Interaction of ribosomal protein L22 with casein kinase 2α: A novel mechanism for understanding the biology of non-small cell lung cancer

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Abstract. Dysfunction of ribosomal proteins (RPs) may play an important role in molecular tumorigenesis, such as lung cancer, acting in extraribosomal functions. Many protein-protein interaction studies and genetic screens have confirmed the extraribosomal capacity of RPs. As reported, ribosomal protein L22 (RPL22) dysfunction could increase cancer risk. In the present study, we examined RPL22-protein complexes in lung cancer cells. Tandem affinity purification (TAP) was used to screen the RPL22-protein complexes, and GST pull-down experiments and confocal microscopy were used to assess the protein-protein interaction. The experiment of kinase assay was used to study the function of the RPL22-protein complexes. The results showed that several differentially expressed proteins were isolated and identified by LC-MS/MS, which revealed that one of the protein complexes included casein kinase 2α (CK2α). RPL22 and CK2α interact *in vitro*. RPL22 also inhibited CK2α substrate phosphorylation *in vitro*. This is the first report of the RPL22-CK2α relationship in lung cancer. Dysregulated CK2 may impact cell proliferation and apoptosis, key features of cancer cell biology. Our results indicate that RPL22 may be a candidate anticancer agent due to its CK2α-binding and -inhibitory functions in human lung cancer.

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Introduction

Lung cancer is the deadliest cancer in the world. Its aggressive biology, resistance to conventional and targeted therapeutic agents, and the absence of effective early detection methods lead to a 5-year survival rate of only 14% (1-3). According to the pathological and histological features, non-small cell lung cancer (NSCLC) is the most prevalent type of lung cancer (4). Despite modest improvements in diagnosis and multimodality therapeutic methods, patients with lung cancer still face a poor prognosis. It is critical to identify molecular events that could lead to advances in lung cancer therapeutics.

Lung tumorigenesis is associated with gene alterations, such as gene dysregulation, mutations or loss of heterozygosity (5,6). Ribosomal proteins (RPs), a major component of ribosomes, are abundant RNA-binding proteins found in every cell (7). Perturbations in ribosomal biogenesis and translation associated with specific genetic defects or syndromes may play a critical role in molecular tumorigenesis (7). An excess of free RPs can lead to cell-cycle arrest and apoptosis and regulate extraribosomal functions, such as ribosomal protein S3 (RPS3) in lymphocytic cells (8), RPL15 in esophageal cancer (9), RPL19 in breast tumors (10), RPL7A in osteosarcoma (11) and RPL41 in breast cancer (12). Another study showed that RPL22 inactivation enhances the transformation potential of lymphoblastic leukemia by inducing the stemness factor, Lin28B (13) suggesting a mechanistic basis by which RPL22 dysfunction could increase cancer risk (14). Thus, RPs may be particularly important regulators of cancer cell function.

In our previous study, we found that RPL22 transcript and protein expression was significantly downregulated in NSCLC and that RPL22 may be involved in carcinogenesis (15); however, RPL22 has not been implicated in any specific lung cancer mechanism.

As the elementary constituents of protein complexes and regulatory pathways, protein-protein interactions are key determinants of function (16,17). Many free RPs including RPL5, L11, L23 and S7 interface with the p53-MdM2 system, leading to cell-cycle arrest or apoptosis and regulation of

extraribosomal functions (18-21). Thus, the characterization of protein-protein interactions is critical to understand protein function and cell biology. Tandem affinity purification (TAP)-tagging methods combined with LC-MS/MS can be used to study protein interactions in eukaryotic cells (16,17,22). In the present study, we characterized RPL22-protein complexes in NSCLC cells. By using a combination of TAP methods and GST pull-down experiments, we demonstrated that RPL22 and casein kinase 2α (CK2α) interact in lung cancer cells *in vitro* and *in vivo*. We also confirmed that RPL22 directly inhibits CK2α substrate phosphorylation through this interaction. To the best of our knowledge, this is the first report of this relationship in lung cancer.

Materials and methods

Cell cultures. Cell lines from HBE, the NSCLC line LTEP-a-2 and control cell line 293T (preserved in our department) were routinely grown in RPMI-1640 medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a moist tissue culture incubator at 37˚C in a 5% CO_2 atmosphere. Cells were seeded ($1x10^8$) and cultured in serum-free medium for 24 h and then in complete medium for 48 h prior to RNA and protein extraction.

Plasmid construction. cDNAs containing full-length wild-type RPL22 and CK2α were obtained by RT-PCR from human bronchial epithelial (HBE) cells. The fidelity of the amplified sequences was confirmed by DNA sequencing. pCeMM-NTAP(GS) was kindly provided by the Austrian Academy of Sciences Center for Molecular Medicine (CeMM). pGEX-6P1 was purchased from GE Healthcare and pcDNA3.1(+) was purchased from Invitrogen. *Not*I and *Xho*I were used for molecular cloning of the *RPL22* sequence into pCeMM-NTAP(GS); *Bam*HI and *Xho*I were used to clone *RPL22* into pGEX-6P1 and pcDNA3.1(+); *Bam*HI and *Eco*RI were used to clone CK2α into pcDNA3.1(+).

Tandem affinity purification. pCeMM-NTAP(GS)-RPL22 was transfected into LTEP-a-2 cells, and GFP-positive cells were selected twice by flow cytometry. RPL22-protein complexes were isolated by the TAP procedure with non-transduced LTEP-a-2 cells as a negative control as previously described (17). Briefly, $4x10^8$ GFP-positive cells were harvested, and total protein lysates were extracted. Lysates (5 ml) were incubated with 200 µl IgG-Sepharose beads (Sigma) for 2 h at 4˚C and then washed 4 times with 10 ml lysate buffer. The beads were incubated in 500 µl TEV cleavage buffer with 100 U TEV protease (Promega) for 1 h at 16˚C. The supernatant was collected by centrifugation at 500 x g; 200 µl streptavidin agarose beads was added and incubated for 4 h at 4˚C. The beads were washed 5 times with 10 ml TEV cleavage buffer. Proteins were heated and detached from the beads with 300 µl 1X Laemmli sample buffer containing 1% β-mercaptoethanol for 5 min at 95˚C. Eluted proteins were separated by 4-15% (Bio-Rad Laboratories) SDS-PAGE and visualized by silver staining. Differentially expressed protein bands were excised for *in situ* tryptic digestion, MALDI-TOF and LC-MS/MS. Mass spectra were analyzed with Flex Analysis software (version 2.4; Bruker Daltonik GmbH, Bremen, Germany) and searched against the NCBInr database by an in-house Mascot (version 2.1; Matrix Science) search engine.

GST pull-down assay. A 50-µl sample of 50% glutathione Sepharose 4B beads (GE Healthcare) was equilibrated in 1X PBS buffer (pH 7.4). The slurry was mixed with 15 µl of 0.4 mg/ml GST-fusion protein (RPL22/CK2α, expressed by pGEX-6P1-RPL22/pGEX-6P1-CK2α) and incubated for 30 min at 4˚C in a rotating incubator to immobilize the fusion protein on the glutathione Sepharose beads. The beads were washed 4 times with 10 ml phosphate-buffered saline (PBS) and then incubated with 1 ml of 2 mg/ml LTEP-a-2 cell lysates for 2 h at 4˚C. The beads were washed 5 times with 10 ml PBS. Laemmli sample buffer (200 µl of 1X) containing 1% β-mercaptoethanol was added and heated at 95˚C for 5 min. Proteins (30 µl) were resolved on precast 4-15% (Bio-Rad Laboratories) SDS-PAGE and visualized by Coomassie blue staining or immunoblotted with the specific antibody.

Subcellular localization. 293T cells were cultured in Millicell® EZ Slide 4-well (Millipore). The full-length cDNAs of RPL22 and CK2α were cloned into the expression vector pcDNA3.1(+). These constructs were transfected into the 293T cells using FuGENE® HD transfection reagent (Roche) according to the manufacturer's instructions. RPL22 proteins were immunostained with polyclonal anti-RPL22 antibody (Abcam) and Alexa Fluor® 488 goat anti-rabbit IgG (H+L) (Invitrogen); CK2α proteins were immunostained with monoclonal anti-CK2α antibody (Sigma) and tetramethyl rhodamine goat anti-mouse IgG (H+L) (Invitrogen). The nucleus was stained by DAPI (Sigma). Fluorescence was visualized on an LSM 710 confocal microscope (Zeiss).

CK2α kinase assay. To evaluate the interaction between RPL22 and CK2α, we analyzed CK2α kinase activity. According to the manufacturer's instructions (Promega), samples (10 µg) were adjusted to a 5-µl final volume in CK2α kinase buffer [100 mM Tris (pH 8.0), 100 mM NaCl, 50 mM KCl, 20 mM $MgCl_2$ and 100 µM Na_3VO_4]. Following addition of 15 µl incubation buffer, including CK2α kinase buffer, 50 µCi [γ-^{32}P]GTP, and 200 ng sample of each reaction group (blank group using CK2α kinase buffer), reactions were incubated at 30˚C for 30 min. RPL22-GST fusion proteins were prepared as previously described. The CK2α-specific peptide substrate RRRADDSDDDDD (1 mM; Promega) was added to the kinase reaction according to the manufacturer's instructions. The reactions included a blank (CK2α kinase buffer), peptide substrate, GST + peptide substrate, GST-RPL22 or GST-RPL22 + peptide substrate. The reactions were stopped by adding 25 µl 100 mM ATP in 4 N HCl. Samples were spotted onto a P81 Whatman filter (Whatman, Inc., Clifton, NJ, USA), washed 4 times in 150 mM H_3PO_4, and incorporated radioactivity was measured by scintillation counting.

Statistical methods. Experiments were performed in triplicate and each experiment was performed 3 times. Data are expressed as mean ± SEM. Statistical analyses were performed by unpaired non-parametric Mann-Whitney test. Statistical significance was indicated at $P<0.05$ (SPSS, Inc., Chicago, IL, USA).

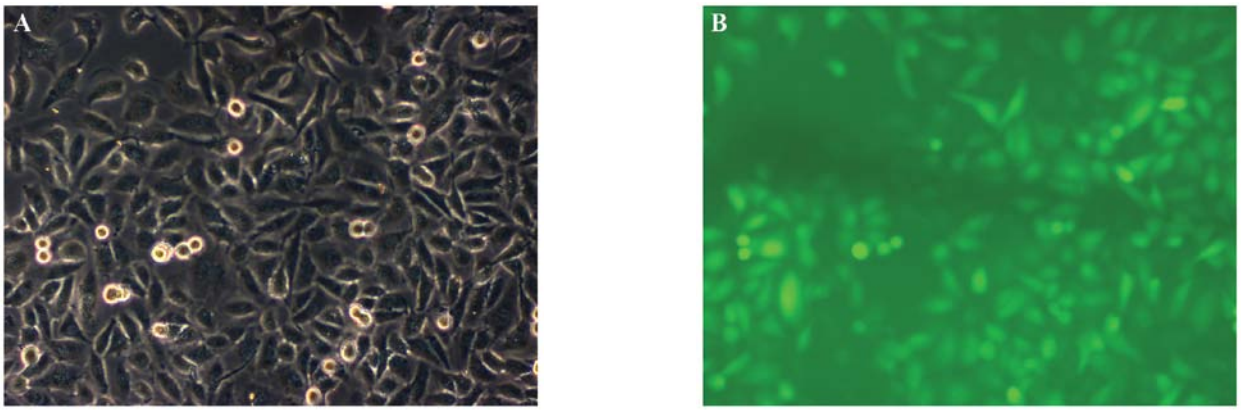

Figure 1. pCeMM-NTAP(GS)-RPL22-positive LTEP-a-2 cells screened by cell flow cytometry. We fused the RPL22 gene in the plasmid pCeMM-NTAP(GS) with tandem affinity peptides (GS), and transfected it into LTEP-a-2 cells. Cells were selected 2 times by cell flow cytometry according to the reporting gene GFP expression. In addition, the positive cells were observed by immunofluorescence microscopy. (A) pCeMM-NTAP(GS)-RPL22-positive LTEP-a-2 cells under white light; (B) under fluorescence (original magnification, x20).

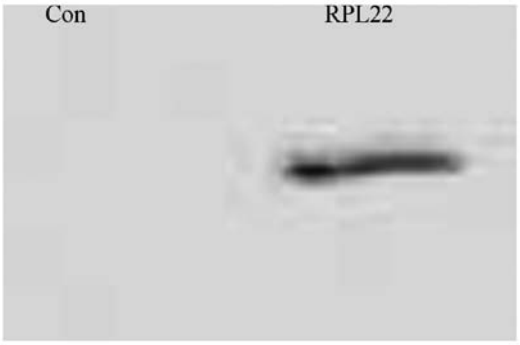

Figure 2. Detection of protein RPL22. Proteins detached from TAP-streptavidin agarose beads were separated by SDS-PAGE and immunoblotted with the antibody against RPL22.

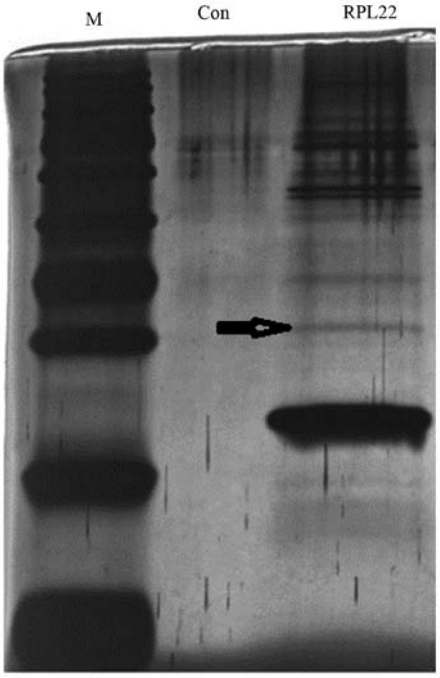

Figure 3. TAP of the RPL22 complex. The GS-RPL22 protein complex was purified from $4x10^8$ GFP-positive LTEP-a-2 cells. Purified proteins were visualized through silver staining method and subsequently identified by LC-MS/MS. The final data set contained six proteins that were interacted with RPL22 compared with the control. The CK2α-RPL22 complex is indicated by the arrow.

Results

Tandem affinity purification of the RPL22-protein complexes. To study RPL22 protein function in lung cancer, we fused the RPL22 gene into pCeMM-NTAP(GS) with tandem affinity peptides (GS) and overexpressed this 'bait' protein in LTEP-a-2 cells. GFP-positive cells were identified by flow cytometry (Fig. 1). We purified RPL22-binding proteins through 2 consecutive affinity steps from $4x10^8$ GFP-positive cells, and lysates containing putative protein complexes were purified over successive affinity columns as previously described (17). Proteins detached from TAP-streptavidin agarose beads were western blotted for RPL22 (Fig. 2). Proteins from the final elution were separated by SDS-PAGE and visualized by silver staining (Fig. 3). Several differentially expressed proteins were isolated and digested *in situ* with trypsin prior to identification by LC-MS/MS, which revealed that one of the protein complexes included casein kinase 2α (CK2α) (arrow in Fig. 3).

GST pull-down assay. GST pull-down experiments were used to verify the interaction between the GST-RPL22 fusion protein (bait) and the CK2α target, and between the GST-CK2α fusion protein (bait) and the RPL22 target. GST-RPL22/GST-CK2α glutathione agarose beads were incubated with LTEP-a-2 cell lysates and then washed. Eluted proteins bound to GST-RPL22/GST-CK2α were identified by western blotting using the antibody against RPL22/CK2α. As shown in Fig. 4, CK2α was detected in the eluent containing the GST-RPL22 fusion protein as bait, and RPL22 was detected in the eluent containing the GST-CK2α fusion protein as bait, thus confirming the interaction between RPL22 and CK2α in lung cancer cells *in vitro*.

Subcellular localization. We used confocal microscopy to characterize the subcellular localization of RPL22 and CK2α. Recombinant plasmids were transfected into 293T cells and

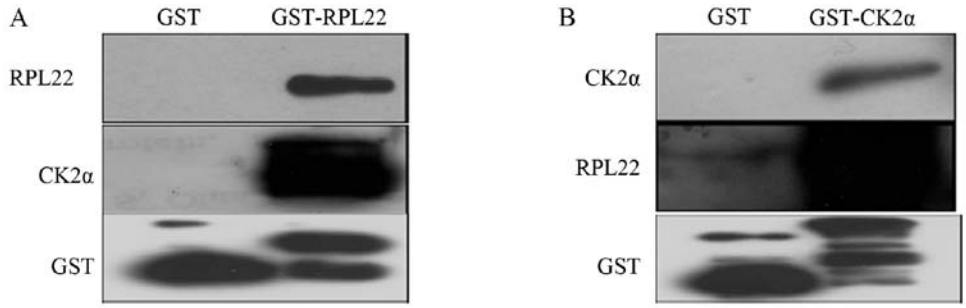

Figure 4. GST pull-down assay to confirm the interaction between RPL22 and CK2α. (A) GST-RPL22 as the 'bait'; (B) GST-CK2α as the 'bait'.

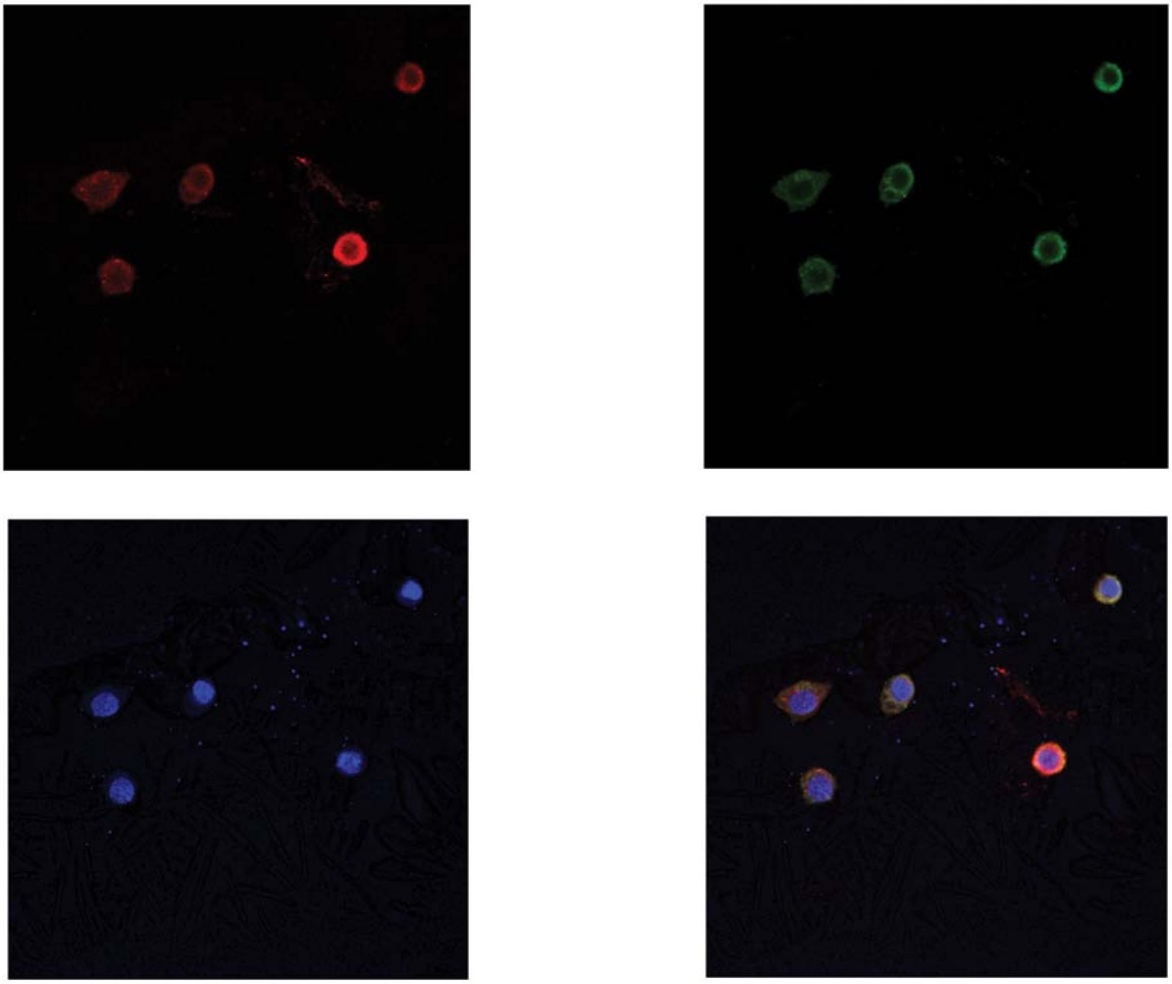

Figure 5. Co-localization of RPL22 with CK2α in 293T cells. Nuclei were indicated using DAPI. Anti-RPL22 fluoresces green and anti-CK2α, red, with the overlay demonstrating co-localization.

proteins were detected with fluorescent antibodies (Fig. 5). RPL22 expression is showed in green, CK2α in red and DAPI-stained nuclei in blue. The results showed that RPL22- and CK2α-specific antibodies produced overlapping signals in the cytoplasm, demonstrating co-localization of the two proteins in cells.

CK2α kinase assay. Protein kinases are central components of signal transduction cascades. CK2α is an important protein kinase in cell proliferation. To explore the potential role of RPL22 and CK2α interaction in lung cancer, we developed a CK2α kinase assay. We measured CK2α activity using blank (CK2α kinase buffer), peptide, GST + peptide, and GST-RPL22 or GST-RPL22 + peptide as kinase reaction substrates (Fig. 6A). Compared to the peptide substrate group, significant downregulation of CK2α activity was observed in the GST-RPL22 + peptide substrate group. Thus, GST-RPL22 inhibited CK2α activity *in vitro* ($P<0.05$). Notably, GST-RPL22 was not phosphorylated by CK2α in comparison to the blank ($P<0.05$; Fig. 6A). GST-RPL22 caused a dose-dependent decrease in CK2α activity with the CK2α-specific peptide substrate RRRADDSDDDDD (Fig. 6B).

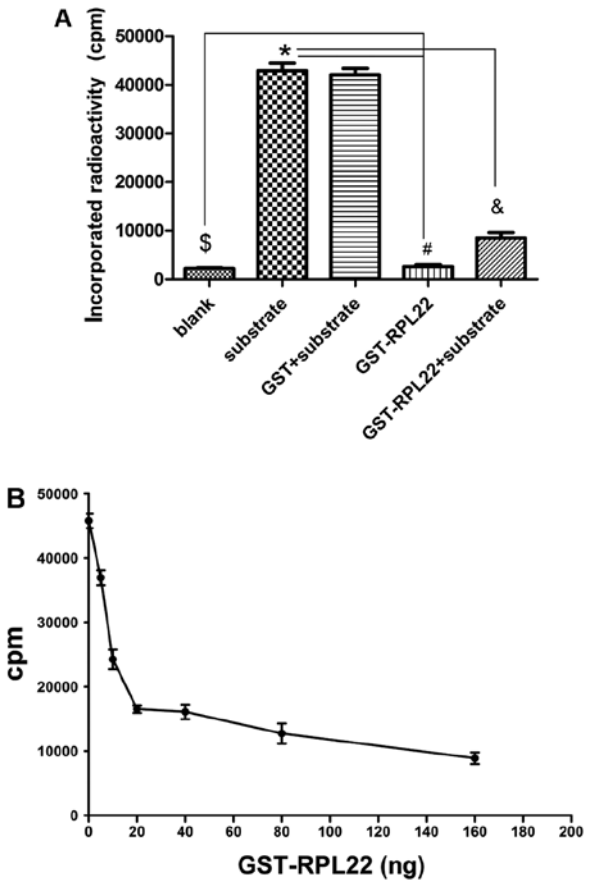

Figure 6. CK2α kinase assay. RPL22 inhibits CK2α activity *in vitro*. (A) CK2α kinase assay used either 200 ng sample of each reaction group (blank group using CK2α kinase buffer) as substrate and 50 μCi [γ-^{32}P] GTP as phosphate donor, and reactions were incubated at 30˚C for 30 min. Where indicated, 1 mM of the CK2α-specific peptide substrate RRRADDSDDDDD was added to the kinase reaction. The reactions included a blank (CK2α kinase buffer), peptide substrate, GST + peptide substrate, GST-RPL22 or GST-RPL22 + peptide substrate. GST-RPL22 inhibited CK2α activity *in vitro* ($^{\&}$P<0.05 vs. *substrate) and GST-RPL22 was not phosphorylated by CK2α ($^{\#}$P<0.05 vs. $^{\$}$blank). (B) Concentration curve of RPL22 inhibition using CK2α-specific peptide substrate. Where indicated, 0, 5, 10, 20, 40, 80 and 160 ng GST-RPL22 and 1 mM of the CK2α-specific peptide substrate RRRADDSDDDDD were added to the kinase reaction. The results showed that GST-RPL22 inhibits CK2α activity *in vitro* in a dose-dependent fashion.

Discussion

In the present study, we confirmed RPL22 and CK2α interactions in lung cancer cells *in vitro* by tandem affinity purification, GST pull-down experiments and confocal microscopy. RPL22 inhibited CK2α substrate phosphorylation through this direct interaction.

RPL22, a small protein consisting of 128 amino acids, is a component of the 60S large ribosomal subunit and co-localizes with ribosomal RNA in the nucleolus and cytoplasm (23,24). Although it is incorporated into the ribosome subunit, RPL22 is not required for protein synthesis (23,25). RPL22 inactivation enhances the transformation potential of lymphoblastic leukemias (13). RPL22 associates with viral RNAs and proteins; it was identified as an EBER-associated protein that protects against tumorigenic transformation of EBV-infected cells (26). Another report showed that RPL22 may act as a repressor of transcription (27). In *Drosophila*, RPL22 interacts with histone H1 and co-localizes on condensed chromatin; upregulation of RPL22 expression inhibits transcription, while depletion of RPL22 reverses this effect (28). RPL22 is also associated with human telomerase (29) and regulates the progress of cell apoptosis in part by selectively regulating p53 expression (25). Our previous study confirmed that RPL22 expression is downregulated in NSCLC cells (15). These data provide insight into the mechanistic basis by which RPL22 may regulate tumorigenesis, particularly in lung cancer.

Virtually, all cellular functions require protein-protein interactions (22,30,31). RPs modulate the trans-activation function of important regulatory proteins (32); they specifically bind to the central regions of the MDM2 oncoprotein and inhibit MDM2 E3 ligase activity towards p53 to regulate cell cycle progression (32,33). The involvement of RPs in tumors represents a new oncogenic pathway associated with their extraribosomal functions through protein-protein interactions with important signal molecules (34).

In the present study, we found that RPL22 binds CK2α in lung cancer cells. Protein kinase CK2 is a highly conserved tetrameric serine/threonine protein kinase (35). It regulates several important signaling pathways including the PI3K/Akt and WNT signaling cascades, NF-κB transcription and the DNA damage response (35,36). CK2α is one of the catalytic subunits of the protein kinase CK2 tetrameric complex. Accumulating evidence confirms that CK2α has a vast range of physiological targets and appears to be highly pleiotropic (37). It is involved in many key biological progresses, including growth and cell cycle control, signal transduction, circadian rhythms and gene expression (36). Upregulation of protein kinase CK2 expression is associated with increased cell growth and proliferation in normal and cancer cells (38). Dysregulated CK2 may impact cell proliferation and apoptosis, key features of cancer cell biology (39). Our results showed that RPL22 protein binds CK2α in lung cancer cells, inhibiting the CK2α phosphorylation reaction in a dose-dependent fashion, and RPL22 expression is downregulated in NSCLC. It remains unclear whether RPL22 could contribute to relatively upregulate the expression and the function of CK2 in lung cancer. The RPL22-CK2α pathway might be associated with lung tumorigenesis.

GST-RPL22 was not phosphorylated by CK2α in the present study, although RPL22 phosphorylation by CK2 has been reported in *Drosophila* (40). It is, therefore, possible that different modifications regulate the RPL22 function in different species. The regulatory motif of the *Drosophila* RPL22 ortholog has a large unique N-terminal extension not present in vertebrates (23,26). The C-terminal acidic region of human RPL22 also differs from *Drosophila* RPL22 and may be used to traffic the protein from the nucleoplasm to the nucleolus (24). The nuclear matrix is a key locus for CK2 signaling in cell proliferation and cell death. RPL22 may be a phosphoregulated substrate of CK2. Our data suggest that RPL22 directly binds and regulates CK2. The impact of CK2 on diverse molecular pathways may control cell proliferation and cell death in cancer (38). Downregulation of CK2 results in induction of apoptosis in cultured cells and xenograft cancer models, suggesting its potential as a therapeutic target (41). Protein-protein interactions generate specificity in signal transduction (42). RPL22 could be a useful anticancer

agent that functions by binding and inhibiting CK2 function in human lung cancer.

In summary, RPL22 and CK2α interact in lung cancer cells. RPL22 inhibits CK2α kinase activity. Given the function of these proteins, we expect the present study will shed light on their regulatory role in lung cancer. We are continuing to characterize the interaction between RPL22 and CK2 and their related signaling pathways in lung cancer.

Acknowledgements

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