control.
RESULTS

Interaction of FBI1 with 14-3-3β in Vivo—In order to identify novel 14-3-3β-interacting proteins, K2 cell cDNA and rat brain cDNA libraries were subjected to yeast two-hybrid screening using 14-3-3β as a bait, and three novel cDNA clones (1-1, 2-3, and 2-6) were obtained. Preliminary Northern blotting showed that the expression level of clone 2-3 mRNA in K2 cells was 24.6-fold higher than that of the normal rat liver and was the highest among these genes. Thus, in this study, we decided to further analyze the oncogenic function of clone 2-3 and designated it as FBI1. The cDNA sequence was deposited in DDBJ/EMBL/GenBank™ under accession number AB234867. FBI1 has a sequence of 1502 nucleotides and encodes 201 amino acid residues (Fig. 1A). FBI1 protein has two putative nuclear localization signals and five putative 14-3-3 recognition sites. A homology search revealed that rat FBI1 shows 99 and 94% identities to mouse and human FBI1s at the amino acid level, respectively, indicating that FBI1 is a highly conserved protein in mammals (Fig. 1B).

To confirm the specific interaction between FBI1 and 14-3-3β in vitro, the GST pull-down assay was carried out. GST-fused FBI1 protein was bound to glutathione-Sepharose beads and then incubated with 35S-labeled 14-3-3β synthesized by an in vitro method. As shown in Fig. 2A, the 28.2-kDa 14-3-3β specifically bound to GST-FBI1 fusion protein. The band detected at 26.9-kDa was perhaps due to an immature product of 14-3-3β. To demonstrate that the interaction between these two proteins also occurs in vivo, K2 cell lysate was immunoprecipitated with anti-FBI1 antibody and subjected to Western blotting with anti-14-3-3β and anti-FBI1 antibodies. As shown in Fig. 2B, endogenous 14-3-3β was co-immunoprecipitated with FBI1. Furthermore, FBI1 expressed in K2 cells was detected as 31.6 and 30.2 kDa bands in the relatively high molecular weight region on SDS-PAGE, probably due to its strong basicity (isoelectric point: 9.12) and the difference in phosphorylation level.

FBI1 protein has five putative 14-3-3 binding sites. To verify the 14-3-3β-binding sites in FBI1, we generated FBI1 point mutants in which Ser31, Thr103, Ser111, Ser119, or Ser131 in the putative 14-3-3-binding sites were converted to Ala and designated as S31A, T103A, S111A, S119A, and S131A, respectively. These mutants were inserted into pACT2, and the interaction with 14-3-3β was analyzed using the yeast two-hybrid system. All mutants did not bind to 14-3-3β (Fig. 2C), suggesting that multiple 14-3-3β-binding motifs of FBI1 are required for the interaction with 14-3-3β.

Overexpression of FBI1 mRNA in Various Tumor Cell Lines—To examine the tissue specificity of FBI1 mRNA expression, total RNAs were extracted from various adult rat tissues and analyzed by Northern blotting with the 1.3-kb fragment of pACT2/FBI1 as a probe. The FBI1 gene was marked by expressed as a 1.8-kb mRNA in the testis, cerebrum, and cerebellum, whereas expression was negligible in the liver, heart, spleen, and muscle (Fig. 3A). The reason for this difference in the expression level of FBI1 transcript among rat tissues is unclear. The FBI1 gene was robustly expressed in rat hepatoma K2 cells compared with normal rat liver. Marked expression of FBI1 mRNA was also detected in other cancer cell lines, including rat hepatomas, glioblastoma cells, pheochromocytoma cells, and embryonic carcinoma cells, compared with the respective normal tissues (Fig. 3B). On the other hand, the...
expression level of FBI1 in non-tumor cell lines, such as COS-7, Balb, and REF52 cells, was very low. These results suggest that overexpression of FBI1 is implicated in malignant transformation.

Specific Inhibition of Colonization in Soft Agar by Forced Expression of Antisense FBI1—It is well known that anchorage-independent growth is one of the hallmarks of malignant cancer cells. Therefore, we analyzed the effect of forced expression of antisense FBI1 on the colony forming ability of K2 cells in semisolid medium. Antisense FBI1 cDNA expression vector was introduced into K2 cells, and two stable clones, K2A1 and K2A2, were selected by Northern blotting (Fig. 3C). The expression levels of endogenous FBI1 mRNA in K2A1 and K2A2 transfectants were decreased to 64 and 65% of the parental K2 cells, respectively. The FBI1 mRNA expression level in vacant vector-introduced K2V1 cells was unaffected. Expression of the neo \(^\text{r}\) gene inserted in the expression vector as a selection marker was clearly detected in all of the transfectants. Since we had previously reported that c-myc and 14-3-3\(\beta\) mRNA were over-expressed in K2 cells (8), the expression levels of these genes in the transfectants were analyzed by Northern blotting. No significant difference in the expression levels of c-myc and 14-3-3\(\beta\) mRNA was observed, suggesting that FBI1

![A New Binding Partner of 14-3-3\(\beta\) Promotes Carcinogenesis](image)

**FIGURE 2. Identification of interaction between FBI1 and 14-3-3\(\beta\) in vitro and in vivo.** A, analysis of direct interaction between FBI1 and 14-3-3\(\beta\) by GST pull-down assay. In vitro translated \(^{35}\text{S}\)methionine-labeled 14-3-3\(\beta\) was incubated with GST-FBI1 or GST and analyzed by autoradiography. B, endogenous association between FBI1 and 14-3-3\(\beta\). K2 cell lysates were immunoprecipitated with anti-FBI1 antibody (α-FBI1) or mouse normal serum as a control, and Western blotting (WB) was performed with anti-14-3-3\(\beta\) antibody (α-14-3-3\(\beta\)). IP, immunoprecipitation. C, identification of the 14-3-3\(\beta\) binding sites in FBI1. 14-3-3\(\beta\) cDNA was ligated in-frame with the GAL4DBD in pAS2-1 and used as a bait. Wild-type FBI1 (WT) or mutant (S31A, T103A, S111A, S119A, and S131A) cDNAs were ligated with the GAL4 activation domain in pACT2. 14-3-3\(\beta\) and mutant FBI1 expression constructs were simultaneously introduced into yeast strain Y153 cells. The cells were streaked on both selection (Leu\(^-\), Trp\(^-\), His\(^-\), X-gal\(^-\)) and nonselection (Leu\(^+\), Trp\(^+\)) media.

![A New Binding Partner of 14-3-3\(\beta\) Promotes Carcinogenesis](image)

**FIGURE 3. Inhibition of anchorage-independent growth of K2 cells by down-regulation of FBI1.** A and B, expression levels of FBI1 mRNA in various rat tissues and cell lines. Total RNAs were extracted from various adult Fisher 344 rat tissues (A) and various cell lines (B), including rat hepatoma cells (K2, AH60tc, AH70Btc, dRL74, and dRLb84), glioblastoma cells (C6), adrenal pheochromocytoma cells (PC12), embryonic carcinoma cells (P19), COS-7, Balb, and rat embryonic fibroblast (REFS2) and analyzed by Northern blotting using a 1.3-kb rat FBI1 cDNA fragment as a probe. A picture of ethidium bromide-stained 28S ribosomal RNAs is shown to allow comparison of the total amount of RNA employed, C, characterization of antisense FBI1 transfectants. K2 cells were transfected with empty or antisense FBI1 cDNA expression vectors. The stable transfectants of empty vector (K2V1) and antisense expression vector (K2A1 and K2A2) were obtained. Total RNAs were extracted from these transfectants and analyzed for the expression level of FBI1, neomycin-resistant gene (neo\(^r\)), c-myc, and 14-3-3\(\beta\) mRNAs. D, expression level of FBI1 protein in transfectants. Cell lysates of transfectants were analyzed by Western blotting with anti-FBI1 and anti-actin antibodies. E, growth ability of the transfectants. Cells were cultivated in monolayer culture for various times and counted for the number of cells. F and G, colony forming ability of the transfectants. Cells were seeded in soft agar medium at \(1 \times 10^5\) cells/35-mm dish and cultivated for 2 weeks. Then the colonies formed were photographed (F) and counted (G) after staining with INT. Scale bar, 5 mm. Each value is the average ± S.E. of triplicate culture dishes. *, \(p < 0.01\) compared with the parental K2 cells.
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does not affect the expression of these genes. To confirm expression level of FBI1 protein in transfectants, cell lysates were analyzed by Western blotting with anti-FBI1 antibody. The expression levels of FBI1 protein in both K2A1 and K2A2 cells were reduced to 59 and 55% compared with that of the parental K2 cells, respectively (Fig. 3D). Next, these transfectants were cultured in soft agar medium containing 10% FCS for 2 weeks, and the number of colonies was counted. The colonization abilities of K2A1 and K2A2 cells were extremely reduced to 3.5 and 11.5% compared with that of the parental K2 cells, respectively (Fig. 3E). These results imply that FBI1 selectively promotes the anchorage-independent growth of K2 cells.

Suppression of Tumorigenicity and Lung Metastasis by Forced Expression of Antisense FBI1—The ability of anchorage-independent growth in vitro correlates with the tumorigenicity and metastatic ability of malignant cancer cells in vivo. To investigate the effects of expression of antisense FBI1 on the tumorigenicity of K2 cells, the transfectants were subcutaneously inoculated into the flank of nude mice, and the sizes of the tumors were estimated after 24 days. K2 cells and empty vector-transfected K2V1 cells formed progressively growing solid tumors in all mice (Figs. 4, A and B). Although solid tumors developed in all mice after inoculation with K2A1 and K2A2 cells, in both cases, the tumors were much smaller than those of K2 and K2V1 cells (Fig. 4, A and B). Since angiogenesis is required for vigorous tumor growth and the expansion and metastasis of solid tumors (19, 20), we studied the angiogenesis of tumors derived from transfectants using anti-von Willebrand factor monoclonal antibody. The anti-von Willebrand factor–positive vessel density was not different among tumors formed by K2, K2V1, K2A1, and K2A2 cells (data not shown). K2-derived tumors in the flanks of nude mice were able to colonize the lung 1 month after subcutaneous injection. Using this assay system, we analyzed the lung metastatic potency of the antisense FBI1 transfectants. The number of lung metastatic foci developed by K2A1 and K2A2 cells was markedly diminished compared with K2 cells (Fig. 4C). Although the reduced number of foci originated from K2V1 cells was also observed, the K2V1-derived foci had a tendency to form larger foci compared with those formed by K2 cells. The reason for this is unknown. To characterize the metastatic tumors, two subcell lines, LM1 and LM2, were established from the K2A2-derived foci developed in different mice. Both cells exhibited the typical morphology of hepatocytes and were indistinguishable from the parental K2A2 cells (data not shown). Expression of the c-myc and 14-3-3β genes was enhanced in LM1 and LM2 cells as observed in K2A2 cells, whereas expression of these genes was negligible in the normal mouse lung (Fig. 4D). However, LM1 and LM2 cell lines acquired potent expression ability for the FBI1 gene compared with K2A2 cells, mainly as a result of the loss of antisense FBI1 RNA expression, confirmed by Northern blotting with the vector-derived BGH fragment as a probe (Fig. 4D). The expression level of FBI1 protein in LM1 and LM2 cells was higher than in K2A2 cells, coinciding with the result of Northern blotting (Fig. 4E). Furthermore, to confirm the effect of antisense FBI1 expression on metastasis, an intravenous injection assay was performed. The lungs were removed and weighed to investigate metastatic foci 7 weeks after the injection. K2 and K2V1 cells developed many metastatic foci in the lungs of nude mice, whereas no obvious nodules were observed with K2A2 transfectant. Consequently, the lung weights from the mice injected with K2A2 were robustly reduced compared with those from nude mice injected with the parental K2 cells (Fig. 4F). These results show that forced expression of antisense FBI1 inhibits the tumorigenicity and metastasis of K2 cells.

Matrix metalloproteinases (MMPs) degrade extracellular matrix, and the expression level of MMPs is correlated with the metastatic ability of cancer cells (21–24). Especially, the activity of MMP-2 and -9 is often found to be elevated in tumor tissues and malignant cancer cells. Therefore, we analyzed MMP-2 and -9 activities in the CMs of K2 transfectants, LM1 and LM2, using gelatin zymography. Active MMP-9 was detected in the CMs of K2 and K2V1 cells, whereas no forms of MMP-2 were observed (Fig. 4G). In K2A1 and K2A2 CMs, active MMP-9 was slightly detected, although the pro-MMP-9 level was comparable with those of K2 and K2V1 cells. Furthermore, LM1 and LM2 cell lines acquired the strong activity of MMP-9. The activity of MMPs is controlled at both transcriptional and posttranscriptional levels (25). To elucidate the reason for the reduced level of active MMP-9 in K2A1 and K2A2 CMs, we analyzed the expression level of MMP-9 by RT-PCR. The expression level of MMP-9 mRNA in K2A1 and K2A2 cells was robustly diminished compared with levels in K2 and K2V1 cells (Fig. 4H). However, the expression levels of MMP-9 in LM1 and LM2 cell lines were equal to those in control cell lines.

Effect of Expression of Antisense FBI1 on MAPK Cascade—MAPK signaling plays a pivotal role in various cellular functions (12–14). Moreover, mutations of the MAPK pathway have been observed in many human cancers (14). Therefore, to elucidate whether overexpression of FBI1 is implicated in MAPK signal transduction, we analyzed the effect of expression of antisense FBI1 on the activation of ERK1/2 by phosphorylation. K2 cells and their antisense FBI1 transfectants were cultivated in serum-free medium for 12 h prior to stimulation with 10% serum. At various times after serum stimulation, levels of phosphorylated ERK1/2 (pERK1/2) were analyzed by Western blotting with anti-pERK1/2 antibody. In all cells tested, within 5 min, ERK1/2 were markedly phosphorylated, and the maximal levels of pERK1/2 were not significantly different among these cells. Even after 30 min, relatively high levels of pERK1/2 were sustained in K2 and K2V1 cells. In contrast, after 10 min, pERK1/2 levels were reduced immediately in K2A1 and K2A2 cells. After 30 min, pERK1/2 levels in the antisense FBI1 transfectants were reduced to their original levels. The expression levels of ERK1/2 proteins in these cells were not altered after stimulation with 10% serum (Fig. 5A).

To elucidate the reason why ERK1/2 activity was shortened in K2A1 and K2A2 cells, we analyzed the expression levels of MKP-1 by Western blotting. MKP-1 directly binds to pERK1/2 and dephosphorylated pERK1/2 (26, 27). Significant induction of MKP-1 protein was observed in K2A1 and K2A2 cells after 30–60 min of serum stimulation (Fig. 5B). In contrast, slight induction was observed in K2 and K2V1 cells. Since some
FIGURE 4. Inhibition of tumorigenicity and metastasis of K2 cells by down-regulation of FBI1. A and B, tumorigenicity of the transfectants. Cells (1 × 10^7) were subcutaneously injected into the flank of nude mice. After 24 days, the tumors were photographed (A). Scale bar, 10 mm. The average volumes of tumors generated in the flank of 5–7 mice were estimated and represented with the mean value ± S.E. *, p < 0.01 compared with the parental K2 cells (B). C, metastatic ability of the transfectants. After 31 days of the subcutaneous injection, the lungs were removed to investigate metastatic foci. The average number of lung metastatic foci of 3–5 mice was estimated and represented with the mean value ± S.E. *, p < 0.05 compared with the parental K2 cells. D, characterization of lung-spread K2A2 transfectant. LM1 and LM2 subcell lines were established from the lung foci, which were metastasized from the original tumors formed by K2A2 transfectant in the flank of nude mice. Expression levels of the c-myc, 14-3-3ε, FBI1, and exogenous antisense FBI1 (AS-FBI1) in LM1 and LM2 were analyzed by Northern blotting. To detect exogenous AS-FBI1 RNA, the 0.23-kb EcoRV-PvuII fragment BGH (bovine growth hormone polyadenylation signal) of pcDNA3 was used as a probe. E, expression level of FBI1 protein in lung-spread K2A2 transfectants. Cell lysates were analyzed by Western blotting with anti-FBI1 and anti-actin antibodies. F, intravenous injection of K2 cells. Cells (1 × 10^6) were inoculated into the tail vein of nude mice. After 7 weeks, the lungs were removed and weighed. *, p < 0.05 compared with the parental K2 cells. G, gelatin zymography of CMs of K2 transfectants and lung-spread K2A2 transfectants. When cells were grown semiconfluently, the medium was exchanged to serum-free medium and cultivated for 18 h. The resulting CM was concentrated to one-thirtieth of its original volume and applied to gelatin zymography. H, RT-PCR analysis of MMP-9 mRNA expression. The expression level of MMP-9 mRNA was analyzed by RT-PCR. The glyceraldehyde 3′-phosphate dehydrogenase (GAPDH) mRNA expression was also analyzed as an internal control.
MKPs are transcriptionally regulated in response to the growth factor, we analyzed the transcription levels of MKP-1 by RT-PCR. As shown in Fig. 5, C and D, the addition of serum led to induction of expression of MKP-1 mRNA shortly after activation of ERK1/2 and reached a peak level at 30 min in all cell lines tested. The relative induction levels of MKP-1 mRNA in antisense FBI1 transfectants were substantially higher than those of K2 and K2V1, although the normal expression levels of MKP-1 mRNA were different to some degree among these cells. The expression levels of MKP-2 and -3 were also analyzed by RT-PCR, and there was no significant difference among antisense FBI1 transfectants (data not shown). These results suggest that FBI1 suppresses MKP-1 gene expression. Sustained ERK activation leads to the induction of cyclin D1 and S phase entry in fibroblasts (28, 29). The expression level of cyclin D1 after serum stimulation was analyzed by RT-PCR. In K2A1 and K2A2 cells, the expression of cyclin D1 was significantly reduced compared with that of K2 and K2V1 cells, suggesting that prolonged ERK activation in K2 cells may play an important role in cell cycle progression.

**FBI1-14-3-3β Complex Has a Transcriptional Silencing Function**—To examine the subcellular localization of FBI1 and 14-3-3β, A, nuclear localization of Flag-FBI1. K2 cells were transfected with Flag-tagged FBI1 expression vector and stained with anti-FLAG antibody. The nuclei were stained with Hoechst 33258 and observed under a fluorescent optics. B, colocalization of FBI1 and 14-3-3β. K2 cells were transfected with enhanced green fluorescent protein-fused FBI1 expression vector and stained with anti-14-3-3β antibody. Signals were visualized as described above. Scale bar, 20 μm (A and B).

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FIGURE 6. Subcellular localization of FBI1 and 14-3-3β. A, nuclear localization of Flag-FBI1. K2 cells were transfected with FLAG-tagged FBI1 expression vector and stained with anti-FLAG antibody. The nuclei were stained with Hoechst 33258 and observed under a fluorescent optics. B, colocalization of FBI1 and 14-3-3β. K2 cells were transfected with enhanced green fluorescent protein-fused FBI1 expression vector and stained with anti-14-3-3β antibody. Signals were visualized as described above. Scale bar, 20 μm (A and B).
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Figure 7. FBI1 harbors a transcriptional silencing function. A, suppression of TK promoter activity by the forced expression of FBI1. pGAL4-TK-Luc (0.25 μg) and β-galactosidase expression plasmid pCDNA3.1/Myc-His/LacZ (0.25 μg) were introduced into K2 cells together with a total of 2 μg of expression vectors of pGAL4DBD-FBI1 and pGAL4DBD in various combinations by lipofection. After 36 h, luciferase activities in the cell lysates were determined according to the manufacturer's protocol and normalized based on β-galactosidase activity as an internal control. β-galactosidase expression plasmid (0.25 μg) was transfected with K2 cells together with total 1.95 μg of pCDNA3-AS-14-3-3β and pCDNA empty vector in various combinations. After 36 h, luciferase activities in the cell lysates were determined. Down-regulation of 14-3-3β was investigated by Western blotting with anti-14-3-3β antibody (bottom). C, effects of FBI1 multiple point mutations in the putative 14-3-3β binding sites on its transcriptional silencing activity. Wild-type (WT) or multiple point mutants (31A/111A/119A, 103A/111A/119A, 111A/119A/131A, and 103A/111A/119A/131A) of pGAL4DBD-FBI1 expression vectors (0.05 μg) were introduced into K2 cells and assayed for luciferase activity as described above. Putative binding sites of FBI1 with 14-3-3β are shown in the bottom panel. Each value indicates the mean ± S.E. of triplicate assays.

* p < 0.01 compared with the K2 cells transfected with only pGAL4DBD (A–C).

The merge of the two images showed that FBI1 and 14-3-3β coexisted in the nuclei. This result is supported, at least to some extent, by the accumulating evidence that 14-3-3 proteins regulate transcription in the nuclei in addition to their roles in various cellular events (30). Taking the result from immunofluorescent studies and the alteration in MMP-9 and MKP-1 mRNA expression in K2A1 and K2A2 cells as described above, we consider that FBI1 could participate in transcriptional regulation. To test this possibility, we constructed a GAL4DBD-fused FBI1 expression vector, pGAL4DBD-FBI1, and the luciferase reporter plasmid pGAL4-TK-Luc containing a GAL4-binding site upstream of the TK basal promoter, and a luciferase reporter assay was performed. These plasmids were cotransfected with K2 cells in various ratios, and luciferase activity was assayed. GAL4DBD-fused FBI1 dose-dependently suppressed TK promoter activity (Fig. 7A). Since FBI1 bound with 14-3-3β that was overexpressed in K2 cells, we examined the effect of forced expression of antisense 14-3-3β on the transcriptional silencing activity of FBI1. The expression of 14-3-3β protein was efficiently down-regulated by the cotransfection with FBI1 multiple point mutants, in which Ser and or Thr in the putative 14-3-3β binding sites were substituted for Ala, on the luciferase activity. As shown in Fig. 7C, FBI1 multiple point mutants 31A/111A/119A, 103A/111A/119A, 111A/119A/131A, and 103A/111A/119A/131A (4A) robustly lost their silencing activities. In particular, the 4A mutant completely lost its transcriptional silencing activity, showing that the interaction with 14-3-3β is required for transcriptional silencing activity of FBI1.

Down-regulation of FBI1 expression in K2 cells altered MKP-1 mRNA expression; therefore, we hypothesized that MKP-1 is one of the targets of FBI1-14-3-3β complex. To analyze the effect of FBI1 and 14-3-3β on the MKP-1 promoter, the luciferase reporter gene plasmid −631Luc, which is driven by the region of the rat MKP-1 promoter from −631 to +240, was constructed according to Ryser et al. (31) with a slight modification (Fig. 8A). The −210 to +240 region as a proximal promoter contains two cAMP-responsive elements, one E box, three GC boxes, and one TATA box. K2 cells transiently transfected with −631Luc displayed substantial luciferase activities under normal conditions, and the activities were dose-dependently suppressed by the cotransfection with FBI1 expression vector (Fig. 8B). When cotransfected with 2 μg of FBI1 plasmid, luciferase activity was suppressed to 46% of the control. To confirm that the silencing activity is specific for the MKP-1 promoter, luciferase assays were performed using oct3/4 and nanog promoters that contain GC boxes. FBI1 had no significant effect on these promoters (data not shown). The inhibition of −631Luc activity by 4A FBI1 mutant, which could not bind to 14-3-3β, was hardly detected (Fig. 8C).

Moreover, −631Luc activity was stimulated similarly in a dose-dependent manner by transfection with antisense 14-3-3β and estimated as 3.4-fold of the control when cotransfected with 2 μg of antisense plasmid (Fig. 8D). These results suggest that the FBI1-14-3-3β complex can act as a transcriptional repressor of MKP-1. To identify the region required for FBI1-14-3-3β repression of the MKP-1 promoter, different MKP-1 promoter constructs were introduced into K2 cells and analyzed for luciferase activities.
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The activity of −90Luc, which contains GC boxes and TATA box, was suppressed by FBI1 expression.

To examine whether the FBI1-14-3-3β complex binds to the MKP-1 promoter region in vivo, a ChIP assay was performed. In quiescent K2 cells prepared by serum starvation for 12 h, a significant amount of FBI1 and 14-3-3β was detected in the −102 to +50 promoter region containing three GC boxes and one TATA box (Fig. 9). Interestingly, the binding level of these proteins began to decrease within 30 min after serum stimulation and reached 30% of the value for quiescent cells at 60 min. We also analyzed the binding levels of HDAC1 and Sp3, since the Sp3 transcriptional complex recruits HDAC1 and suppresses rat MKP-1 promoter activity via GC box1 (31). In a manner similar to the response profile of FBI1 and 14-3-3β, the amount of HDAC1 and Sp3 bound to the −102 to +50 promoter region in quiescent K2 cells was diminished by serum stimulation (Fig. 9).

DISCUSSION

14-3-3 proteins bind to various target proteins. Many of the target proteins (e.g. Raf-1, Bcr-Abl, TERT, and p53) act as oncogenes or tumor suppressor genes, and their functions are regulated by 14-3-3 proteins (32–35). 14-3-3σ is well known as a tumor suppressor gene; in many kinds of cancer tissues, 14-3-3σ is down-regulated by promoter methylation or degradation (36, 37). On the other hand, several studies have reported that some isoforms of 14-3-3 are overexpressed in specific cancer cells and have provided some correlative information between 14-3-3 proteins and oncogenesis (38–40). However, there are few reports that demonstrate the molecular mechanisms of 14-3-3 in oncogenesis. We previously reported that 14-3-3β is over-expressed in various cancer cells and that down-regulation of 14-3-3β proteins results in suppression of anchor-deendant growth, tumorigenicity, and angiogenesis of rat hepatoma K2 cells (8). Thus, 14-3-3β may act as an oncogene. In this study, we attempted to demonstrate the mechanisms of 14-3-3β-induced cell growth and tumorigenesis by screening binding partners. We identified FBI1 as a novel binding partner of 14-3-3β and FBI1 promoted anchorage-independent growth, tumorigenicity, and metastasis. In addition, luciferase reporter and ChIP assays revealed that 14-3-3β and FBI1 formed a repressor complex on the MKP-1 promoter.

The immunoprecipitation and pull-down analyses demonstrated that FBI1 binds to 14-3-3β directly in vitro and in vivo. Luciferase assay showed that multiple mutations of binding sites were required to completely abrogate the silencing activity of FBI1. This result suggests FBI1 binds to 14-3-3β through multiple sites. 14-3-3 proteins are dimeric proteins; thus, they can interact with a single FBI1 protein as well as Raf-1, Bad, and Cdc25B, which also contain some 14-3-3 binding sites (41–43). Cdc25B contains three 14-3-3 binding sites. Although single mutations of these binding sites reduce the interaction with
During metastasis, cancer cells must cross several extracellular matrix barriers. MMPs are activated in several stages of metastasis and degrade extracellular matrix components (21, 25). MMP-9 degrades type IV collagen, which constitutes the major component of the basement membrane and promotes metastasis (22, 23), indicating that the activity of MMP-9 plays an important role in metastasis. The expression of MMP-9 was reduced in antisense FBI1 transfecteds. However, in metastatic cell lines, which acquired high expression of FBI1 protein, the expression of MMP-9 was comparable with that in K2 cells. It is possible that FBI1 may regulate the expression level of MMP-9 and promote metastasis.

Luciferase reporter and ChIP analyses revealed that the FBI1-14-3-3β complex could act as a transcriptional silencer and that MKP-1 is one of the target genes. Several reports have demonstrated that 14-3-3 proteins regulate transcription. In most cases, the interaction between 14-3-3 proteins and transcriptional factors leads to sequestration in the cytoplasm, away from their transcriptional targets (30, 46). However, our studies revealed that FBI1 formed a complex with 14-3-3β on the MKP-1 promoter and that the complex suppressed MKP-1 transcription in K2 cells. Thus, these results propose a novel function of 14-3-3 in transcriptional regulation. Luciferase reporter analysis using different MKP-1 promoter constructs showed that the region from −90 to +240 containing GC boxes and one TATA box is important for suppression of the FBI1-14-3-3β complex. In addition, Sp3 and HDAC1 bound to the MKP-1 promoter in the absence of serum stimulation. Sp3, which can recruit HDAC1/2, suppresses MKP-1 promoter activity via the GC box1 (GGACCGCCCC) in the −203 to +19 proximal promoter region (31). It is possible that the FBI1-14-3-3β complex suppresses MKP-1 promoter activity via the GC box 1 in cooperation with the Sp3 transcriptional repressor complex containing HDAC1.

There are at least seven isoforms of 14-3-3 in mammals. 14-3-3ζ is also highly expressed in some cancer tissues (38). A recent report has demonstrated that knockdown of 14-3-3ζ in a lung cancer cell line induces anoikis (47). The anoikis induction is mediated by reduction of Akt activity and induction of BH3-only proapoptotic proteins. On the other hand, in our studies, the activity of Akt was not detected in K2 cells, and there was no significant difference in apoptosis induction by some apoptotic inducers (data not shown). Therefore, 14-3-3β induces cell growth, tumorigenicity, and metastasis by different molecular mechanisms from that of 14-3-3ζ, suggesting that 14-3-3β is a unique isoform among 14-3-3 family proteins.

We demonstrated that FBI1 plays a pivotal role in metastasis of K2 cells as well as tumor growth. Metastasis is the cause of 90% of human cancer deaths. However, little is known about its genetic and biochemical determinants. Since FBI1 was overexpressed in several cancer cell lines, identification of FBI1 target genes and investigation of the molecular mechanism of FBI1 transcriptional regulation may help in further understanding the mechanism of metastasis and in the search for new therapeutic targets.

Acknowledgments—We thank Prof. K. Oda for encouragement throughout this study and critical reading of the manuscript and Y. Iwahana for technical assistance.
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