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 G1/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

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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 241A to 255A, and anti-HA antibody (Santa Cruz Biotechnology, Santa Cruz, USA), or followed by incubation with corresponding 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'-ATGCTGACTGCTGGCCGG-3') and ORF3 (5'-TAAAGCCATCCCCATTTGCTT-3'). Amplification was carried out at 94 ºC for 30 s, 60 ºC for 30 s, and 72 ºC for 30 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% CO₂ 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 Axiover 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 (5×10³ 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 (1×10³ 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.

Tumorigenicity in xenograft models

The B11, B14 or SPC-HA cells were injected subcutaneously into 6-week-old male BALB/c nude mice (3×10⁶ 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 G2/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 G2/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-
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 tumorigenicity 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 acids...
acids might be essential for the oncogenetic activities of CT120A and that the proportion of CT120A/CT120B might determine the potential of cell proliferation.

References


