Phosphorylation and Activation of Cell Division Cycle Associated 8 by Aurora Kinase B Plays a Significant Role in Human Lung Carcinogenesis

Satoshi Hayama,1,2 Yataro Daigo,1 Takumi Yamabuki,1 Daizaburo Hirata,1 Tatsuya Kato,1 Masaki Miyamoto,2 Tomoo Ito,3 Ejiu Tsuchiya,4 Satoshi Kondo,2 and Yusuke Nakamura1

1Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, The University of Tokyo, Tokyo, Japan; Departments of Surgical Oncology and Surgical Pathology, Hokkaido University Graduate School of Medicine, Sapporo, Japan; and Kanagawa Cancer Center Research Institute, Kanagawa, Japan

Abstract

Through genome-wide gene expression analysis of lung carcinomas, we detected in the great majority of lung cancer samples cotransactivation of cell division cycle associated 8 (CDCA8) and aurora kinase B (AURKB), which were considered to be components of the vertebrate chromosomal passenger complex. Immunohistochemical analysis of lung cancer tissue microarrays showed that overexpression of CDCA8 and AURKB was significantly associated with poor prognosis of lung cancer patients. AURKB directly phosphorylated CDCA8 at Ser154, Ser219, Ser275, and Thr278 and seemed to stabilize CDCA8 protein in cancer cells. Suppression of CDCA8 expression with small interfering RNA against CDCA8 significantly suppressed the growth of lung cancer cells. In addition, functional inhibition of interaction between CDCA8 and AURKB by a cell-permeable peptide corresponding to 20-amino acid sequence of a part of CDCA8 (11R-CDCA8 261–280), which included two phosphorylation sites by AURKB, significantly reduced phosphorylation of CDCA8 and resulted in growth suppression of lung cancer cells. Our data imply that selective suppression of the CDCA8-AURKB pathway could be a promising therapeutic strategy for treatment of lung cancer patients. [Cancer Res 2007;67(9):4113–22]

Introduction

Lung cancer is one of the most common cancers in the world, and non–small cell lung cancer (NSCLC) accounts for 80% of those cases (1). Many genetic alterations involved in development and progression of lung cancer have been reported, but the precise molecular mechanisms still remain unclear (2). Over the last decade, newly developed cytotoxic drugs, several molecular targeted agents, such as paclitaxel, docetaxel, gemcitabine, and vinorelbine, have emerged to offer clinically applicable anticancer drugs. In fact, cyclin-dependent kinase inhibitors (flavopiridol, roscovitine, and BMS-387032), specific KIF11 inhibitors for EGFR tyrosine kinase (i.e., gefitinib and erlotinib), and histone deacetyltransferase inhibitors for EGF tyrosine kinase, were developed and are applied in clinical practice (5). However, each of the new regimens can provide survival benefits to a small subset of the patients (6, 7). Hence, new therapeutic strategies, such as development of more effective molecular targeted agents applicable to the great majority of patients with less toxicity, are eagerly awaited.

Systematic analysis of expression levels of thousands of genes using cDNA microarrays is an effective approach for identification of unknown molecules involved in carcinogenic pathways and can effectively screen candidate target molecules for development of novel therapeutics and diagnostics. We have been attempting to isolate potential molecular targets for diagnosis and/or treatment of NSCLC by analyzing genome-wide expression profiles of 101 lung cancer tissue samples on a cDNA microarray containing 27,648 genes (8–12). To verify the biological and clinicopathologic significance of the respective gene products, we have established a screening system by a combination of the tumor tissue microarray analysis of clinical lung cancer materials and RNA interference (RNAi) technique (13–24). This systematic approach revealed that a cell division cycle associated 8 (CDCA8) was frequently overexpressed in primary lung cancers and was essential for growth/survival and malignant nature of lung cancer cells.

Recently, CDCA8 was identified as a new component of the vertebrate chromosomal passenger complex. CDCA8 was suggested to be phosphorylated in vitro by aurora kinase B (AURKB), but the precise phosphorylated sites and its functional importance in cancer cells as well as in normal mammalian cells remain unclear (25, 26). The chromosome passenger complex consists of at least four proteins: AURKB, inner centromere protein (INCENP), BIRC5/survivin, and CDCA8 (27), each of which shows a dynamic cellular localization pattern during mitosis (28). Because several mitotic functions of the chromosomal passenger complex have been reported, such as the regulation of metaphase chromosome alignment, sister chromatid resolution, spindle checkpoint signaling, and cytokinesis (29), this complex was likely to be categorized in a group of mitotic regulators. Activation of AURKB and BIRC5 was reported in some of human cancers (30, 31), and many other mitotic and/or cell cycle regulators are also aberrantly expressed in tumor cells and are considered to be targets for development of promising anticancer drugs. In fact, cyclin-dependent kinase inhibitors (flavopiridol, UCN-01, E7070, R-Roscovitine, and BMS-387032), specific KIF11 inhibitor (monastrol), and histone deacetyltransferase inhibitors revealed anticancer activity and have applied in preclinical or clinical phases (32–34).
We here describe the novel mechanism of oncogenic activation of CDCA8 by AURKB that is important for lung cancer growth and progression. We also show that functional inhibition of the CDCA8/AURKB interaction would lead to potential strategies for treatment of lung cancer patients.

Materials and Methods

Cell lines and tissue samples. The human lung cancer cell lines used in this study were as follows: lung adenocarcinomas, A427, A549, LC319, PC14, and NCI-H137L; lung squamous cell carcinomas (SCLC), SK-MES-1, EBC-1, and NCI-H226; and SCLC, DMS114, DMS273, SBC-3, and SBC-5. Human lung-derived cells used in this study were as follows: MRC-5 (fibroblast), CCD-19Lu (fibroblast), and BEAS-2B (lung epithelia: bronchus); these cells were purchased from the American Type Culture Collection (Manassas, VA). All cells were grown in monolayers in appropriate medium supplemented with 10% FCS and were maintained at 37°C in atmospheres of humidified air with 5% CO2. Human small airway epithelial cells (SAEC) were grown in optimized medium (SAGM) purchased from Cambrex BioScience, Inc. Primary lung cancer samples had been obtained with written informed consent as described previously (15). A total of 273 NSCLC and adjacent normal lung tissue samples for immunostaining on tissue microarray analysis were also obtained from patients who underwent surgery at Hokkaido University and its affiliated hospitals (Sapporo, Japan). This study and the use of all clinical materials were approved by individual institutional ethical committees.

Semiquantitative reverse transcription-PCR. Total RNA was extracted from cultured cells and clinical tissues using Trizol reagent (Life Technologies, Inc.) according to the manufacturer’s protocol. Extracted RNAs and normal human tissue polyadenylic acid RNAs were treated with DNase I (Nippon Gene) and reversely transcribed using oligo(dT) primer and SuperScript II reverse transcriptase (Invitrogen). Semiquantitative reverse transcription-PCR (RT-PCR) experiments were carried out with the following synthesized CDCA8-specific primers, AURKB-specific primers, E2F1-specific primers, or with β-actin (ACTB)–specific primers as an internal control: 5′-CATCTGGCAATTCGTCCCTGCT-3′ and 5′-CTACGG-GAAGAGAGATAAAGAC-3′ (CDCA8); 5′-CCACCCTGCACTGTCC- CAT-3′ and 5′-AACAGATAAAGCACTTGGAGA-3′ (AURKB); 5′-GAGGATGTTGTTGTTTAGTGTT-3′ and 5′-GGAGAACAGCATGGTGGAG-3′ (E2F1); and 5′-GAGGATGATAGCTGGTCTTGC-3′ and 5′-CAAGT- CAGTGTCACAGGAAAC-3′ (ACTB). PCRs were optimized for the number of cycles to ensure product intensity within the logarithmic phase of amplification.

Northern blot analysis. Human multiple-tissue blots containing 23 tissues (BD Biosciences Clontech) were hybridized with a 32P-labeled PCR product of CDCA8. The cDNA probe of CDCA8 was prepared by RT-PCR using the primers described above. Prehybridization, hybridization, and washing were done according to the supplier’s recommendations. The blots were autoradiographed at room temperature for 30 h with intensifying BAS screens (Bio-Rad Laboratories).

Antibodies. To obtain anti-CDCA8 antibody, we prepared plasmids expressing full-length of CDCA8 that contained His-tagged epitopes at their N- and C-termini with pET28 vector (Novagen). The recombinant proteins were expressed in Escherichia coli, BL21 codon-plus strain (Stratagene), and purified using TALON resin (BD Biosciences Clontech) according to the supplier’s protocol. The protein, extracted on a SDS-PAGE gel, was incoated into rabbits; the immune sera were purified on affinity columns according to standard methodology. Affinity-purified rabbit polyclonal anti-CDCA8 antibodies were used for Western blotting and immunostaining. A rabbit polyclonal anti-AURKB antibody was purchased from Abcam, Inc. On Western blots, we confirmed that the antibody was specific to CDCA8 or AURKB, using lysates from NSCLC cell lines that either expressed CDCA8 and AURKB endogenously or not or from cells transfected with CDCA8 or AURKB expression vector.

Western blot analysis. Cells were lysed in lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.5% NP40, 0.1% sodium deoxycholate, 0.1% SDS, protease inhibitor (Protease Inhibitor Cocktail Set III, Calbiochem)]. We used an enhanced chemiluminescence Western blot analysis system (GE Healthcare Biosciences) as described previously (15, 16).

Immunocytochemistry. Cultured cells were washed twice with PBS(−), fixed in 4% formaldehyde solution for 60 min at room temperature, and rendered permeable for treatment for 1.5 min with PBS(−) containing 0.1% Triton X-100. Cells were covered with 3% bovine serum albumin in PBS(−) for 60 min to block nonspecific binding before the primary antibody reaction. Then, the cells were incubated with antibody to human CDCA8 or AURKB protein or with anti-FLAG mAb (Sigma-Aldrich Co.). The immune complexes were stained with a goat anti-rabbit secondary antibody conjugated to Alexa 594 (Molecular Probes) and viewed with a laser confocal microscope (TCS SP2 AOBS, Leica Microsystems). To determine the cell cycle–dependent expression and localization of wild-type (WT) or mutant CDCA8 that was stably expressed in A549 cells, synchronization at the G1–S boundary was achieved by aphidicolin block as described previously (18). Cells were treated with 1 μg/mL aphidicolin (Sigma-Aldrich) for 24 h and released from the cell cycle arrest by washes in PBS for four times. These cells were cultured in medium and harvested for analysis at 1.5 and 9 h after the release of the cell cycle arrest and were used for immunoblotting and immunostaining.

Immunohistochemistry and tissue microarray analysis. To investigate the CDCA8/AURKB protein in clinical materials, we stained tissue sections using EnVision+ kit (horseradish peroxidase (HRP; DakoCytomation). Affinity-purified anti-CDCA8 antibody or anti-AURKB antibody was added after blocking of endogenous peroxidase and proteins, and each section was incubated with HRP-labeled antirabbit IgG as the secondary antibody. Substrate-chromogen was added and the specimens were counterstained with hematoxylin.

Tumor tissue microarrays were constructed as published elsewhere, using formalin-fixed NSCLCs (35–37). Positivity for CDCA8 and AURKB was assessed semiquantitatively by three independent investigators without prior knowledge of the clinical follow-up data. The intensity of histochemical staining was recorded as absent (scored as 0), weak (1+), or strong (2+). When all scorers judged as strongly positive, the cases were scored as 2+.

Statistical analysis. We attempted to correlate clinicopathologic variables, such as age, gender, and pathologic tumor-node-metastasis stage, with the expression levels of CDCA8 and AURKB protein determined by tissue microarray analysis. Tumor-specific survival curves were calculated from the date of surgery to the time of death related to NSCLC or to the last follow-up observation. Kaplan-Meier curves were calculated for each relevant variable and for CDCA8 or AURKB expression in survival times among patient subgroups were analyzed using the log-rank test. Univariate analysis was done with the Cox proportional hazard regression model to determine associations between clinicopathologic variables and cancer-related mortality.

RNAi assay. We had established previously a vector-based RNAi system, psiH1B3x3.0, which was designed to synthesize small interfering RNAs (siRNA) in mammalian cells (13, 15, 16, 18–24). Ten micrograms of siRNA expression vector were transfected using 30 μL LipofectAMINE 2000 (Invitrogen) into lung cancer cell lines, LC319 and SBC-5. The transfected cells were cultured for 4 days in the presence of appropriate concentrations of geneticin (G418), and the number of colonies was counted by Giemsa staining, and viability of cells was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (cell counting kit-8 solution; Dojindo), at 7 days after the G418 treatment. To confirm suppression of CDCA8 protein expression, Western blotting was carried out with affinity-purified polyclonal antibody to CDCA8 according to the standard protocol. The target sequences of the synthetic oligonucleotides for RNAi were as follows: control 1 (enhanced green fluorescent protein (EGFP) gene), a mutant of Aequorea victoria GFP); 5′-CAGCGAGCAGACTTTCC-3′; control 2 (Luciferase: Phutinus pyralis luciferase gene), 5′-CGTACGGGAAATCT- TCCA-3′; si-CDCA8#1, 5′-CAAGCAGAAGATCTTACGAC-3′; and si-CDCA8- r2, 5′-GGCTGCTACACTTACGAC-3′.

Luciferase assay. Human genomic DNA was extracted from LC319 cells and used as templates for PCR. 5′ Flanking region of the human CDCA8 or AURKB gene was amplified by PCR with the following synthesized 5′ region
of CDCA8-specific primers (5'-CGGGGTACCCGACAAGGCCCTGCCGG-GAGTAGT-3' and 5'-CCCAAGCTTGGGCGAATCTGTGCAGCTCGTGTC-3') and 5' region of AURKB-specific primers (5'-AACGTAGGCATGTA-GAGGCTC-3' and 5'-CGGGGAAGAAAGTGCTTAAAGGA-3'). The fragments of promoter region were excised with KpnI and HindIII restriction enzymes and inserted into the corresponding enzyme sites of pGL3-Basic vector. The entire coding sequence of E2F1 was cloned into the appropriate site of pcDNA3.1/myc-His plasmid vector (Invitrogen) to achieve pcDNA3.1-E2F1.

The plasmids containing the 5' flanking region of the CDCA8 gene and phRL-SV40 (Promega) were cotransfected into LC319 cells with pcDNA3.1-E2F1 or mock vector using LipofectAMINE Plus (Invitrogen). Firefly luciferase activity values were normalized by comparing Firefly luciferase activity with Renilla luciferase activity, expressed from phRL-SV40 to allow variation in transfection efficiency.

**Recombinant CDCA8 proteins.** We prepared 13 plasmids expressing WT CDCA8 or various deletion mutant CDCA8 proteins (CDCA8Δ1–Δ12), each of which was mutated at serine/threonine to alanine and contained His-tagged epitopes at their NH2 termini using pET28 vector. The recombinant proteins were expressed in *E. coli*, BL21 codon-plus strain, and purified using TALON resin according to the supplier's protocol.

**In vitro kinase assay.** Purified recombinant WT or mutated CDCA8 proteins were incubated with recombinant AURKB (rhAURKB; Upstate) and [γ-32P]ATP in kinase buffer [20 mmol/L Tris (pH 7.5), 10 mmol/L MgCl2, 2 mmol/L MnCl2, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L DTT] supplemented with a mixture of protease inhibitors, 10 mmol/L NaF, 5 mmol/L microcystin LR, and 50 μmol/L ATP. The reaction was terminated by the addition of a 0.2 volume of 5× protein sample buffer and the proteins were analyzed by SDS-PAGE.

---

**Figure 1.** Activation of CDCA8 and AURKB in lung tumor samples. **A,** expression of CDCA8 and AURKB in clinical samples of 14 NSCLC (T) and corresponding normal lung tissues (N), examined by semiquantitative RT-PCR. We prepared appropriate dilutions of each single-stranded cDNA prepared from mRNAs of clinical lung cancer samples, taking the level of 18S (ACTB) expression as a quantitative control. **B,** expression of CDCA8 and AURKB proteins in 11 lung cancer cell lines, examined by Western blot analysis. **C,** subcellular localization of endogenous CDCA8 (top, red) and endogenous AURKB (bottom, red) in LC319 cells, detected by rabbit polyclonal antibodies to CDCA8 or AURKB. Both were triple-stained with α-tubulin (TUBA; green) and 4,6-diamidino-2-phenylindole (see merged images).
Inhibition of AURKB activity. To inhibit the AURKB activity in mammalian cells, we constructed siRNA oligonucleotides against AURKB (si-AURKB-#1 and si-AURKB-#2), as well as control siRNA oligonucleotides for EGFP. Cells seeded onto 10-cm dishes were incubated in mixtures of both LipofectAMINE and control (5'-GAAGCAGCACGACUCUUUC-3') or si-AURKBs (si-AURKB-#1, 5'-CCAAACUGUCAGGCAUAUA-3'; si-AURKB-#2, 5'-ACGCGGCACUUCACAAUUG-3') in a final concentration of 100 nmol/L. At 4 h after transfection, the siRNA-LipofectAMINE mixtures were replaced with fresh medium. The cells were collected and analyzed after additional 72-h culture.

Synthesized cell-permeable peptide. Nineteen- or 20-amino acid peptide sequences corresponding to a part of CDCA8 protein that contained possible phosphorylation sites by AURKB were covalently linked at its NH2 terminus to a membrane transducing 11 poly-arginine sequence(11R;refs.21,38). Three cell-permeable peptides were synthesized: 11R-CDCA8147–165, RRRRRRRRRRR-GGG-PSKKRTIQQGKKGKRS; 11R-CDCA8209–228, RRRRRRRRRRR-GGG-ERIYNISGNSPLADEIF; and 11R-CDCA8261–280, RRRRRRRRRRR-GGG-NIKKLSNRSKTHK. Scramble peptides derived from the most effective 11R-CDCA8261–280 peptides were synthesized as a control: Scramble, RRRRRRRRRRR-GGG-TSAQIRHKCSSQIKLSNKRNRL. Peptides were purified by preparative reverse-phase high-pressure liquid chromatography.

LC319 and SBC-5 cells, as well as human lung-derived MRC-5, CCD19-Lu, and BEAS-2B cells, were incubated with the 11R peptides at the concentration of 2.5, 5, and 7.5 \( \mu \text{mol/L} \) for 7 days. The medium was exchanged at every 48 h at the appropriate concentrations of each peptide and the viability of cells was evaluated by MTT assay at 7 days after the treatment. To confirm the transduction efficiency of the peptides, we synthesized the 11R-CDCA8261–280 and its control peptides labeled with FITC at NH2 terminus. Transduction of the peptides (2.5–7.5 \( \mu \text{mol/L} \)) was monitored by fluorescence microscopic observation after a 3-h incubation of the peptides with the cell lines at 37°C. The 11R-CDCA8261–280 as well as its control peptides was transduced into almost all of cultured cells as reported elsewhere (38).

Results

Coactivation of CDCA8 and AURKB in lung cancers. Using a cDNA microarray representing 27,648 genes of 101 lung cancer tissues, we identified CDCA8 to be overexpressed in a large proportion of lung cancers. Subsequently, we confirmed its transactivation in 11 of 14 additional NSCLC cases (four of seven adenocarcinomas; all of seven SCCs) by semiquantitative RT-PCR experiments (Fig. 1A, top). We further confirmed high levels of endogenous CDCA8 expression in all of 11 lung cancer cell lines by Western blot analysis using a rabbit polyclonal anti-CDCA8 antibody (Fig. 1B, top). Northern blot analysis using CDCA8 cDNA as a probe identified a 2.5-kb transcript, exclusively in the testis among 23 human tissues examined (Supplementary Fig. S1A).

To determine the subcellular localization of endogenous CDCA8 in lung cancer cells, we did immunocytochemical analysis for LC319 cells using anti-CDCA8 antibody; CDCA8 proteins were mainly detected at nucleus in cells at the G1-S phase and at nucleus and contractile ring in cells at the G2-M phase (Fig. 1C, top). CDCA8 was isolated previously as a new member of a vertebrate chromosomal passenger complex (25), and phosphorylation of COOH-terminal region of glutathione S-transferase-tagged CDCA8 by rhAURKB was shown by in vitro kinase assay (26). However, the precise phosphorylation site(s) and its functional significance in cancer cells remain unclear. To elucidate the biological role of

![Figure 2](https://www.aacrjournals.org)
CDCA8 activation in lung cancer cells, we first examined the expression status of AURKB using our gene expression database (8–12) and found that AURKB was frequently overexpressed in lung cancers compared with normal lung cells. Furthermore, our data indicated that the levels of AURKB expression seemed to be correlated with those of CDCA8. We subsequently reexamined primary lung cancer tissues and found increase of AURKB expression in 12 of 16 NSCLC clinical samples (five of seven adenocarcinomas and all of seven SCCs) by semiquantitative RT-PCR experiments (Fig. 1A, middle). The expression patterns of AURKB in lung cancers were very similar to those of CDCA8. We further confirmed by Western blot analysis that CDCA8 and AURKB proteins were coactivated in almost all of the lung cancer cell lines examined (Fig. 1B, middle). Immunocytochemical analysis using a rabbit polyclonal anti-AURKB antibody detected AURKB proteins to be located mainly at nucleus in cells at the G1-S phase and at nucleus and contractile ring in cells at the G2-M phase; the subcellular localization of AURKB in lung cancer cells was very similar to that of CDCA8 as reported elsewhere (Fig. 1C, bottom; ref. 25).

Association of CDCA8 and AURKB positivity with poor prognosis of NSCLC patients. Using tissue microarrays prepared from 273 surgically resected NSCLCs, we did immunohistochemical analysis with affinity-purified anti-CDCA8 and anti-AURKB polyclonal antibodies. We classified patterns of CDCA8/AURKB expression as absent (scored as 0), weak (scored as 1+), or strong (scored as 2+). Of the 273 NSCLC cases examined, 113 (41%) cases were positive (scored as 1+ to 2+) for both CDCA8 and AURKB, and 33 were negative for the both proteins. Cases (30 of the 273) were positive for only CDCA8 and 27 were positive for only AURKB. The expression pattern of CDCA8 protein was significantly concordant with AURKB protein expression in these tumors (P < 0.0001, χ² test) as similar to the results by RT-PCR and Western blotting. We found that strong expression (scored as 2+) of CDCA8 in NSCLCs was significantly associated with tumor size (P(T1 versus P(T2–4; P = 0.0414, χ² test)), lymph node metastasis (P(N0 versus P(N1–2; P = 0.0005, χ² test)), and with shorter tumor-specific 5-year survival times (P = 0.0009, log-rank test; Fig. 2B, left). Strong expression (scored as 2+) of AURKB in NSCLCs was significantly associated with tumor size (P(T1 versus P(T2–4; P = 0.0361, χ² test)), lymph node metastasis (P(N0 versus P(N1–2; P = 0.0004, χ² test)), and 5-year survival (P = 0.0001, log-rank test; Fig. 2B, right). NSCLC patients without either CDCA8 or AURKB expression in their tumors could reveal the longest survival period, whereas those with strong positive staining for both markers showed the shortest tumor-specific survival (P < 0.0001, log-rank test; Supplementary Fig. S1B). Using univariate analysis, we found that lymph node metastasis (P(N0 versus P(N1 and N2 P < 0.0001, score test)), tumor size (P(T1 versus P(T2, T3, and T4; P < 0.0001, score test)), and high CDCA8/AURKB expression (P = 0.0009 and = 0.0001, respectively, score test) were important correlative features for poor prognoses of patients with NSCLC.

Growth inhibition of lung cancer cells by specific siRNA against CDCA8. To assess whether CDCA8 is essential for growth or survival of lung cancer cells, we constructed plasmids to express siRNA against CDCA8 (si-CDCA8-#1 and si-CDCA8-#2), using siRNAs for EGFP and Luciferase as controls. Transfection of si-CDCA8-#1 or si-CDCA8-#2 into LC319 or SBC-5 cells significantly suppressed expression of endogenous CDCA8 proteins in comparison with the two controls and resulted in significant decreases in cell viability and colony numbers measured by MTT and colony formation assays (representative data of LC319 were shown in Fig. 3).

Simultaneous activation of CDCA8 and AURKB regulated by E2F1. The concordant activation of CDCA8 and AURKB in lung cancers suggested that these two genes might be regulated by the same transcription factor(s). To validate this hypothesis, we examined the DNA sequences of the CDCA8 and AURKB promoter regions and found the cell cycle–dependent element (CDE) and cell cycle gene homology region (CHR) consensus sequences (CDE-CHR), by which transcription of AURKB as well as cyclin A
(CCNDA) and CDC25 is regulated (Supplementary Fig. S2A). Among the transcription factors that could bind to the CDE-CHR of the AURKB gene, we confirmed that E2F1 was coactivated with CDCA8 and AURKB as detected by semiquantitative RT-PCR analysis of NSCLC cases (Supplementary Fig. S2B). To investigate the direct transcriptional regulation of the CDCA8 gene promoter by E2F1, we transiently cotransfected LC319 cells with E2F1 or mock vector, along with CDCA8 or AURKB (positive control) promoter constructs containing putative regulatory elements (CDE-CHR) fused to a luciferase reporter gene. Expectedly, both CDCA8 and AURKB promoter functions were activated by E2F1 (Supplementary Fig. S2C). The induction of endogenous CDCA8 and AURKB was further confirmed by introduction of exogenous E2F1 into LC319 cells (data not shown).

Phosphorylation of CDCA8 by AURKB in lung cancer cells. Western blot analysis detected two different sizes of CDCA8 protein (Fig. 1B, top). To examine a possibility, the CDCA8 phosphorylation, we incubated extracts from LC319 cells in the presence or absence of protein phosphatase (Bio-Rad Laboratories) and analyzed the molecular weight of CDCA8 protein by Western blot analysis. Because the measured weight of the majority of CDCA8 protein in the extracts treated with phosphatase was smaller than that in the untreated cells (Fig. 4A), we considered that CDCA8 was phosphorylated in lung cancer cells. We then examined whether AURKB could phosphorylate CDCA8 in vitro as reported previously (26). When full-length recombinant CDCA8 (rhCDCA8) protein was incubated with rhAURKB protein in kinase buffer, including [γ-32P]ATP, CDCA8 was phosphorylated in an AURKB dose-dependent manner (Fig. 4B). To assess whether the abundant expression of endogenous AURKB is critical for phosphorylation of endogenous CDCA8 in cancer cells, we selectively knocked down with siRNA against AURKB (si-AURKB) the AURKB expression in LC319 cells, in which these two genes were expressed abundantly. Reduction of AURKB protein by si-AURKBs (si-AURKB-#1 and si-AURKB-#2) dramatically decreased the amount of CDCA8 protein, whereas a level of CDCA8 transcripts in the same cells was not influenced by si-AURKBs (Fig. 4C). Hence, we hypothesized that endogenous CDCA8 protein may be stabilized when it is phosphorylated by endogenous AURKB and/or incorporated in some protein complex that are possibly regulated by the AURKB signaling.

Because our data imply that human CDCA8 and AURKB were coactivated in lung cancer cells and that CDCA8 phosphorylation by AURKB might play a significant role in pulmonary carcinogenesis, we then investigated the phosphorylation sites of CDCA8 by AURKB. We prepared six His-tagged CDCA8 proteins (CDCA8 knock-in mutant CDCA8 constructs (CDCA8–Δ12), in which either of the six serine/threonines was substituted to an alanine (Fig. 5A, bottom). In vitro kinase assay of these six mutants revealed the reduction of phosphorylation levels in four mutated constructs, including a substitution at either of four serine/threonine residues, Ser154, Ser219, Ser275, and Thr278 (CDCA8–Δ8, CDCA8–Δ9, CDCA8–Δ11, and CDCA8–Δ12; Fig. 5B, bottom). We subsequently made a mutant construct (CDCA8–Δ13), in which all of these four serine/threonines were substituted to an alanine, and did in vitro kinase assay using AURKB. The result showing complete disappearance of CDCA8 phosphorylation by AURKB (Fig. 5C) clearly showed that CDCA8 was phosphorylated by AURKB.
at four serine/threonine residues at Ser154, Ser219, Ser275, and Thr278 by AURKB.

**Growth inhibition of lung cancer cells by cell-permeable peptides.** To investigate the functional significance of interaction between CDCA8 and AURKB as well as CDCA8 phosphorylation for growth or survival of lung cancer cells, we developed bioactive cell-permeable peptides that were expected to inhibit the in vivo phosphorylation of CDCA8 by AURKB. We synthesized three different peptides of 19- or 20-amino acid sequence that included the four CDCA8 phosphorylation sites (Ser154, Ser219, Ser275, and Thr278). These peptides were covalently linked at its NH2 terminus to a membrane transducing 11 arginine-residues (11R). We first investigated by in vitro kinase assay the effect of the three 11R-CDCA8 peptides on the phosphorylation level of rhCDCA8 by rhAURKB (each of substituted residue was indicated as bold character on underline), whereas CDCA8D1, CDCA8D3, and CDCA8D4 represented the same levels of phosphorylation compared to WT CDCA8. 

**Figure 5.** Identification of the cognate phosphorylation sites on CDCA8 by AURKB. A, top, six full-length rhCDCA8 mutants that were substituted at putative serine/threonine phosphorylated sites to alanines; each construct contained two or three substitutions (CDCA8D1–D6); bottom, additional six full-length rhCDCA8 mutants that were substituted at either of six serine/threonine residues to an alanine residue (CDCA8D7–D12). B, top, in vitro kinase assays incubating WT and mutant CDCA8 proteins with rhAURKB. CDCA8D2, CDCA8D5, and CDCA8D6 constructs resulted in a reduction of phosphorylation levels by AURKB (each of substituted residue was indicated as bold character on underline), whereas CDCA8D1, CDCA8D3, and CDCA8D4 represented the same levels of phosphorylation compared with WT CDCA8. Bottom, CDCA8D8, CDCA8D9, CDCA8D11, and CDCA8D12 resulted in a reduction of phosphorylation, whereas CDCA8D7 and CDCA8D10 showed the same levels of phosphorylation compared with WT, indicating that CDCA8 was phosphorylated at Ser154, Ser219, Ser275, and Thr278 (indicated as bold character with underline) by AURKB. C, in vitro kinase assays incubating WT and mutant CDCA8 protein (CDCA8D13), in which all of the four serine/threonines were substituted to an alanine, with rhAURKB. CDCA8D13 construct resulted in complete diminishment of CDCA8 phosphorylation by AURKB.

www.aacrjournals.org 4119 Cancer Res 2007; 67: (9). May 1, 2007
Figure 6. Inhibition of growth of lung cancer cells by cell-permeable CDCA8 peptides. A, reduction of the AURKB-dependent CDCA8 phosphorylation by cell-permeable CDCA8 peptides (11R-CDCA8261–280), detected by in vitro kinase assay. B, top, the expression levels of endogenous CDCA8 protein, detected by Western blot analysis of LC319 cells transfected with the 11R-CDCA8261–280; bottom, the expression levels of endogenous CDCA8 transcript, detected by semiquantitative RT-PCR analysis of LC319 cells transfected with the 11R-CDCA8261–280. C, top, MTT assay of LC319 cells, detecting a growth-suppressive effect of 11R-CDCA8261–280 peptides or Scramble peptides. D, left, expression of CDCA8 protein in normal human lung fibroblast-derived MRC-5 and CCD19-Lu cells compared with lung cancer cell line LC319, examined by Western blot analysis; right, MTT assay, detecting no significant off-target effect of the 11R-CDCA8261–280 peptides on MRC-5 cells that scarcely expressed CDCA8 and AURKB protein.
Addition of the 11R-CDCA8261–280 into the culture medium of LC319 cells inhibited the phosphorylation and decreased stability of endogenous CDCA8 protein, whereas no effect on a level of CDCA8 transcript was observed (Fig. 6B). The 11R-CDCA8261–280 treatment of LC319 cells resulted in significant decreases in cell viability as measured by MTT assay (representative data in Fig. 6C, top). To clarify the mechanism of tumor suppression by the 11R-CDCA8261–280 peptide, we did flow cytometric analysis of the tumor cells treated with these peptides and found the significant increase in sub-G, fraction at 48 h after the treatment of 11R-CDCA8261–280 (Fig. 6C, bottom). 11R-CDCA8261–280 revealed no significant effect on cell viability of human lung fibroblast-derived MRC-5 or CCD19-Lu or human bronchial epithelia-derived BEAS-2B cells, in which CDCA8 and AURKB expression were hardly detectable (representative data of MRC-5 and BEAS-2B cells were shown in Fig. 6D; Supplementary Fig. S3). The data indicate that 11R-CDCA8261–280 Could specifically inhibit an enzymatic reaction of AURKB for CDCA8 phosphorylation and have no or minimum toxic effect on normal human cells that do not abundantly express these proteins.

Discussion

Toward the goal of developing novel therapeutic anticancer drugs with a minimum risk of adverse reactions, we established a powerful screening system to identify proteins and their interacting proteins that were activated specifically in lung cancer cells. The strategy was as follows: (a) identification of up-regulated genes in 101 lung cancer samples through the genome-wide cDNA microarray system coupled with laser microdissection (8–12); (b) verification of very low or absent expression of such genes in normal organs by cDNA microarray analysis and multiple-tissue Northern blot analysis (39, 40); (c) confirmation of the clinicopathologic significance of their overexpression using tissue microarray consisting of hundreds of NSCLC tissue samples (12, 14–24); and (d) verification of the targeted genes whether they are essential for the survival or growth of lung cancer cells by siRNA (13, 15, 16, 18–24). By this systematic approach, we identified that CDCA8 and AURKB are cooverexpressed in the great majority of clinical lung cancer samples as well as lung cancer cell lines and that these two proteins are indispensable for growth and progression of lung cancer cells.

CDCA8 was shown to be phosphorylated in vitro by AURKB previously (26), but its significance in development and/or progression of human cancer has been never described. CDCA8 was indicated recently to be one of new components of the vertebrate chromosomal passengers, such as AURKB, INCENP, and BIRC5 (25), which are considered to be key regulators of mitotic events responsible for correcting the error of bipolar attachments that inevitably occur during the ‘search-and-capture’ mechanism (41–43). In this study, we have shown that the CDCA8 protein is likely to be stabilized by its AURKB-dependent phosphorylation at Ser446, Ser446, Ser275, and/or Thr278. Depletion of AURKB function by RNAi or the 11R-CDCA8261–280 that could inhibit phosphorylation of CDCA8 significantly decreased the level of endogenous CDCA8 protein. Phosphorylation is an important posttranslational modification that regulates the protein stability, function, localization, and binding specificity to target proteins. For example, MKP-7 phosphorylated at Ser246 or p27 phosphorylated at Ser10 has a longer half-life than unphosphorylated form; when at the sites were dephosphorylated, the amount of these proteins was promptly decreased in cells (44, 45). The evidence suggests that the stability of CDCA8 protein could be tightly regulated by the AURKB signaling in cancel cells. AURKB was shown to be overexpressed in many tumor cell lines, and its overexpression was noted to be involved in chromosome number instability and tumor invasiveness (30, 31). AURKB is one of the cancer-related kinases and therefore was thought to be a promising target for anticancer drug development. Indeed, two AURKB inhibitors have been described recently: ZM447439 and Hesperadin (46, 47). Our study has indicated that CDCA8 is a putative oncogene that is aberrantly expressed in lung cancer cells along with AURKB. We found by tissue microarray analysis that CDCA8 and AURKB were cooverexpressed and that patients with NSCLC showing higher expression of these proteins represented a shorter tumor-specific survival period, doubtlessly suggesting that CDCA8, along with AURKB, plays a crucial role for progression of lung cancers. Furthermore, we showed at the first time that growth of lung cancer cells overexpressing CDCA8 and AURKB could be suppressed effectively by blocking the AURKB-dependent CDCA8 phosphorylation by means of the 20-amino acid cell-permeable peptide that corresponds to a part of CDCA8 protein and included two phosphorylation sites, Ser275 and Thr278, by AURKB. We detected a significant increase in the sub-G, fraction after the treatment of 11R-CDCA8261–280 peptide, suggesting that the cell-permeable polypeptides induced apoptosis of the cancer cells. Because the phosphorylation of CDCA8 at these sites was likely to be indispensable for the growth/survival of lung cancer cells and CDCA8 could belong to cancer-testis antigens, selective targeting of CDCA8-AURKB enzymatic activity could be a promising therapeutic strategy that is expected to have a powerful biological activity against cancer with a minimal risk of adverse events. Further analyses of the mechanism of growth suppression by specific inhibiting of CDCA8 phosphorylation by AURKB may be of the great benefit toward the development of new types of anticancer agents.

In summary, we have found that CDCA8 is coactivated with and phosphorylated/stabilized by AURKB in lung cancer cells and that phosphorylated CDCA8 plays a significant role in growth and/or survival of cancer cells. The data strongly imply the possibility of designing new anticancer drugs targeting the CDCA8-AURKB association as a promising therapeutic strategy for lung cancer.

Acknowledgments

Received 12/21/2006; revised 2/13/2007; accepted 2/20/2007.

Grant support: The Japan Society for the Promotion of Science “Research for the Future” Program grant 00L01402 (Y. Nakamura).

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.

References

4. Kelly K, Crowley J, Bunn PA, Jr., et al. Randomized phase III trial of paclitaxel plus carboplatin versus...
Phosphorylation and Activation of Cell Division Cycle Associated 8 by Aurora Kinase B Plays a Significant Role in Human Lung Carcinogenesis

Satoshi Hayama, Yataro Daigo, Takumi Yamabuki, et al.


Updated version Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/67/9/4113

Supplementary Material Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2007/05/01/67.9.4113.DC1

Cited articles This article cites 47 articles, 22 of which you can access for free at: http://cancerres.aacrjournals.org/content/67/9/4113.full.html#ref-list-1

Citing articles This article has been cited by 20 HighWire-hosted articles. Access the articles at: /content/67/9/4113.full.html#related-urls

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.