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High-level Expression of Functional Tumor Suppressor LKB1 in *Escherichia coli*

Jun'e LIU[1,2], Tingmao HU[1], and Xin HOU[1]*

[1] *College of Life Sciences, Inner Mongolia University, Hohhot 010021, China;*
[2] *Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China*

Abstract The human LKB1 tumor suppressor has been implicated as an important regulator of many cellular processes and signaling pathways, indicating that it could be a good candidate for anticancer drugs. The failure of its obtain high-level expression has been a major obstacle to study its protein structure and function *in vitro*. Here, we describe the high-level expression of human LKB1 in *Escherichia coli* and show its kinase activity and anticancer effects on a tumor cell line. The gene encoding LKB1 was optimized by replacing rare codons with codons frequently used in *E. coli* and synthesized with overlapping primers. The recombinant His-LKB1 was expressed in hosts BL21(DE3) (BL) and Rosetta-gami(DE3)pLysS (RG). His-LKB1 from BL was present mainly as inclusion body. The soluble His-LKB1 from RG accounted for 34.1% of total proteins and the yield of purified His-LKB1 was approximately 92 μg/ml. Purified His-LKB1 protein from both hosts was functionally active, as shown by reversible autophosphorylation and kinase activity in the absence of any other associated kinase. The growth inhibitory ratio of the purified BL-derived and RG-derived His-LKB1 on hepatic carcinoma SMMC-7721 cells was 24.97% and 45.68%, respectively, and both could produce significant cell-cycle arrest.

Keywords LKB1; synthetic gene; tumor suppression; kinase activity

Human LKB1 (also called serine/threonine kinase 11, or STK11) was first cloned by two independent groups through linkage analysis of patients affected by Peutz-Jeghers syndrome [1,2]. Approximately 80% of families with Peutz-Jeghers syndrome and a variety of carcinomas harbor germline LKB1-inactivating mutations, defining LKB1 as a tumor suppressor protein [3–5]. Human LKB1 has been implicated as an important regulator of many cellular processes and signaling pathways, including the control of cell-cycle arrest [6], cell apoptosis [7,8], Wnt signaling [9,10], transforming growth factor-β signaling [11], Ras-induced cell transformation [12], AMPK pathway and energy metabolism [8,13], and cell polarity [14,15]. These findings suggested LKB1 as a new choice for anticancer therapy. However, the failure to obtain high-level expression of active LKB1 in any system has been a major obstacle to therapeutic research and determination of the protein's structure and function.

In this study, the gene encoding LKB1 was optimized by replacing rare codons with high-frequency codons preferred in *E. coli*, and the whole gene was synthesized with 38 overlapping primers by polymerase chain reaction (PCR). Recombinant His-LKB1 in the soluble form was expressed in *E. coli* hosts BL21(DE3) (BL) and Rosetta-gami(DE3)pLysS (RG). The soluble His-LKB1 from RG accounted for 34.1% of total proteins and the yield of purified His-LKB1 was approximately 92 μg/ml. The purified protein showed autophosphorylation and kinase activities in the absence of any other associated kinase. Tumor cell suppression by recombinant His-LKB1 was verified in hepatic carcinoma SMMC-7721 cells.

Materials and Methods

Cells and plasmids

E. coli strain DH5α was used for molecular cloning and plasmid propagation, and BL and RG (Novagen, San

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*Corresponding author: Tel, 86-471-4991483; Fax, 86-471-4992442; E-mail, houxinliu@yahoo.com.cn

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Diego, USA) were used for recombinant protein expression. Vector pMD18-T (TaKaRa, Dalian, China) was used for cloning the optimized gene and pET-44a(+) (Novagen) for expression of His-LKB1. Hepatic carcinoma SMMC-7721 cells and human embryonic kidney 293 cells from our laboratory were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Carlsbad, USA) supplemented with 10% fetal bovine serum (HyClone, Logan, USA) and antibiotics (100 mg/ml streptomycin and 100 U/ml penicillin) at 37 ºC with 5% CO_2.

Design and assembly of the optimized *LKB1* gene

A codon-optimized, double-stranded cDNA of human *LKB1* was designed with software Vector NTI (Invitrogen, Carlsbad, USA). The wild-type *LKB1* cDNA sequence (GenBank accession No. AF035625) was altered by replacing rare codons with high-frequency codons preferred in *E. coli*, and a His_6 tag was inserted after the ATG start codon to facilitate detection and purification. A total of 38 long-overlap oligonucleotides (45–51 bp) were synthesized by Sangon (Shanghai, China). The first and last primers contained an *Nde*I and an *Xho*I restriction site, respectively, to facilitate cloning.

The optimized gene was obtained by a two-step procedure. First, the 5′ part (685 bp) was synthesized by PCR using the first 20 oligonucleotides and the 3′ part (632 bp) was obtained using the other 18 oligonucleotides; there were 15 bp overlaps between these two fragments. Second, the above PCR products were used as templates and the first and last oligonucleotides were used as primers to carry out the PCR. The product was purified with a PCR purification kit (TaKaRa) and cloned directly into the pMD18-T vector by a T-A cloning strategy. Recombinant clones were screened and positive plasmids were identified by nucleotide sequencing analysis. The positive plasmid was named pMD-LKB1.

Construction of recombinant vector and expression of His-LKB1

The full-length *LKB1* cDNA was excised from pMD-LKB1 with *Nde*I and *Xho*I, and subcloned into a similarly digested pET-44a(+) vector. There was another His_6 tag after the *Xho*I site, which could be fused at the C-terminal of recombinant protein, further facilitating efficient purification. Recombinant plasmids were sequenced and the positive plasmid was named pET-LKB1.

pET-LKB1 was transformed into BL and RG competent cells. Single colonies were grown overnight at 37 ºC in Luria Broth medium supplemented with antibiotics, then diluted 100-fold in fresh medium and grown at 37 ºC to mid-log phase (A_{600}=0.5–0.7). Induction was initiated by the addition of 0.8 mM IPTG (final concentration) (Promega, Madison, USA) at 28 ºC. For BL and RG, cells were harvested by centrifugation after 4 h and 8 h, respectively. After resuspension in 0.05 culture volume of phosphate-buffered saline (PBS), cells were disrupted by three freeze-thaw cycles followed by sonication on ice (50–100 cycles of 10 s on/10 s off), and the soluble and insoluble fractions were separated by centrifugation. Proteins were prepared under denaturing [0.1% sodium dodecyl sulfate (SDS), 100 ºC, 5 min] and reducing (140 mM 2-mercaptoethanol) conditions, and separated by SDS-polyacrylamide gel electrophoresis (PAGE) using standard methods [16]. The expression level was calculated by UVIband software (UVItec, Cambridge, UK).

Purification of His-LKB1

In a typical preparation, cells were harvested from a 500 ml culture by centrifugation at 6000 *g*, and the pellets were washed with cold PBS and resuspended in 50 ml buffer A (50 mM NaH_2PO_4, 20 mM imidazole, 1 M NaCl, pH 7.4), and lysed by sonication. After centrifugation at 13,000 *g* for 30 min, the supernatant containing the soluble fraction was loaded onto a 5 ml Ni-NTA column (Pierce, Rockford, USA) that had been equilibrated with buffer A. The column was washed with 25 ml buffer A containing 40 mM imidazole and then with buffer A containing 60 mM imidazole. The proteins were eluted by 25 ml buffer A containing 200 mM imidazole, analyzed by SDS-PAGE, and fractions containing His-LKB1 were pooled. The purified protein was identified by anti-LKB1 antibody (Upstate Biotechnology, Lake Placid, USA) by Western blot analysis.

Production of polyclonal antibody and identification of its specificity by Western blot analysis

The purified His-LKB1 was used to produce anti-LKB1 ascitic polyclonal antibody in mice. The specificity of this polyclonal antibody was shown by Western blot analysis, using LKB1 protein (Upstate Biotechnology; produced in insect cells) and endogenetic LKB1 in 293 cells as antigens.

For Western blot analysis, equal amounts of total protein were separated by SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane (Amersham, Solna, Sweden), then blocked with 5% skimmed milk in PBS containing 0.05% Tween-20 for 1 h at room temperature. The PVDF membrane was incubated with anti-His monoclonal antibody (Novagen; 1:1500) or anti-LKB1 polyclonal antibody (1:1000; produced by our laboratory) for 4 h at room temperature and washed three times with PBS. Subsequently, the membrane was probed with horse-

radish peroxidase-labeled secondary antibody (1:5000) for 1 h at room temperature and then visualized using 3,3′-diaminobenzidine-tetrachloride as the substrate (Novagen).

Assay of His-LKB1 autophosphorylation activity

Purified His-LKB1 (1 μg) was incubated in 30 μl kinase buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM Na_2EDTA, 5 mM dithiothreitol, 2 mM $MnCl_2$) and 10 μCi of [γ-^{32}P] ATP (Furi, Fuzhou, China) at 30 ºC for 30 min. The reaction was terminated with SDS-PAGE loading buffer. Proteins were separated by SDS-PAGE, transferred onto a PVDF membrane, and detected by exposure to X-ray film for 8 h [17].

To identify the specificity of autophosphorylation, 1 μg purified His-LKB1 was incubated in 30 μl kinase buffer at 30 ºC for 30 min as described above. The protein was inactivated at 75 ºC for 15 min and 200 units of lambda protein phosphatases (Sigma, St. Louis, USA) was added. After incubation for 3 h at 30 ºC, the reaction was terminated and proteins were detected as described above. After exposure, the PVDF membrane was analyzed by Western blot with anti-LKB1 polyclonal antibody.

Assay of His-LKB1 kinase activity by LKBtide

Kinase activity of His-LKB1 towards LKBtide (Upstate Biotechnology) was measured according to the kinase assay protocol of the manufacturer. Briefly, after LKBtide and Triton X-100 were mixed with reaction buffer, purified His-LKB1 was diluted and added to the mixture, then 10 μl diluted [γ-^{32}P]ATP was added. After incubation for 10 min at 30 ºC with constant agitation, the reaction was stopped by adding 5 μl of 3% phosphoric acid. A 20 μl aliquot was spotted onto the center of a P81 paper square (Upstate Biotechnology), allowed to dry, then washed three times with 0.75% phosphoric acid and methanol. After transferring the paper square to a sealable plastic bag and adding 4 ml scintillation cocktail, radioactivity was measured in a scintillation counter (MicroBeta 1450; PerkinElmer, Waltham, USA). Active LKB1/STRADα/MO25α (Upstate Biotechnology) was used as the positive control and samples containing all assay components plus 2 μl of 30% phosphoric acid were used as negative controls.

Growth suppression assay of His-LKB1

The growth suppression of SMMC-7721 cells by His-LKB1 was tested by the MTT assay. Chariot (Active Motif, Carlsbad, USA) was used as a carrier for the delivery of biologically active proteins into mammalian cells [18,19]. Exponentially growing SMMC-7721 cells were digested and seeded in 96-well flat-bottom plates with a final volume of 200 μl containing 2×10^3 cells per well and incubated for 24 h at 37 ºC. Cells were divided into five groups: Chariot was added to the negative control, named the Chariot group; purified His-LKB1 from BL mixed with Chariot was added to four groups with the concentration of His-LKB1 varying from 1.0 to 10.0 μg/ml. After treatment for 24–96 h, 20 μl MTT (0.1 mg; Sigma) was added to each well and incubated for 4 h at 37 ºC. Medium was removed and 150 μl dimethylsulfoxide was added. After the plate was shaken thoroughly for 5 min, the absorbance at 570 nm was measured with a SPECTRAMax Plus spectrophotometer (Molecular Devices, La Jolla, USA). The assay was carried out in triplicate, and the growth inhibition ratio (IR%) was calculated as: IR%=(1−A_{570} of test group/A_{570} of blank control)×100%. The same assay was carried out with RG-derived His-LKB1.

Cell-cycle analysis

Flow cytometry analysis was carried out to evaluate whether the recombinant His-LKB1 would exert any effect on the cell-cycle distribution of SMMC-7721 cells. Briefly, SMMC-7721 cells were plated on 60 mm plates and incubated at 37 ºC till 40% confluent. Cells were divided into four groups: blank control, only PBS was added; Chariot control, only Chariot was added; BL and RG groups, recombinant BL-derived and RG-derived His-LKB1 mixed with Chariot were added to each plate, respectively (the final concentration was 10.0 μg/ml). After 72 h, cells were trypsinized, washed in PBS, fixed in 70% cold ethanol, and incubated for 2 h on ice. Cells were washed again, then stained for 30 min at room temperature with 10 μg/ml propidium iodide, 0.1% Trito X-100, and 0.1% RNase A in PBS in the dark. Stained cells were then analyzed using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, USA) The assay was carried out in triplicate.

Statistical analysis

Comparisons between means among the various treatment groups were analyzed by Student's *t*-test with $P<0.05$ set as statistically significant.

Results

Expression and purification of recombinant His-LKB1 from *E. coli*

The optimized full-length cDNA (GenBank accession

No. EF051031) corresponding to human LKB1 protein was obtained by two-step PCR and subcloned into the pET-44a(+) vector. The positive recombinant plasmid was named pET-LKB1. A 60-kDa recombinant protein was detected by SDS-PAGE and Western blot analysis after induction (**Fig. 1**). The expression level and solubility of His-LKB1 varied in the two hosts. In BL, the recombinant His-LKB1 accounted for 34.8% of total proteins and was mostly insoluble. The soluble His-LKB1 accounted for only 8.1% of total soluble proteins. In RG, however, the recombinant His-LKB1 accounted for 39.3% of total proteins and the soluble His-LKB1 accounted for 34.1% of total soluble proteins.

The high-level expression of human LKB1 made it easier to be purified by affinity chromatography. Two His_6 tags fused at the N- and C-terminus of LKB1 also contributed to the high efficiency of purification. The recombinant His-LKB1 proteins were purified under native conditions from soluble fractions of cell lysate and confirmed by SDS-PAGE and Western blot analysis (data not shown). There was no significant loss or detectable degradation of protein during the process of purification. The yield of the purified recombinant protein was approximately 92 μg/ml from RG and 20 μg/ml from BL.

Production of polyclonal antibody and identification of its specificity

Purified His-LKB1 from RG could be recognized specifically by anti-LKB1 antibody [**Fig. 2(A)**], so it was used as the antigen to produce ascitic polyclonal antibody in mice. The obtained ascitic could specifically recognize our purified His-LKB1, as well as the insect cell-produced LKB1 protein and endogenetic LKB1 protein in 293 cells in Western blot analysis [**Fig. 2(B)**].

Autophosphorylation activity of recombinant His-LKB1

The first-line assay to determine the LKB1 kinase activity is to identify its autophosphorylation activity. It was reported that the purified GST-LKB1, which was recovered from insect cells without chaperone proteins, was able to autophosphorylate in a specific and reversible way [17]. Here, we tested the autophosphorylation activity of His-LKB1 from *E. coli*. The autophosphorylation of His-LKB1 was shown by the incorporation of [γ-^{32}P]ATP (**Fig. 3**). When equal amounts of proteins were used in this assay, the signal of phosphorylated LKB1 from RG was approximately 10 times stronger than that from BL, indicating that the autophosphorylation activity of recombinant His-LKB1 from RG was higher than that from BL. This autophosphorylation could be reversed specifically by treatment with lambda phosphatase. The identity of LKB1 was confirmed by Western blot analysis (**Fig. 3**).

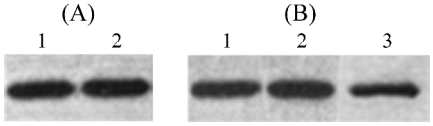

Fig. 2 Identification of purified His-LKB1 and the specificity of polyclonal antibody by Western blot analysis

(A) Detected with anti-LKB1 antibody. 1, purified His-LKB1; 2, LKB1. (B) Detected with ascitic polyclonal antibody. 1, purified His-LKB1; 2, LKB1; 3, total proteins of 293T cells.

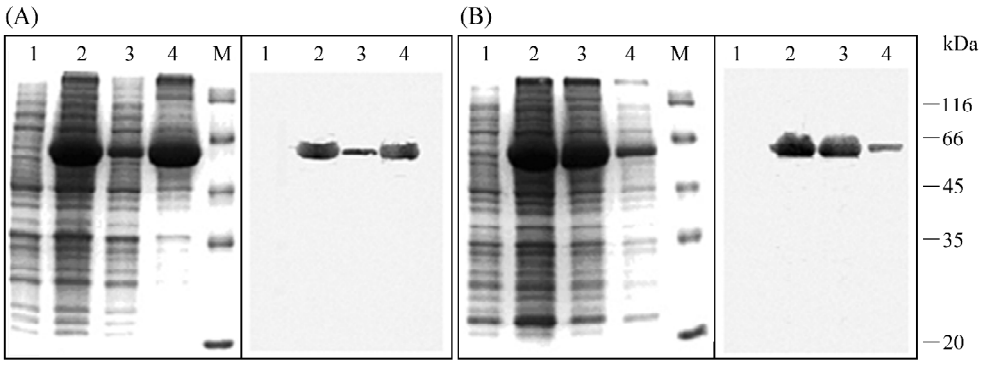

Fig. 1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis of soluble and insoluble fractions after expression of His-LKB1 in BL21(DE3) (A) and Rosetta-gami(DE3)pLysS (B) hosts

1, total fraction before induction; 2, total fraction after induction; 3, soluble fraction; 4, insoluble fraction; M, protein molecular mass marker. The numbers on the right-hand side of the panels indicate the molecular mass of markers (in kDa). The left-hand panels of each figure part are SDS-PAGE analyses; the right-hand panels are the corresponding Western blot analysis with anti-His monoclonal antibody.

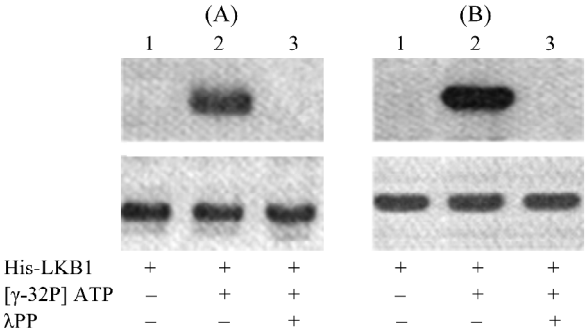

Fig. 3 Autophosphorylation activity of BL21(DE3)-derived His-LKB1 (A) and Rosetta-gami(DE3)pLysS-derived His-LKB1 (B)

The upper panels are the autokinase assays and the lower panels are the Western blot analyses of the autophosphorylated then dephosphorylated His-LKB1 with an LKB1-specific polyclonal antibody. 1, His-LKB1 alone (negative control); 2, His-LKB1 was incubated for 30 min with [γ-^{32}P]ATP, resolved by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane, then detected after exposure to X-ray films for 8 h; 3, His-LKB1 treated as in 2, then dephosphorylated with lambda protein phosphatase (λPP). +, component is included; –, component is excluded.

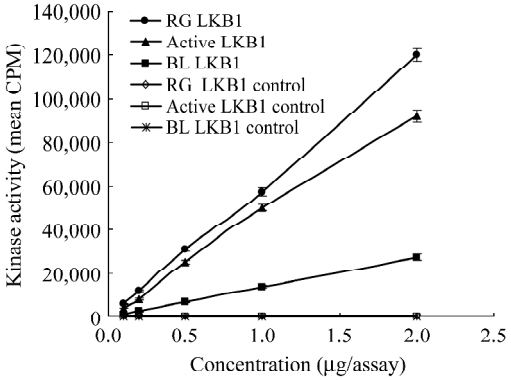

Fig. 4 Kinase activity of purified His-LKB1

Kinase activity of His-LKB1 towards LKBtide was measured according to manufacturer's protocol. Purified His-LKB1 was diluted and added to reaction buffer mixed with LKBtide (Upstate Biotechnology) and Triton X-100, then 10 μl diluted [γ-^{32}P]ATP was added. After incubation for 10 min at 30 °C the reaction was stopped by adding 5 μl of 3% phosphoric acid. A 20 μl aliquot was spotted onto a P81 paper square (Upstate Biotechnology), dried, washed and sealed in a plastic bag with 4 ml scintillation cocktail. Radioactivity was then measured in a scintillation counter (MicroBeta 1450; PerkinElmer, Waltham, USA). Active LKB1/STRADα/MO25α was used as the positive control and samples containing all assay components plus 2 μl of 30% phosphoric acid were used as negative controls. Data are presented as the mean±SE. BL LKB1, BL21 (DE3)-derived His-LKB1; CPM, counts per minute; RG LKB1, Rosetta-gami (DE3)pLysS-derived His-LKB1.

Kinase activity of recombinant His-LKB1

The kinase activity of recombinant His-LKB1 was further investigated *in vitro* by LKBtide. The activity of RG-derived His-LKB1 was approximately 5 times higher than that of BL-derived His-LKB1 (**Fig. 4**). Together with the data of the autophosphorylation activity assay, the kinase activity of RG-derived His-LKB1 was proved to be much higher than that of BL-derived His-LKB1.

Growth suppression assay of His-LKB1 on SMMC-7721 cells

Evidence showed that the growth suppression activity of LKB1 is dependent on its functional kinase domain [6]. As the autophosphorylation and kinase activities of purified His-LKB1 were confirmed by the above analysis, the growth suppression on cells of His-LKB1 was assayed further. The low-level expression of LKB1 in SMMC-7721 cells, compared with 293 cells, was detected by Western blot (**Fig. 5**), so SMMC-7721 cells were used to check the growth suppression function of purified His-LKB1. Chariot, a carrier for the delivery of biologically active proteins into mammalian cells [18,19], was used to transfer purified His-LKB1 into SMMC-7721 cells. The transfer efficiency was confirmed by Western blot (**Fig. 5**). The growth inhibitory ratios of 10 μg/ml His-LKB1 from BL and RG after 96 h were 24.97% and 45.68%, respectively (**Fig. 6**). Compared with the Chariot control group,

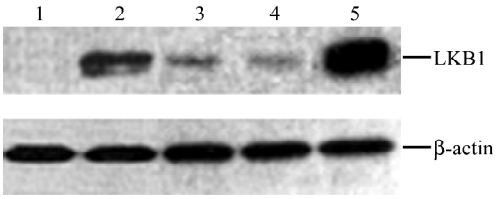

Fig. 5 Western blot analysis of internal LKB1 protein in human embryonic kidney 293 cells and hepatic carcinoma SMMC-7721 cells and the transfer efficiency of His-LKB1 by Chariot

1, Control; 2, 293 cells; 3, SMMC-7721 cells; 4, SMMC-7721 cells treated with Chariot; 5, SMMC-7721 cells treated with His-LKB1 carried by Chariot. β-actin was the internal reference.

recombinant His-LKB1 showed significant growth inhibition on SMMC-7721 cells (P<0.05).

Cell-cycle arrest of His-LKB1 on SMMC-7721 cells

The observed growth suppression by LKB1 could be caused by increased apoptosis or by inhibition of cell proliferation. Tiainen *et al.* [6] had reported that no increase in the rate of apoptosis in LKB1-expressing cells was detected. Therefore, possible cell-cycle arrest of SMMC-7721 cells by treatment with recombinant His-

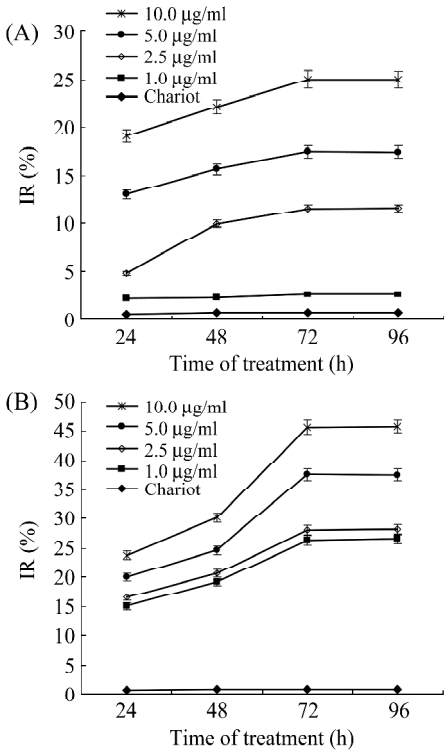

Fig. 6 Growth inhibitory ratio (IR%) of BL21(DE3) (BL)-derived His-LKB1 (A) and Rosetta-gami(DE3)pLysS (RG)-derived His-LKB1 (B) on SMMC-7721 cells by MTT assay

Exponentially growing hepatic carcinoma SMMC-7721 cells were divided into five groups: Chariot was added to the negative control, named the Chariot group; purified His-LKB1 from BL mixed with Chariot was added to four other groups with the concentration of His-LKB1 varying from 1.0 to 10.0 μg/ml, as indicated in the figure keys. After treatment for 24–96 h, MTT assay were carried out. IR% was calculated as: IR%=(1−A_{570} of test group/A_{570} of blank control)×100%. The assays were carried out in triplicate and data are presented as the mean±SE.

LKB1 was examined using flow cytometry (**Table 1**). G_1 fractions of the BL and RG groups were 59.17% and 63.28%, respectively, compared with 52.23% of the blank control and 52.06% of Chariot control groups, indicating statistically significant differences. These results proved that recombinant His-LKB1 inhibited cell growth by G_1 cell-cycle arrest.

Discussion

Human LKB1 has been discovered to play a major role in tumor suppression, and is implicated in the regulation of many important cellular processes [3–15]. These findings prompted scientists to explore how a protein could have so many important functions, and to find the structural basis of these functions. Moreover, recombinant active LKB1 could be a candidate for tumor therapy. However, the failure to obtain high-level expression of functional LKB1 in any expression system has precluded studies of this protein. Although Martinez-Torrecuadrada *et al.* tried different strategies, they could not recover active LKB1 from *E. coli*, and only recovered functional GST-LKB1 from insect cells when it was co-expressed with chaperone Hsp70 and its cofactors [17]. The expression level was as low as 3 μg/ml, and the process was time-consuming and expensive. Here, we have successfully expressed functional human LKB1 in *E. coli* and purified it using simple methodology. The functional human LKB1 provides a basis for tumor therapy research and structural analysis.

We tried to express His-LKB1 with the wild-type-encoded cDNA in BL and RG, but failed to obtain detectable recombinant protein. We speculated that the poor expression level of LKB1 in *E. coli* was probably due to the rare codons in the wild-type LKB1 gene. Therefore, we constructed a synthesized cDNA of LKB1, in which all the rare codons were replaced with high-frequency codons according to the *E. coli* codon bias. As

Table 1 Influence of His-LKB1 on cell cycle of SMMC-7721 cells

Group	G_1 (%)	G_2/M (%)	S (%)
Blank control	52.23±5.94	18.83±2.15	28.94±3.35
Chariot control	52.06±5.53	19.63±2.07	28.31±3.27
BL (BL-derived His-LKB1+Chariot)	59.17±5.88*	18.74±2.36	22.09±3.44*
RG (RG-derived His-LKB1+Chariot)	63.28±6.15*	18.17±1.83	18.55±2.85*

Flow cytometry analysis was carried out to evaluate the effect of His-LKB1 on the cell-cycle distribution of SMMC-7721 cells. SMMC-7721 cells (40% confluent) were divided into four groups: blank control, PBS was added; Chariot control, Chariot was added; BL21(DE3) (BL), BL-derived His-LKB1 (final concentration was 10.0 μg/ml) was mixed with Chariot and added; and Rosetta-gami(DE3)pLysS (RG), RG-derived His-LKB1 (final concentration was 10.0 μg/ml) was mixed with Chariot and added. After 72 h, cells were stained and analyzed using a FACScan flow cytometer The assay was carried out in triplicate and data are presented as the mean±SE. *P< 0.05.

a result, the expression level was improved significantly and soluble His-LKB1 accounted for 34.1% of total soluble proteins in RG. This result provides an example of the use of optimized codons for high-level expression of a eukaryotic protein (60 kDa) in *E. coli*.

When the optimized *LKB1* gene was expressed in two *E. coli* hosts, BL and RG, the total amounts of recombinant His-LKB1 were similar, but the solubility differed widely. The recombinant His-LKB1 accounted for 34.8% and 39.3% of total proteins in BL and RG, respectively, but the soluble His-LKB1 accounted for 8.1% and 34.1% of total soluble proteins in each of them. It seems that the host is the most important factor when expressing soluble His-LKB1 in *E. coli*. The RG host contains the *trxB* and *gor* mutations, allowing enhanced disulfide bond formation and soluble expression of eukaryotic proteins, whereas BL is a widely used expression host without these mutations. Ten discontinuous cysteine residues exist in the LKB1 protein, and the formation of disulfide bonds between them is highly possible. This might be the reason for the different solubility of His-LKB1 in the two hosts, as RG is more favorable for the formation of disulfide bonds. Once correct disulfide bonds were formed, the solubility of the protein was improved and the formation of the desired conformation was facilitated.

As reported elsewhere, when expressed in insect cells, purified GST-LKB1 alone was able to autophosphorylate in a specific and reversible way [17]. When expressed in BL, however, the MBP-LKB1 fusion protein was not able to autophosphorylate. We determined the autophosphorylation and kinase activities of the purified His-LKB1 protein. In the autokinase assay, the autokinase activity of RG-derived His-LKB1 was much higher than BL-derived His-LKB1. The kinase activity of RG-derived His-LKB1 towards LKBtide was approximately 5 times higher than that of BL-derived His-LKB1. These results indicated that the kinase activity of RG-derived His-LKB1 was much higher than that of BL-derived His-LKB1, making RG a good candidate for the expression of active His-LKB1. This result is reasonable when we consider that RG has an advantage over BL in the expression of soluble proteins. We conjectured that part of the soluble His-LKB1 from BL was not in the desired conformation, whereas most of the protein from RG was in the desired conformation because of the correct formation of disulfide bonds.

As shown by our results and those of others [17], the purified recombinant LKB1 protein alone has autokinase and phosphotransferase activity, showing that this recombinant protein could be used for functionality studies. Mammalian LKB1 has been implicated as a regulator of multiple biological processes and signaling pathways, and many of them are related to tumor suppression, so this is one of the most promising aspects of its clinical application. It has been reported that the growth suppression activity of LKB1 requires a functional kinase domain. When cells with impaired LKB1 activity were transfected with the LKB1 expression vector, the restored LKB1 activity induced growth suppression [6]. In our study, when recombinant His-LKB1 was carried by Chariot into SMMC-7721 cells, in which the internal LKB1 expression level is low, a significant growth inhibition of cells (IR%=45.68%) was observed. This finding showed that this recombinant protein expressed in *E. coli* could be a promising candidate for antitumor drugs.

The detailed molecular mechanisms by which LKB1 exerts its tumor-suppressing activities remain uncertain. There was evidence showing that growth suppression was mediated through G_1 cell-cycle arrest and did not involve an increas in apoptosis [6]. Our results proved that the recombinant His-LKB1 induced G_1 cell-cycle arrest when it was carried into cells by Chariot. At a final concentration of 10 μg/ml, His-LKB1-induced G_1 cell-cycle arrest was obvious even without synchronization of mitosis.

In summary, we have shown that the expression level of recombinant His-LKB1 in *E. coli* was improved significantly after codon optimization, and the recombinant protein showed a high level of autokinase activity when it was expressed in RG. When recombinant His-LKB1 was carried into SMMC-7721 cells by Chariot, cell growth suppression and cell-cycle arrest were observed. These results suggested that the recombinant His-LKB1 protein from *E. coli* could be helpful in future biochemical and structural studies, and is a prospective candidate for anti-tumor drugs.

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Edited by
Bingwei LIU