The Transmembrane Adaptor Cbp/PAG1 Controls the Malignant Potential of Human Non–Small Cell Lung Cancers That Have c-Src Upregulation

Takashi Kanou1,2, Chitose Oneyama1, Kunimitsu Kawahara3, Akira Okimura3, Mitsunori Ohta4, Naoki Ikeda5, Yasushi Shintani6, Meinoshin Okumura2, and Masato Okada1

Abstract

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Introduction

Lung cancer is the leading cause of cancer death among elderly men and is the second leading cause of cancer death among elderly women in Japan. From 2000 to 2007, the mortality rate due to lung cancer among the elderly population was 272.41/100,000 and 62.45/100,000 in men and women, respectively (1). Previous reports have demonstrated that lung cancer development involves both environmental and genetic factors (2). Over the past few decades, the methods for the diagnosis and treatment of lung cancer have improved. Recently, targeted molecular therapy in the treatment for non–small cell lung cancer (NSCLC) has received significant attention. Although epidermal growth factor receptor (EGFR) inhibitors have been successfully developed, NSCLC patients still have unfavorable prognoses (3). Therefore, the development of novel therapeutic strategies for the treatment of NSCLC is urgently needed.

c-Src was the first identified proto-oncogene product, and extensive research has shown that c-Src plays crucial roles in various intracellular signaling pathways that are implicated in cell growth, adhesion, and migration (4, 5). Several reports have shown that the overexpression or hyperactivity of c-Src is common in human lung cancers, especially in adenocarcinomas (6–10); however, the C-SRC gene is rarely mutated in human cancers, and the involvement of c-Src protein expression or enzymatic activation in the etiology of human cancer remains obscure (11). The kinase activity of c-Src is tightly regulated by several factors. A critical protein that negatively regulates c-Src activation is C-terminal Src kinase (Csk), which phosphorylates the negative regulatory tyrosine residue of c-Src (Tyr529; refs. 12, 13). Csk expression has been reported to be reduced in hepatocellular carcinoma in comparison with normal tissue, and this alteration may be correlated with c-Src activation (14). These observations suggest that Csk has a tumor-suppressive activity and that its reduced expression may facilitate the malignant cell behavior that is promoted by c-Src; however, the mechanisms underlying the downregulation of Csk in cancer cells are unclear.

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Csk is a cytoplasmic protein (15), and, thus, it has been proposed that Csk requires an appropriate membrane adaptor protein to access the membrane-anchored c-Src. We and another group previously identified the Csk-binding protein (Cbp), which is also known as PAG1 (16), as a specific membrane adaptor of Csk (17). Cbp exclusively localizes in lipid rafts and is involved in regulating the activity of Src family kinases (SFK) through the recruitment of Csk to lipid rafts where SFKs accumulate (17, 18). Several recent studies have implicated Cbp in cancer progression. In colon cancer patients, Cbp expression was significantly downregulated in some tumors in comparison with adjacent normal tissues (19), suggesting that Cbp has a tumor suppressor function for some types of cancer; however, other studies have shown that Cbp is overexpressed in renal cell carcinoma (RCC) and serves as a positive regulator of tumor malignancy in RCC patients (20). Thus, it has been suggested that Cbp plays distinct roles in controlling tumor progression depending on the cancer type.

In this study, we investigated the function of Cbp in several human lung cancer cell lines and tissues to address the precise in vivo role of Cbp. We show that Cbp expression is significantly downregulated in NSCLC cells and that the overexpression of Cbp in NSCLC cells, in which c-Src is highly activated, results in the suppression of tumor growth by promoting Csk-mediated c-Src inactivation. In addition, we show that Cbp inhibits both the in vitro invasiveness and the in vivo metastasis of a NSCLC cell line, and that there is a significant correlation between the Cbp expression level and lymph node metastasis. These findings suggest that Cbp can suppress the malignant potentials of NSCLC by attenuating the c-Src-mediated tumorigenic pathway.

Materials and Methods

Cell lines and reagents
The human NSCLC cell lines (A549, PC9, PC10, Lu65, Lu99, Lc4a, Lc3, Lc2, Lc28, EBC-1, and RERF-LC-99Lu) were obtained from the European Collection of Cell Cultures (ECACC). A549 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) and all other cancer cells were cultured in RPMI1640 (Nacalai). Normal human lung cells (13Lu, 39Lu, and 888Lu) were obtained from the Osaka Prefectural Medical Center for Respiratory and Allergic Diseases. All gene transfer and expression protocols were performed using previously described methods (21). Briefly, the cDNAs that encode wild-type human Cbp and its mutant, which had a substitution of Tyr314 to Phe (Y314F), were subcloned into the retroviral vectors pCX4puro and pCX4bleo, respectively. All constructs were generated using a PCr-based procedure. The retroviral vectors were transfected into Plat-E, which is an ecotropic murine leukemia virus-packaging cell line, using Fugene6 (Boehringer) according to the manufacturer’s directions. To transfer the gene into human cells, we used pGP + pE-Ampho (Takara Bio), which is an amphotropic retrovirus-packaging construct. Infected cell populations were selected with puromycin or bleomycin, and the mixed cell populations were subjected to each assay that is described in this study.

Antibodies
We generated an anti-human Cbp antibody by immunizing rabbits with a GST-Cbp (residues 331–430) fusion protein. We acquired the anti-Src antibody (Ab-1) from Calbiochem; the anti-Src pY418, anti-Src pY529, and anti-FAK pY397 antibodies from Biosource; the anti-FAK, anti-Csk, and anti-β-tubulin antibodies from Santa Cruz Biotechnology; the anti-Caveolin antibody from BD Transduction Laboratories; the anti-Transferrin receptor from Zymed; and the anti-Akt, anti-Akt pS473, anti-ERK, and anti-Erk pT202/pY204 antibodies from Cell Signaling. Horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG (Zymed) was used as the secondary antibody.

Western blot and immunoprecipitation
Cells were washed and lysed in ODG buffer (50 mmol/L of Tris-HCl, pH 7.4, 1 mmol/L of EDTA, 0.25 mol/L of NaCl, 20 mmol/L of NaF, 1 mmol/L of Na3VO4, 1% Nonidet P-40, 2% Octyl-p-glucoside, 5 mmol/L of β-mercaptoethanol, 0.1% aprotinin, 0.1% leupeptin, 1 mmol/L of PMSF, and 5% glycerol). Equal amounts of total protein were separated using SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked and probed with primary antibodies, followed by incubation with HRP-conjugated secondary antibodies. For immunoprecipitation, the precleared lysate (1-mg protein) was incubated with the indicated antibodies for 1 hour and protein G-Sepharose (GE Healthcare) for 3 hours at 4°C. The immunoprecipitates were washed with ODG buffer and analyzed by Western blotting.

Cancer specimens
NSCLC specimens and paired noncancerous tissues were snap-frozen in liquid nitrogen immediately after resection. The resected lung tissues were divided upon visual inspection into tumor (T) and nontumor (N) regions, which were then histologically confirmed. To compare the Cbp mRNA expression level in each NSCLC case, we used formalin-fixed, paraffin-embedded (FFPE) samples of human primary lung adenocarcinoma. Sections (10 μm) were cut from each block and placed in a 1.5-ml test tube. Total RNA was then extracted using the High Pure FFPE RNA Micro Kit (Roche). This study was approved by the ethical review board of the Osaka Prefectural Medical Center for Respiratory and Allergic Diseases.
**In vitro proliferation assay**

In vitro anchorage-dependent growth was assessed by using an assay that is based on the cleavage of the tetrazolium salt WST-1 into formazan by cellular mitochondrial dehydrogenases (Roche). Each cancer cell line was plated in 96-well microplates at a density of 1,000 cells per well. Cells were allowed to grow for 72 hours, and 10 μL of WST-1 reagent was added to each well followed by 1-hour incubation. The absorbance at 450 nm was read with a microplate reader, and the average of 3 wells was determined. Each experiment was repeated 3 times.

**Soft agar assay**

Single-cell suspensions of 1 × 10⁴ cells in 1.5 mL of DMEM that contained 10% FBS and 0.36% agar were plated onto a layer of 2.5 mL of the same medium that contained 0.7% agar in 6-well culture dishes. Viable colonies were stained with MTT 10 to 14 days after plating. Colonies larger than approximately 0.1 mm in diameter were scored.

**Invasion assay**

Invasion assays were conducted using a BioCoat Matrigel Invasion Chamber (BD Bioscience) according to the manufacturer’s instructions. A cell suspension (5 × 10⁴ cells) in serum-free medium was added to the inserts, and each insert was placed in the lower chamber, which contained a culture medium that was supplemented with 10% FBS. After a 24-hour incubation, invasiveness was evaluated by staining the cells that migrated through the extracellular matrix layer.

**Tumor growth and metastasis in nude mice**

Female BALB/c athymic nude mice (4 weeks old) were purchased from Japan CLEA. Cancer cells were grown, harvested, washed with PBS, and resuspended in serum-free DMEM. For the analysis of tumor growth, 1 × 10⁶ cancer cells (0.2 mL) were subcutaneously inoculated into nude mice. Tumor sizes were monitored weekly, and tumor volume was calculated on the basis of the following formula: volume = 0.5 × length × (width)². For the lung metastasis model, 5 × 10⁸ cells were intravenously injected into mice via the tail vein. After 1 month, mice were euthanized using diethyl ether, and the number of metastatic nodules was determined in lungs that were fixed in Bouin’s solution (22). Mice were handled and maintained according to the Osaka University guidelines for animal experimentation.

**Preparation of detergent-resistant membrane domains**

The separation of detergent-resistant membrane domains (DRM) was performed as previously described (23). Briefly, cells were lysed in a buffer that contained 0.25% Triton X-100, and the lysate was placed at the bottom of a discontinuous sucrose gradient (40%/35%/5%). After centrifugation at 40,000 rpm for 6 hours, fractions were collected from the top of the gradient, and aliquots were analyzed by Western blotting. The separation of DRMs and non-DRMs was confirmed by detecting caveolin-1 and transferrin receptor (TFR) as markers for DRMs and non-DRMs, respectively.

**Real-time quantitative PCR**

Total RNA was extracted from cancer cell lines and frozen tissue samples using Sepasol (Nacalai). The High Pure FFPE RNA Micro Kit (Roche) was used to extract total RNA from the FFPE tissue samples. The Transcriptor First Strand cDNA Synthesis Kit (Roche) was used to reverse transcribe all samples for qPCR. The mRNA levels of Cbp (ID: 179693), matrix metalloproteinase (MMP)-2/9 (ID: 234422, 957562), and glyceraldehydes-3-phosphate dehydrogenase (GAPDH; ID: 99999915) were quantified using TaqMan gene expression assays (Applied Biosystems). PCR was performed using an Applied Biosystems 7900HT Fast Real-time PCR System and Sequence Detection System, Software v2.2.1. The expression of the target genes was normalized to that of GAPDH.

**Immunohistochemistry**

Cancer cells were cultured on fibronectin-coated plates and fixed with 4% PFA (paraformaldehyde) for 15 minutes at room temperature. After blocking with 1% BSA (bovine serum albumin), the cells were incubated with the primary antibodies overnight at 4°C. The cells were then incubated with secondary antibodies for 1 hour at room temperature, and coverslips were mounted on the glass slides. Alexa Fluor 488 phalloidin (Invitrogen) was used to stain the F-actin. Images were photographed using a fluorescent confocal microscope (Olympus, FV-1000). Immunohistochemistry was performed on FFPE tumor samples. Sections from cancerous and corresponding normal tissue were stained with an anti-Cbp antibody or a neutralized antibody. The detection of immunoreactivity was performed using an iVIEW DAB Detection Kit on Benchmark (Ventana Medical Systems).

**Wound-healing assay**

A549 cells were seeded into 6-well plates and incubated in a medium that was supplemented with 10% FBS until the cells reached confluence. The confluent monolayer cultures were then scratched with a pipette tip. After incubation for 24 hours, the wounded areas were examined with a light microscope. The wound gap distances were then measured. All experiments were performed in triplicate.

**Results**

**Cbp expression is downregulated in NSCLC cells**

As an initial experiment, we compared, by Western blotting, the expression levels of Cbp and c-Src proteins in normal lung cells and in a variety of NSCLC cells. As shown in Figure 1A, the expression of Cbp was markedly downregulated in NSCLC cells in comparison to those in normal lung cells. In contrast, the expression of c-Src varied depending on cell types; higher levels of c-Src expression...
were detected in A549 and Lu99 cells. These suggest that the expression level of Cbp is not necessarily correlated with the level of c-Src. However, the majority of NSCLC cells exhibited a higher ratio of the signal intensity of pY418 (an autoactivation site) to that of total Src protein (Fig. 1A, top right) than normal cells. In addition, the phosphorylation status of pY529 (a negative regulatory site that is phosphorylated by Csk) in several NSCLC cells was lower than that in normal cells (Fig. 1A, bottom right). These observations demonstrate that c-Src is widely activated in NSCLC cells, although the expression of Csk in NSCLC cells is similar to that in normal lung cells (Fig. 1A, left).

To examine whether Cbp expression was altered in human cancer tissues, the expression of Cbp mRNA in frozen samples from 4 pairs of NSCLC tissues and their matched noncancerous control tissues was analyzed by quantitative real-time (RT) PCR. The relative levels of Cbp mRNA, which were normalized to GAPDH mRNA expression, in lung tumors (black bars) and matched normal tissues (white bars) are shown. C, immunohistochemical staining was performed for Cbp in lung tissues. Sections of cancerous tissue and normal tissue were stained with anti-Cbp and eosin and observed using light microscopy at 400× magnification.
The expression of Cbp mRNA was significantly downregulated in all human lung cancer samples in comparison to that in their adjacent normal controls (Fig. 1B). Immunohistochemistry also indicated that the tumor cells very weakly expressed the Cbp protein, although the Cbp protein was readily detectable in the normal bronchiolar epithelia (Fig. 1C). These results indicate that Cbp expression is generally downregulated in NSCLC cells as well as in NSCLC tissues.

The ectopic expression of Cbp inhibits anchorage-independent growth and tumor growth of NSCLC cells in which c-Src is upregulated

To investigate the role of Cbp downregulation in NSCLC cells, we ectopically expressed Cbp in A549, Lu99, PC9, and Lu65 cells; A549 and Lu99 cells have the highest levels of c-Src upregulation, whereas PC9 and Lu65 cells express normal levels of c-Src. The expression levels of Cbp in the transfected cells substantially exceeded those in the normal lung cells (Fig. 2A); however, because
the A549 and Lu99 cells exhibited a marked (>10-fold) upregulation of the c-Src protein in comparison to normal lung cells (Fig. 1A), the ratio of overexpressed Cbp to c-Src in these cells appeared to be at a comparable level to that in normal cells. Western blot analysis revealed that the phosphorylation of c-Src Tyr418 in all 4 cell lines was suppressed by the overexpression of Cbp; although, the levels of total c-Src and pY418 in PC9 and Lu65 cells were much lower than those in A549 cells (Fig. 2A). These results indicate that the kinase activity of c-Src is inhibited by the expression of Cbp in these cells.

We next examined the effects of Cbp expression on the growth ability of these NSCLC cells. In A549 and Lu99 cells, the expression of Cbp significantly suppressed both anchorage-dependent and anchorage-independent growth, as assessed by a colony assay and a soft agar colony formation assay (Fig. 2B and C); however, in PC9 and Lu65 cells, the expression of Cbp had little effect on both anchorage-dependent growth and anchorage-independent growth. The suppressive effect of Cbp on tumor growth was further examined in a xenograft mouse model. Nude mice were subcutaneously injected with Cbp-overexpressing A549 and PC9 cells or control cells, and tumor formation was monitored weekly. Similar to anchorage-independent growth, the expression of Cbp significantly suppressed the tumor growth of A549 cells but not PC9 cells (Fig. 2C). These data suggest that Cbp can suppress the tumor growth of NSCLC cells, which have upregulated c-Src.

The Cbp-mediated suppression of tumor growth signaling is associated with a reduction in active c-Src in non–raft compartments.

To elucidate the molecular mechanisms by which Cbp suppresses tumor growth, we investigated the activity status of several Src downstream molecules, including FAK, Akt, and Erk, in A549 cells. Despite the reduction of total c-Src activity in these cells (Fig. 2A), the expression of Cbp preferentially suppressed the activity of FAK (pY379), whereas the activities of Akt and Erk were unchanged (Fig. 3A). To address the mechanism for the inactivation of FAK, we examined the membrane distribution of c-Src, FAK, and Csk by separating DRMs, in which major components of lipid rafts are concentrated (24). In Cbp-expressing cells, Cbp was enriched in the DRMs (Fig. 3B, right), representing its preferential localization to lipid rafts. In these cells, the activity of c-Src (pY418 signals) was substantially reduced in the non-DRMs, whereas the level of inactive c-Src (pY529) rather increased (Fig. 3B). Quantitative analysis revealed that the ratio of active c-Src (pY418) in the DRMs to that in the non-DRMs significantly increased in Cbp-expressing cells (Fig. 3C, left), whereas the levels of inactive c-Src (pY529) in the DRMs decreased (Fig. 3C, right). These findings demonstrate that the expression of Cbp can induce not only the suppression of c-Src activity but also the accumulation of active c-Src in lipid rafts. Consistent with the reduction of c-Src activity in the non-DRMs, the activity of FAK (pY397) that is present in the non-DRMs was appreciably suppressed (Fig. 3B).

This raises the possibility that the Cbp-mediated elimination of active c-Src from the non–raft compartments may interfere with the access of active c-Src to FAK, which is crucial for tumor growth (18).

Cbp-Csk association and inhibition of c-Src activity are involved in the growth suppression of A549 cells

To further elucidate the mechanisms for the suppression of c-Src activity by the expression of Cbp, we examined the interaction between c-Src, Csk, and Cbp in A549 cells. Figure 3B demonstrates that Csk was detected in the DRMs from Cbp-expressing cells but was absent in the DRMs from the mock-treated cells. These data indicate that a part of Csk can be recruited to lipid rafts by the expression of Cbp. The immunoprecipitation assays for Cbp and c-Src revealed that Cbp coimmunoprecipitated with both c-Src and Csk (Fig. 4A), indicating that Cbp, Csk, and c-Src are able to form a ternary complex in lipid rafts. These results suggest that active c-Src and Csk are recruited to Cbp, which allows for an efficient inactivation of c-Src by Csk. To verify the importance of the formation of the Cbp-Csk complex in c-Src–induced tumor growth, we expressed a Cbp mutant (Cbp Y314F) with a mutation in the Csk-binding site in A549 cells; the mutant was expressed at a level that was comparable to that of wild-type Cbp (Fig. 4B). The expression of Cbp Y314F failed to suppress anchorage-independent growth in soft agar and tumor formation in a xenograft model (Fig. 4B and C), indicating that Cbp–Csk association is crucial for the tumor-suppressing effect of Cbp.

To examine whether the effects of Cbp expression on cell growth were mediated through suppressing c-Src activity, we further investigated the effects of Src inhibitors, including PP2, Dasatinib, and Saracatinib, on the growth of A549 cells. As shown in Figure 4D, the addition of each inhibitor decreased the phosphorylation levels of c-Src Tyr418. Consistent with the activity status of c-Src, the Src inhibitors dose dependently repressed both anchorage-dependent and anchorage-independent growth of these cells, as assessed by a growth assay and a soft agar colony formation assay (Fig. 4E and F). These data demonstrate that the upregulation of c-Src activity is indeed involved in the enhanced growth ability of A549 cells and suggest that the inhibition of c-Src by the Cbp–Csk regulatory circuit can also contribute to the suppression of tumor growth of A549 cells.

The expression of Cbp affects the cytoskeletal organization and motility of lung cancer cells

We next examined the effects of Cbp expression on the organization of the actin cytoskeleton because the actin cytoskeleton is crucial for regulating the invasion and metastasis of cancer cells (25). Although the control A549 cells had prominent actin stress fibers, Cbp expression substantially attenuated stress fiber formations (Fig. 5A). As detected by a wound-healing assay, cell motility was significantly repressed by Cbp expression (Fig. 5B). In
contrast, the expression of Cbp Y314F had little effect on both the actin cytoskeleton and cell motility (Fig. 5A and B), indicating that the Cbp-Csk association and c-Src activity are crucial for controlling these cellular functions as well. The significant reduction in the invasiveness that is associated with Cbp expression was also observed in a Matrigel invasion assay (Fig. 5C). We further examined the expression of invasion-related metalloproteases (MMP-2, MMP-9), which degrade extracellular matrices. The expression of MMP-9 was significantly reduced in Cbp-expressing cells compared with control cells, whereas the expression of MMP-2 was only moderately repressed (Fig. 5D). These results suggest that Cbp can suppress the invasive potential of lung cancer cells by modulating cytoskeletal organization, cell motility, and the expression of MMPs via the inhibition of a c-Src–mediated pathway (5).

The expression of Cbp affects the metastatic ability of lung cancer cells

The observations that Cbp may control the invasive potential of lung cancer cells in vitro directed us to examine the suppressive role of Cbp in tumor metastasis in vivo.

Figure 3. Cbp suppresses Src signaling by sequestering active c-Src into DRMs. A, total cell lysates from mock and Cbp-expressing A549 cells were analyzed by Western blotting with the indicated antibodies (left). The activity status of FAK was assessed by quantifying the relative intensity of the signal for phosphorylated forms in comparison to that of the nonphosphorylated forms. Relative specific activities (mean ± SE) of FAK in Cbp-expressing cells in comparison to those in mock cells were obtained from 3 independent experiments. B, DRMs and non-DRMs were separated from mock cells and Cbp-expressing cells, and each fraction was analyzed by Western blotting with the indicated antibodies. Caveolin 1 and TFR were detected as markers of DRMs and non-DRMs, respectively. C, the intensity of signals for Src pY418 (left) and pY529 (right) was quantified, and the ratios of the signals in DRMs to those in non-DRMs were compared between mock cells and Cbp-expressing cells. The relative values (mean ± SE) that were obtained from 3 independent experiments are shown.
Figure 4. Cbp-Csk association and inhibition of c-Src are involved in the growth suppression of A549 cells. A, cell lysates from mock and Cbp-expressing A549 cells were immunoprecipitated with anti-Cbp antibody, and the immunoprecipitates were analyzed by Western blotting with anti-Src, anti-Csk, or anti-Cbp antibody. To detect Csk in the immunoprecipitates, the sample was not heat denatured so as to avoid an overlap with the IgG heavy chain on the gel. *, the location of the IgG heavy chain; **, the non-specific bands. B, the anchorage-independent growth of mock cells, Cbp-expressing cells, and Cbp mutant-expressing cells (Cbp Y314F) were examined by a colony formation assay in soft agar. Colonies were stained with MTT 14 days after plating. The colony numbers per cm² ± SE that were obtained from 3 independent experiments are shown (left). The expression levels of Cbp wild type and Cbp mutants were assessed by Western blotting. C, the ability of tumor formation was compared in mouse xenograft models. The tumor volumes (mean ± SE) that were obtained from 4 mice are plotted as a function of days after inoculation. D, A549 cells were incubated with increasing concentrations of PP2, Dasatinib, Saracatinib or DMSO for 24 hours, and the total cell lysates were subjected to Western blotting with the indicated antibodies. E, in vitro proliferation assays were performed for A549 cells incubated with the indicated Src inhibitor. The relative growth rate (mean ± SE) was obtained from 3 independent experiments. F, colony formation assays were performed for A549 cells incubated with DMSO, PP2, Dasatinib, or Saracatinib at the concentrations indicated. Colonies were stained with MTT 10 days after plating. The colony numbers per cm² (mean ± SE) were obtained from 3 independent experiments. Student’s t tests: *, P < 0.05.
A549 cells, with or without Cbp expression, were intravenously injected into nude mice, and the pulmonary metastatic burden was assessed by counting the number of tumor nodules on the surface of the lung. Mice in the control groups displayed numerous distinguishable pulmonary nodules, whereas mice that were injected with Cbp-expressing cells exhibited fewer visible nodules (Fig. 6A).

To examine whether the levels of Cbp expression in lung cancer affected the degree of tumor progression, the expression of Cbp mRNA was examined in primary lung adenocarcinoma by quantitative PCR using total RNA from FFPE tissue samples as templates. The lowest score of Cbp mRNA in 12 cases was arbitrarily set as 1.0, and the relative level of Cbp mRNA in each cancer specimen was evaluated. As shown in Figure 6B, appreciable differences in the levels of Cbp mRNA were observed among individual samples, although there was no significant relationship between the Cbp expression levels and the histologic tumor grade; however, Cbp expression could be significantly correlated with lymph node involvement. The mean Cbp score for tumors without lymph node metastases was 7.71 ± 4.38, whereas the mean Cbp score for tumors with lymph node metastases was 3.20 ± 2.05 (Fig. 6C). These findings suggest that the level of Cbp in lung cancer may be correlated with metastatic potential to the lymph node rather than primary tumor grade.
Discussion

In this study, we found that the expression of Cbp is significantly downregulated in various human NSCLC cell lines and tissues. Due to its role as a scaffold for both active c-Src and Csk, Cbp expression was observed to suppress the tumor growth of A549 and Lu99 cells, which have substantially upregulated c-Src. In addition, we have shown that the expression of Cbp suppresses cell motility, in vitro invasiveness, and the in vivo metastatic potential of A549 cells, and the expression level of Cbp was significantly correlated with lymph node involvement in human lung cancers. These findings suggest the important role of Cbp in controlling the malignant potential of NSCLC, particularly those that have upregulated c-Src activities.

Recently, several studies have implicated a role for Cbp in tumor malignancy. Cbp may function as a negative regulator of EGF-induced cell transformation (26). In colon cancer cells, Cbp is downregulated and the introduction of Cbp suppresses tumorigenesis (19). In this study, we have shown that Cbp acts as a suppressor of NSCLC tumor progression by controlling c-Src signaling. On the other hand, it has been reported that the expression of Cbp is increased in a significant majority of diffuse large B-cell lymphomas (27). In RCC, Cbp is overexpressed in cancer tissues and enhances cell motility and invasiveness, wherein it regulates RhoA activity via its PDZ-binding domain (20). These observations suggest that Cbp has distinct functions depending on the cancer type. To elucidate the molecular basis for the distinct functions of Cbp, a more extensive analysis of Cbp function in each case will be necessary.

In the immune system, lipid rafts have been thought to play a positive role in signaling that is initiated through the T-cell, B-cell, and Fc receptors (28, 29). In contrast, we previously proposed a role for Cbp in suppressing c-Src-mediated transformation using a model system (19). In this study, we provide firm evidence that Cbp in lipid rafts plays a suppressive role in controlling the c-Src–induced malignant potential of human cancer cells. Biochemical analysis demonstrated that Cbp recruits active c-Src and Csk into
lipid rafts by forming a Cbp–Csk–c-Src complex and serves as a scaffold where the efficient inactivation of c-Src by Csk can be achieved. As a consequence, active c-Src is eliminated from the non–raft compartments, and this elimination potentially contributes to the suppression of the non–raft FAK that is crucial for tumor growth. These findings indicate that the function of c-Src may be regulated not only on the activity level but also by influencing the intracellular distribution that is determined by the specific scaffold Cbp. In contrast to the overexpression of Cbp, the overexpression of Csk failed to suppress the tumor growth of A549 cells (data not shown). This intriguing result further supports the requirement of Cbp in Csk-mediated c-Src regulation as well as in the control of malignancy of A549 cells.

Recent reports have shown that c-Src is altered and highly activated in NSCLC, especially in adenocarcinomas (7, 9, 30). The level of c-Src kinase activity has been reported to correlate with tumor size in NSCLC (8). Furthermore, the mitogenic effect of both nicotine and asbestos in NSCLC cells is likely to involve the activation of c-Src (31, 32). These observations suggest that c-Src activity is frequently associated with the malignant progression of a variety of NSCLC cells. Because c-Src is also known to be an important mediator of upregulated receptor tyrosine kinases, including HER2 and EGFR, therapeutic strategies to inhibit c-Src activity are currently being developed (33). Recently, several inhibitors of activated c-Src in cancer cells have been examined in clinical trials for the treatment of not only lung but also breast, colon, and prostate cancer (34). In preclinical studies, NSCLC cells that were treated with dasatinib (BMS-354825) demonstrated decreased cell growth and changes in downstream signaling that resulted in a reduced capability for invasion (35, 36). These reports suggest that c-Src could be an attractive target for treatment in a particular subset of patients with NSCLC. Therefore, the c-Src regulatory circuit that consists of Csk, Cbp, and lipid rafts may offer new targets that can prevent the progression of NSCLC by controlling c-Src function.

In addition to tumor growth, the in vivo invasiveness of A549 cells was also significantly inhibited by the overexpression of Cbp. The attenuation of actin stress fiber formation and the downregulation of MMP-9 expression may explain the lower invasiveness of Cbp-expressing cells. This result was consistent with the previous observation that c-Src activation promoted tumor invasiveness (25). Recently, it has been demonstrated that Src-FAK signaling through JNK (c-jun NH kinase) alters the transcriptional regulation of MMP-9 (37), and that the pharmacologic blockade of Src activity suppresses VEGF-induced MMP production in squamous carcinoma cells (38). These lines of evidence support that the inhibition of c-Src and FAK functions through the overexpression of Cbp leads to the modulation of cytoskeletal organization and MMP production. We have also found that the expression levels of Cbp mRNA that were detected in FFPE NSCLC samples (39) in cases with lymph node involvement were lower than those in cases without lymph node involvement. This raises the possibility that the level of Cbp in primary tumors is generally relevant to the metastatic potential of NSCLC; however, the molecular mechanism by which the expression of Cbp is specifically downregulated in NSCLC remains to be addressed. Because there was no apparent correlation between c-Src upregulation and Cbp downregulation, pathways other than c-Src signaling may also contribute to Cbp downregulation mechanisms. The elucidation of the pathway(s) that lead to Cbp downregulation may shed light on the mechanisms of tumor progression and offer potential new opportunities for therapeutic intervention in NSCLC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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