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Inhibitory Effect of CT120B, an Alternative Splice Variant of CT120A, on Lung Cancer Cell Growth

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Abstract The expression product of *ct120a*, a novel gene isolated from human chromosome 17p13.3 in our laboratory, was predicted to have seven transmembrane domains and could cause malignant transformation of mouse NIH3T3 cells. There existed an mRNA splicing variant of *ct120a*, namely *ct120b*, which had a 96-nucleotide deletion and produced an in-frame loss of 32 amino acids from codon 136 to codon 167 of CT120A. The CT120B protein was predicted to have six transmembrane domains. In this study, we observed that the green fluorescent protein-tagged CT120B was localized on plasma membrane and in cytoplasm in SPC-A-1 cells. The expression of CT120B/A in normal lung tissue and in lung cancer cells was also examined. Results showed that the stable CT120B overexpression in SPC-A-1 cells resulted in a reduction of cell growth rate, and inhibited tumorigenecity and anchorage-independent growth in nude mice. The functions of CT120A and CT120B for cell growth appeared antagonistic. We suggested that the delayed G_1/S phase transition might contribute to the inhibitory activities of CT120B on cell growth and that the deleted 32 amino acids missing in CT120B might be essential for the oncogenetic activities of CT120A.

Key words CT120B; alternative splicing; cell growth; lung cancer

The novel human plasma membrane-associated gene *ct120a* (GenBank accession No. AF477201), was previously isolated from chromosome 17p13.3 by positional cloning and rapid amplification of cDNA ends (RACE) in our laboratory [1]. The full-length cDNA sequence of *ct120a* had five exons and encoded a protein with 257 amino acids. A BLAST search in the human genome database of the National Center for Biotechnology Information (NCBI) revealed that there existed an mRNA splicing variant, *ct120b* (GenBank accession No. BC026023), which was isolated from large cell lung carcinoma tissue [2]. *ct120b*, with the absence of the fourth exon compared with *ct120a*, had a 96-nucleotide deletion and produced an in-frame loss of 32 amino acids from codon 136 to codon 167 of CT120A.

Transcript of *ct120a* was undetectable in normal lung tissue, but could be detected in the SPC-A-1 (human lung adenocarcinoma) cells; thus it was suggested that *ct120a* might be involved in lung cancer development [1,3]. The ectopic expression of CT120A caused the malignant transformation of NIH3T3 cells. The overexpression of CT120A by cDNA transfection in the A549 (human lung adenocarcinoma) cells could promote tumor growth in a xenograft model [3]. However, little is known about the function of CT120B and the effects of CT120B expression on lung cancers.

In this report, we have established a stable CT120B-overexpressed SPC-A-1 cell line. It was observed that CT120B suppressed cell proliferation, clonal expansion and tumorigenecity, which indicated that the overexpression of CT120B could inhibit the growth of lung cancer cells. The delayed G_1/S phase transition might contribute to the growth inhibitory activities of CT120B.

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Materials and Methods

Western blotting

Cells and tissue samples were lysed in T-PER tissue protein extraction reagent (Pierce, Rockford, USA) containing proteinase inhibitor cocktail (Roche, Basel, Switzerland). The extracted protein (10 μg from cell culture and 30 μg from tissue sample) was analyzed by 15% SDS-PAGE and then transferred onto a nitrocellulose membrane (Schleicher & Schuell BioScience, Keene, USA). Membranes were incubated with chicken anti-CT120A/B antibody, which was prepared by immunization of chickens with synthesized C-terminal 15-mer oligopeptide (CRKAVRLFDTPQAKK) of CT120A from ^{241}A to ^{255}A, and anti-HA antibody (Santa Cruz Biotechnology, Santa Cruz, USA), or followed by incubation with correspoding second antibody HRP-conjugated (Santa Cruz Biotechnology). Detection was performed using an enhanced chemiluminescent (ECL) kit (Pierce).

cDNA clone of *ct120b*

The *ct120b* open reading frame (ORF) fragment was amplified by reverse transcription-polymerase chain reaction (RT-PCR) from the cDNA library of human lung adenocarcinoma cell line A549 (Cell Bank of the Chinese Academy of Sciences, Shanghai, China), with primers ORF5 (5′-ATGCTGCTGACGCTGGCCGG-3′) and ORF3 (5′-TTAGCCATCCTTTTTGGCTT-3′). Amplification was carried out at 94 ºC for 30 s, 60 ºC for 30 s, and 72 ºC for 50 s, for 35 cycles. The products were examined by automated sequencing. A pcDNA3.1-HA vector with a hemagglutin (HA) tag in the N-terminal of the expression product was used for transfection.

Cell culture and stable transfection

SPC-A-1 cells (Cell Bank of the Chinese Academy of Sciences) and A549 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, USA) supplemented with 10% newborn bovine serum (Invitrogen), penicillin and streptomycin in humidified 5% CO_2 at 37 ºC. The SPC-A-1 cells were transfected by the pcDNA3.1-HA/ct120b or the null vector plasmid using Lipofectamine reagent (Invitrogen) according to the manufacturer's protocol. Stable transfectants were selected for neomycin resistance in the medium containing 1.0 mg/ml G418 and later maintained in the medium containing 0.4 mg/ml G418.

Subcellular localization

The *ct120b* gene was subcloned into pEGFP-N1 vector (Clontech, San Jose, USA) and the recombinant pEGFP/ct120b was transiently transfected into the SPC-A-1 cells in 24-well tissue culture plates with Lipofectamine as well as control pEGFP-N1 vector. After 48 h, cells were trypsinized and transferred to glass slides, then grown on the slides for approximately 12 h. These transfectants were fixed with 3.7% formaldehyde and permeabilized in 0.2% Triton X-100 (FisherBiotech, New Jersey, USA). After blocking with 3% (*W*/*V*) bovine serum albumin for 30 min, the cells were incubated with anti-GFP antibody (1:25; Santa Cruz Biotechnology) at 4 ºC overnight. Subsequently the cells were incubated with the FITC-coupled anti-mice IgG (1:25; Santa Cruz Biotechnology) for 30 min at room temperature. Immunofluorescent cells were observed and scanned with the LSM510 Axiovert 200M confocal microscopy system (Carl Zeiss, Jena, Germany) assembled on an inverted microscope

In vitro cell proliferation assay

Cell proliferation was assessed by colorimetric measurement of a BrdU-TdR incorporation kit (Roche) [4] with a little modification. The stable cell transfectants (B11, B14 or SPC-HA cells) were cultured in a 96-well plate (5×10^3 cells/well) and incubated with BrdU for 3 h. Anti-BrdU-POD antibody bound to the BrdU was incorporated in newly synthesized DNA. The immune complexes were detected by the subsequent substrate reactions and quantitated by measuring the absorbance at 450 nm on a Bio-Rad Model 550 microplate reader (BD Bioscience, San Jose, USA).

Soft agarose colony formation assay

Soft agarose assay was essentially performed according to previous methods [5,6]. The B11, B14 or SPC-HA cells (1×10^3 cells/well) were suspended in complete medium containing 0.3% agarose (Gibco BRL, Grand Island, USA). Triplicate of cells were seeded in complete medium containing 0.6% agarose in a 6-well plate. After 14 d, the cells were stained overnight with the vital dye piodonitrotetrazolium violet (Sigma, St. Louis, USA) and colonies containing more than 50 cells were counted.

Tumorigenecity in xenograft models

The B11, B14 or SPC-HA cells were injected subcutaneously into 6-week-old male BALB/c nude mice (3×10^6 cells/mouse). The developed tumors were dissected and

weighed 24 d after injection.

Cell cycle assay

The B11, B14 or SPC-HA cells were synchronized to G_2/M phase with nocodazole treatment according to previous methods [7]. Cells were incubated with 0.2 μg/ml nocodazole (Sigma) for 20 h to induce G_2/M arrest. After being washed with phosphate-buffered saline (PBS), cells were cultured in nocodazole-free growth medium. At indicated time points, cells were harvested, fixed with 70% cold ethanol and stained with propidium iodide to analyze cell cycle distribution with a FACSCalibur flow cytometer (BD Bioscience).

Results

ct120b expression in normal lung tissues and the SPC-A-1 cells

The expression of CT120B in normal lung tissue and in the SPC-A-1 cells was studied by Western blotting. The A549 cell lysate was loaded as positive control. As shown in **Fig. 1**, the expression level of CT120B was higher than that of CT120A in normal lung tissue samples, whereas it was much lower than that of CT120A in SPC-A-1 and A549 cells.

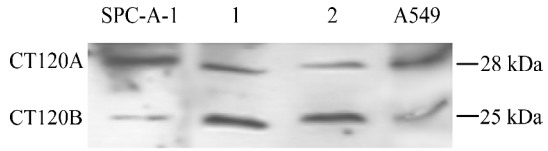

Fig. 1 Expression of CT120B in normal lung tissue and SPC-A-1 cells detected by Western blotting

1 and 2, two samples of normal human lung tissue (30 μg protein per lane). The A549 cell lysate was used as positive control. The chicken anti-CT120A/B antibody was used for Western blotting analysis.

cDNA cloning of *ct120b*

The *ct120b* ORF fragment was cloned by RT-PCR from the A549 cells. Although the 96th nucleotide altered from C to T, the corresponding amino acid remained unchanged and was identified as CT120B by automated sequencing. The full-length cDNA sequence of CT120B encoded a protein with 225 amino acids. Functional predictions based on the amino acid sequence of CT120B with TMHMM programs (http://www.cbs.dtu.dk/services/TMHMM/) revealed that CT120B had six transmembrane domains with intracellular N-terminus and C-terminus, two intracellular loops and three extracellular connecting loops.

Localization of CT120B to both cytoplasm and plasma membrane

We subcloned the *ct120b* gene into the pEGFP-N1 vector to construct the recombinant pEGFP/ct120b, which was transiently transfected into SPC-A-1 cells. After 48 h, immunofluorescent staining was performed to increase the sensitivity of GFP detection with the monoclonal anti-GFP antibody and the FITC-coupled anti-mouse IgG. Observed with confocal microscopy, the CT120B/EGFP fusion protein exhibited a staining pattern of plasma membrane and cytoplasm [**Fig. 2(A)**], but the EGFP protein presented in both the cytoplasm and the nucleus of the SPC-A-1 cells in the control group [**Fig. 2(B)**].

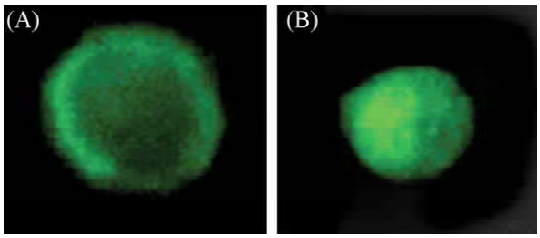

Fig. 2 Subcellular localization of CT120B in SPC-A-1 cells

Confocal images of SPC-A-1 cells transiently transfected with the CT120B/EGFP plasmid and the EGFP-N1 vector. (A) The CT120B/EGFP fusion protein exhibited a plasma membrane and cytoplasm staining pattern. (B) The vector EGFP protein in the SPC-A-1 cells presented in both the cytoplasm and the nucleus. Magnification, 1000×.

Inhibition of CT120B overexpression in SPC-A-1 cells

To explore the function of CT120B relating to cell growth, we constructed the CT120B expression plasmid with an HA tag to transfect the SPC-A-1 cells. According to the results of Western blotting with anti-HA antibody, the G418 resistant clones B11 and B14 were chosen for further studies, which expressed a relatively high level of CT120B [**Fig. 3(A)**].

BrdU incorporation and soft agarose colony formation assay were performed to determine the effects of CT120B on cell growth *in vitro*. The growth rate of the CT120B-overexpressed cells, B11 and B14, was decreased 32% and 26% (P<0.05 vs. that of control SPC-HA cells) respectively [**Fig. 3(B)**]. The abilities for anchorage-

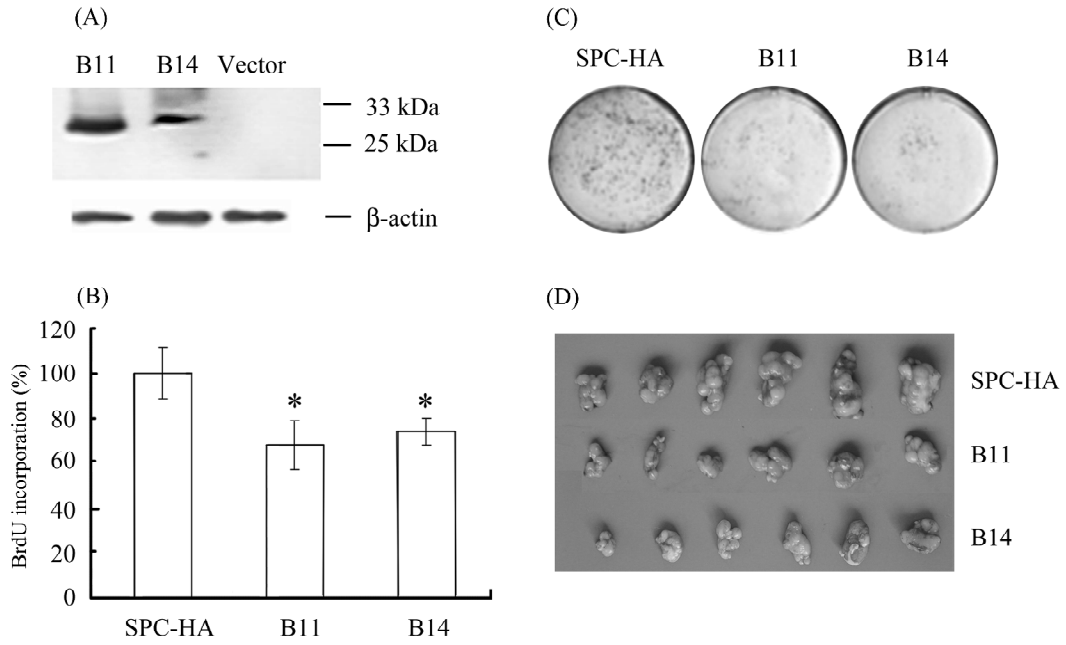

Fig. 3 Inhibitory effect of the overexpressed CT120B on SPC-A-1 cell growth

(A) The overexpression of HA-tagged CT120B in several transfected cells was examined by Western blotting with anti-HA antibody. The molecular weight of the fusion protein of CT120B and HA tag was approximately 28 kDa. (B) Cell proliferation was determined by BrdU incorporation assay. The growth rate of the B11 and B14 cells was lower than that of the SPC-HA cells (*P<0.05 vs. SPC-HA group). (C) Soft agarose assay was performed to determine the capability of anchorage-independent colony formation. After 14 d of incubation, the cells were stained overnight with the vital dye p-iodonitrotetrazolium violet and the number of colonies containing more than 50 cells was counted. (D) The size of tumors derived from the SPC-HA, B11 and B14 cells in tumorigenicity assay.

independent growth of the B11 and B14 cells were also significantly decreased. The clone number of the B11 and B14 cells reduced to only 35% and 50% of that of the SPC-HA cells, respectively (P<0.01) [**Fig. 3(C)**].

To further explore the effects of CT120B on tumorigenicity *in vivo*, the B11, B14 and SPC-HA cells were injected subcutaneously into nude mice. After 24 d, a decrease of tumor weight was observed in groups of the B11 and B14 cells and the average tumor weight (n=6) of the two groups (0.320±0.146 g, 0.507±0.143 g) decreased to 65% and 44% compared with that of the vector transfectant group (0.902±0.365 g) (P<0.05) [**Fig. 3(D)**].

Delayed G_1/S phase transition and the growth inhibition induced by CT120B

Fluorescence-activated cell sorting (FACS) analysis was applied to study the cell cycle profile. The percentage of the B11 and B14 cells in G_1 phase was a little higher than that of the SPC-HA cells (64% and 63% vs. 57% respectively) under conditions without treatment. We then synchronized the cells to the G_2/M phase by exposure to nocodazole for 20 h. Approximately 60% of the cells were arrested in the G_2/M phase after nocodazole treatment. When cells were cultured in fresh complete medium and released from the G_2/M block, most of the arrested cells gradually re-entered the cell cycle. Twenty-four hours after nocodazole removal, the B11 and B14 cells showed dramatically delayed G_1/S phase transition (59% and 50% in G_1 phase, 36% and 28% in S phase, respectively). However, at this time the majority of the SPC-HA cells had entered into the S phase (29% in G_1 phase and 57% in S phase) (**Fig. 4**). Our data implied that the delayed G_1/S phase transition might contribute to the growth inhibitory activities of CT120B.

Discussion

Our previous data indicated that CT120A could transform NIH3T3 cells and increase the tumorigenecity of lung cancer cells [3]. In this study, the overexpression of CT120B was implicated to inhibit lung cancer cell growth, and the effects of CT120A and CT120B appeared antagonistic. The CT120B protein was short of 32 amino acids of CT120A from codon 136 to codon 167. Based on these results, we estimated that the deleted 32 amino

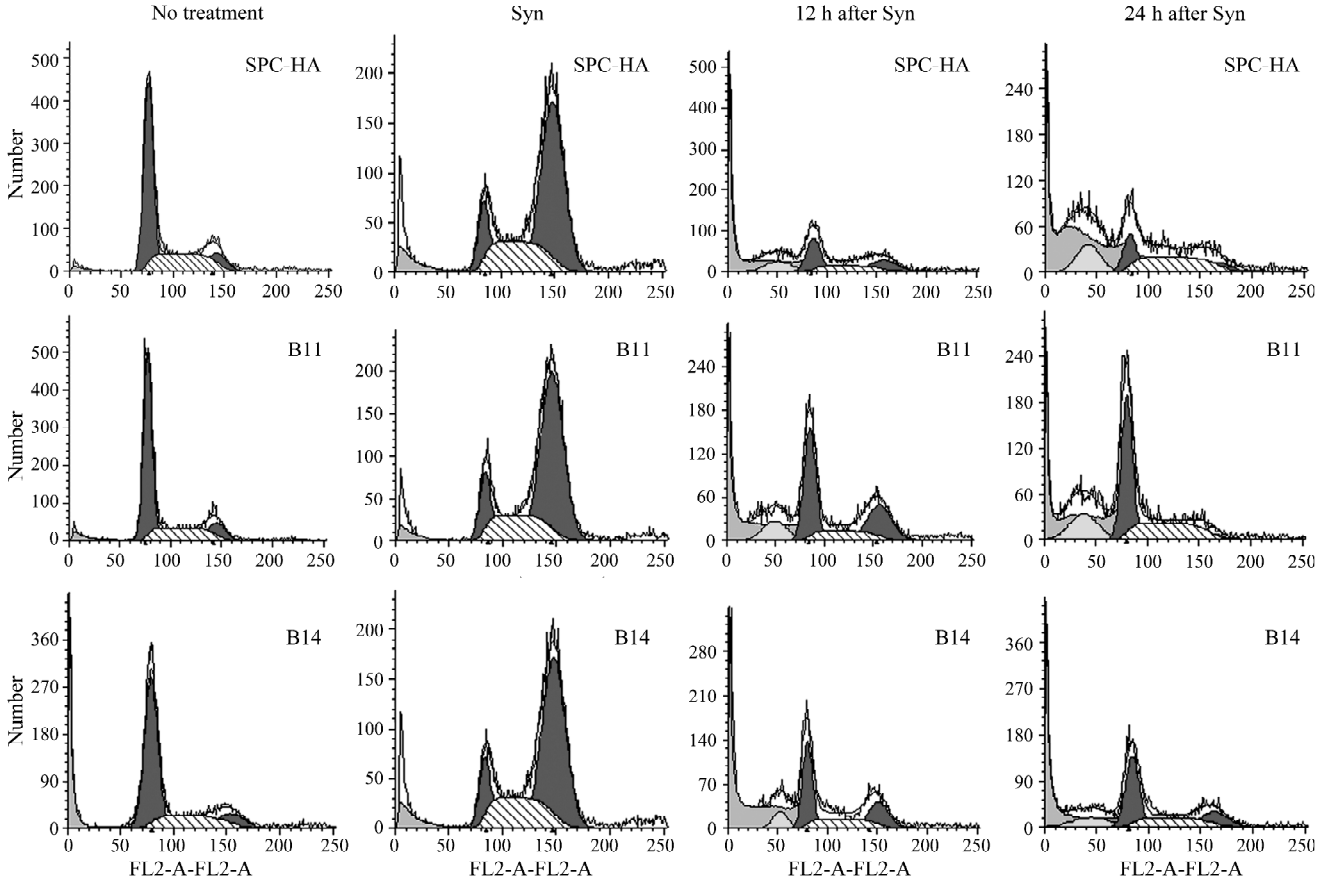

Fig. 4 Delayed G_1/S phase transition in CT120B transfected SPC-A-1 cells

The B11, B14 and SPC-HA cells were synchronized to G_2/M phase by exposure to 0.2 μg/ml nocodazole for 20 h. Washed with PBS, the cells were incubated with fresh medium to analyze the cell cycle profiles. Twenty-four hours after synchronization, the B11 and B14 cells showed dramatically delayed G_1/S phase transition compared with the SPC-HA cells. Syn, synchronization.

acids might be essential for the oncogenetic activities of CT120A and that the proportion of CT120A/CT120B might determine the potential of cell proliferation.

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