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ORIGINAL ARTICLE

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Cloning and characterization of a novel gene which encodes a protein interacting with the mitosis-associated kinase-like protein NTKL

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Abstract NTKL is an evolutionarily conserved kinase-like protein. The cell-cycle-dependent centrosomal localization of NTKL suggested that it was involved in centrosome-related cellular function. The mouse NTKL protein is highly homologous with human NTKL. A novel mouse protein was identified as an NTKL-binding protein (NTKL-BP1) by yeast two-hybrid screening, and the full-length cDNA was amplified based on the result of a sequence data analysis cloning strategy. The full-length cDNA sequence of the *NTKL-BP1* gene consists of 2,537 bp, which encode 368 amino acids. A database search revealed that homologues of NTKL-BP1 exist in different organisms, including *Arabidopsis thaliana, Drosophila melanogaster, Plasmodium falciparum, Geobacter metallireducens, Anopheles gambiae* and human. It suggests that NTKL-BP1 is an evolutionarily conserved protein. The expression of *NTKL-BP1* was observed in multiple normal mouse tissues. The interaction of the two proteins was confirmed by co-immunoprecipitation. Moreover, immunofluorescent staining indicated that NTKL and NTKL-BP1 were all localized in the cytoplasm.

Keywords NTKL · NTKL-BP1 · Yeast two-hybrid · Co-immunoprecipitation

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Introduction

The N-terminal kinase-like protein (NTKL) is found in *Drosophila melanogaster, Caenorhabdatis elegans, Arabidopsis thaliana,* mouse and human. In 2000, Liu and co-workers cloned the cDNA of mouse *NTKL* (GenBank accession no. AF276514), encoding a 105-kDa protein (Liu et al. 2000). The protein was widely expressed in mouse tissues and concentrated in the cytosol and the fraction of low-density microsomes in 3T3-L1 adipocytes. These fractions contained Golgi apparatus, some cytoskeletons and other small cellular compartments (Liu et al. 2000). The human *NTKL* gene, whose mRNA is expressed ubiquitously in human tissues, is located on chromosome 11q13 and maps around chromosomal breakpoints found in several carcinomas, suggesting that *NTKL* dysfunction may be involved in carcinogenesis. Alternative splicing generates two variant forms of *NTKL* mRNA that encode protein isoforms with internal deletions. Variant 2 *NTKL* mRNA was localized to the centrosome during mitosis; however, variant 1 was found in the cytoplasm. This suggested that *NTKL* variant 2 might have mitosis-related function, such as spindle formation or segregation of condensed chromosomes (Kato et al. 2002). Human *NTKL* is most closely related to the mouse homologue (identity 90% in amino acid level) (Liu et al. 2000; Kato et al. 2002).

Centrosome plays an essential role in mitosis. In interphase cells, centrosome-anchored microtubules serve as tracks for molecular motor-based transport and positioning of vesicles and organelles. In mitotic cells, centrosome become incorporated into spindle poles and organized into bipolar spindles. The centrosome functions as a microtubule-organizing centre and has a critical role in accurate chromosome segregation (Kukri and Holzbaur 1999, Compton 1998). The centrosome is composed of a pair of perpendicularly aligned centrioles, surrounded by amorphous pericentriolar materials (PCM) (Kukri and Holzbaur 1999). The PCM contains

many proteins, such as γ-tubulin (Compton 1998), pericentrin, PCM-1 (Zimmerman et al. 2000), NuMA (Zeng 2000), and TACCtics (Fanni 2002). Both centrosomes and spindle poles recruit and anchor components that regulate a growing list of cellular activities, including spindle organization and function, cell cycle progression, protein degradation and centrosome duplication (Zimmerman et al. 1999; Sluder and Hinchcliffe 1999; Rieder et al. 2001). The NTKL protein may play important roles in these aspects.

The mouse NTKL protein contains three protein kinase domains in the N-terminal region, and may be involved in the mitosis process through regulating phosphorylation or dephosphorylation of other mitosis-associated proteins. We searched for the NTKL-binding proteins by yeast two-hybrid screening in a mouse fetal cDNA library. The obtained positive clones encode one novel NTKL-binding protein. The interaction of the two proteins was confirmed by co-immunoprecipitation assay. The function of the novel gene was unclear. We tentatively named the novel protein as NTKL-BP1 (NTKL-binding protein 1). A database search revealed that the protein is evolutionarily conserved, existing in different organisms from plants to animals. The NTKL-BP1 protein was expressed in multiple mouse normal tissues and localized in cytoplasm. We predicted it might also be involved in the cell mitosis progression through binding NTKL.

Materials and methods

Yeast two-hybrid analysis

The ProQust (Invitrogen) yeast two-hybrid system was used in this study. The ORF of the *NTKL* gene was inserted into the "bait" vector, pDB-Leu. A 10.5-day-old fetal mouse cDNA library was used for screening. More than 1×10^6 cDNA colonies were screened. After the positive colonies had grown out on the leucine-, tryptophan-, histidine-lacking medium, 3AT (3-amino-1, 2,4-triazole) was added to the plate, the prey plasmids were isolated and their cDNA inserts were sequenced. Homology algorithm comparisons were performed using BLAST algorithm through NCBI web site (www.ncbi.nlm.nih.gov/BLAST).

Sequence data analysis cloning and full-length cDNA amplification

Via a sequence BLAST search at the NCBI web site, a highly matched cDNA sequence was obtained (GenBank accession no. XM_129584). RACE primers G1 (5′-cgaacctcggcacttttgtgacca-3′) and G2 (5′-ggaagaggtgggtgtgaggggac-3′) were designed based on the XM_129584 sequence. The mouse liver Marathon Ready cDNA kit (Clontech) was used to conduct the 5′RACE experiments. Amplifications were performed according to manufacturer's instructions. The primary PCR was performed with primer AP1 (adaptor primer 1) and the gene-specific antisense primer G1, followed by secondary PCR using AP2 (adaptor primer 2) and the antisense-specific primer G2. The PCR products were cloned into pT-Adv (Clontech) vector and sequenced. The *NTKL-BP1* forward primer (5′-gaaggaacccggatgtgcctgtggag-3′) and *NTKL-BP1* reverse primer (5′-aaacttatcagacttgttaaataagac-3′) were designed based upon the XM_129584 sequence. Amplifications were performed from a mouse fetal cDNA library with the primers. The PCR products were cloned into vector pMD-T 18 and sequenced.

Northern blot analysis

Total RNA of eight normal mouse tissues was extracted with TRIZOL reagent (Life Technologies, Gaithersburg, Md.) according to the manufacturer's protocol. A 15-μg portion of total RNA was loaded per lane on a 1.2% denatured formaldehyde agarose gel. After electrophoresis, RNA was transferred to Hybond-N^+ nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) and immobilized by UV cross-linking. The cDNA probe was generated by amplification with primers BPs (5′-gtcgaccggatggctcaggattggg-3′) and BPa (5′-gttaacacactggccgctcatgtgg-3′) and labeled with [α-^{32}P] dCTP by random prime labeling system (Amersham, Arlington Heights, Ill.). The hybridization solution contained 6×SSC, 5×Denhardt's solution, 50% deionized formamide, 0.5% SDS, and 100 μg/ml denatured sheared salmon sperm DNA. Hybridization, membrane washing, and autoradiography were done according to the manufacturer's instructions.

Plasmid construction

To construct eukaryotic expression plasmids, the open reading frame of *NTKL* was inserted between the *Sal*I/*Not*I sites of vector pCMV-Myc (Clontech), and the open reading frame of *NTKL-BP1* was amplified with HA-BP1 sense and antisense primers, and inserted between the *Xho*I/*Hpa*I sites of eukaryotic expression vector pcDNA3.1/HA2.

Co-immunoprecipitation

For immunoprecipitation, COS-7 cells were co-transfected with plasmids pCMV-Myc-NTKL and pcDNA3.1/HA2-NTKL-BP1. Forty-eight hours after transfection, the cells were collected in lysis buffer (Roche) for 15 min at room temperature. The lysate supernatant was incubated with protein A/G agarose (Santa Cruz) and rabbit anti-HA antibody or mouse monoclonal antibody anti-c-myc for 4 h at 4 °C. The pellets were washed four times with lysis buffer. The precipitated proteins were eluted from the beads with protein loading buffer, separated on a 12% SDS-PAGE gel, transferred to PVDF-plus membranes (Bio-Rad) by Western blotting and, after blocking, detected with mouse monoclonal antibody anti-c-myc (diluted 1:2,000) (Invitrogen) or mouse monoclonal antibody anti-HA. Specific bands were visualized by the enhanced chemoluminescence system (Pierce).

Immunofluorescent staining

SMMC-7721 cells were cultured on glass cover slips in six-well plates. Cells were transfected with the Myc-NTKL and HA2-NTKL-BP1 plasmids independently. Forty-eight hours later, the cells were washed twice with cold PBS, fixed at 4 °C in freshly prepared 4% paraformaldehyde (pH 7.4) for 30 min, and permeabilized with 0.2% Triton X-100 in PBS for 10 min. Samples were reacted with monoclonal antibody anti-c-myc or rabbit polyclonal antibody anti-HA as primary antibody (1:100) at room temperature for 1 h. Cells were rinsed in PBS and stained with FITC-conjugated anti-mouse IgG (Dako) and Alexa 594-conjugated goat anti-rabbit IgG (Molecular probes) (1:500) at room temperature for 25 min. Cells were rinsed three times with 0.5% Tween-20 in PBS. Fluorescent image analyses were performed on an Axioskop 2 universal microscope with the ISIS system (Carl Zeiss).

Results

Yeast two-hybrid screening, full-length NTKL-BP1 cDNA cloning and sequence analysis

Using the mouse NTKL protein as the "bait" to screen a mouse 10.5-day-old fetal cDNA library with yeast two-hybrid system, we obtained four positive clones. The cDNAs of the positive clones were sequenced and the sequences were used as queries to search the GenBank database. Homology searches showed that these clones encoded one novel protein. The full-length of *NTKL-BP1* cDNA was cloned from a mouse fetal library. The gene encoding NTKL-binding protein is a novel mouse gene (GenBank accession no. XM_129584). The full length of the cDNA sequence contains 2,537 bp, encoding a 368-amino-acid protein. Fig. 1 represents the cDNA sequence of *NTKL-BP1* and the derived amino acid sequence.

One human gene (XM_044455.2) shares high sequence similarity with *NTKL-BP1*. It has 70% identity and 77% positives at the amino acid level, and 87% homology at the nucleotide level, respectively. The human homologous gene encoded a 394-amino-acid protein. In other species, some proteins share conserved amino acids with the NTKL-BP1. We aligned and compared the amino acid sequences of these proteins from *A. thaliana* EAA02935, *Geobacter metallireducens* ZP_00081191, *D. melanogaster* AE003524_32, *Anopheles gambiae* EAA10459, human XP_044455 and mouse XP_129584 (Fig. 2A). The NTKL-BP1 protein was also evolutionarily conserved. The protein is also similar to dynactin 1 of mouse (Fig. 2B).

Function predictions based on the amino acid sequence analysis of the NTKL-BP1 protein with SMART

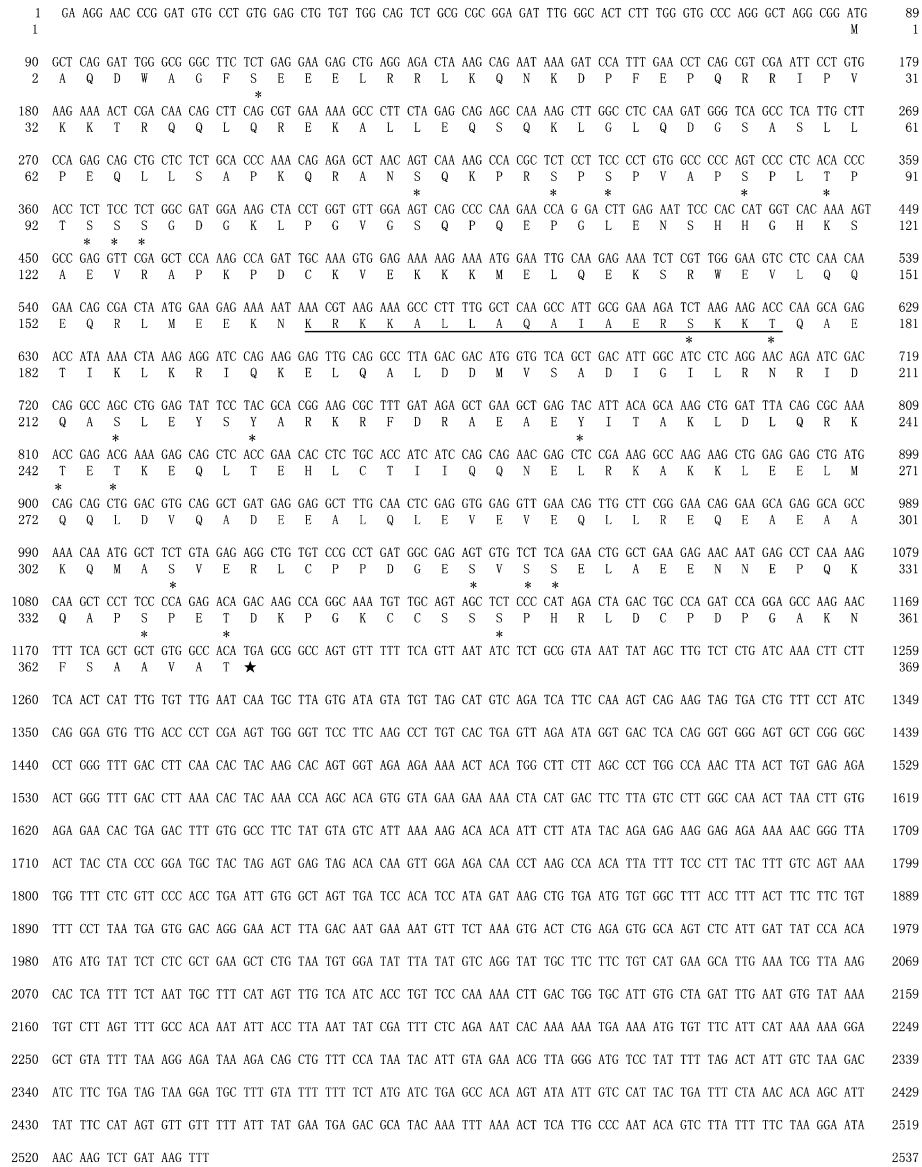

Fig. 1 Nucleotide and deduced amino acid sequences for the *NTKL-BP1* gene. The two coiled-coil domains were assigned on the basis of SMART analysis and were highlighted in *gray*. The predicted bipartite nuclear localization signal is *underlined*. The *asterisk* indicates the predicted phosphorylation site

(http://smart.em-heidelberg.de/) programs revealed that there is a predicted bipartite nuclear localization signal (161–178 aa), a BRCT (breast cancer carboxy-terminal domain, 70–343 aa) and two coiled-coil domains. Prositescan software analysis (http://www.cbs.dtu.dk/services/NetPhos/) showed that there are 23 predicted phosphorylation sites (Ser: 16, Thr: 5, Tyr: 2).

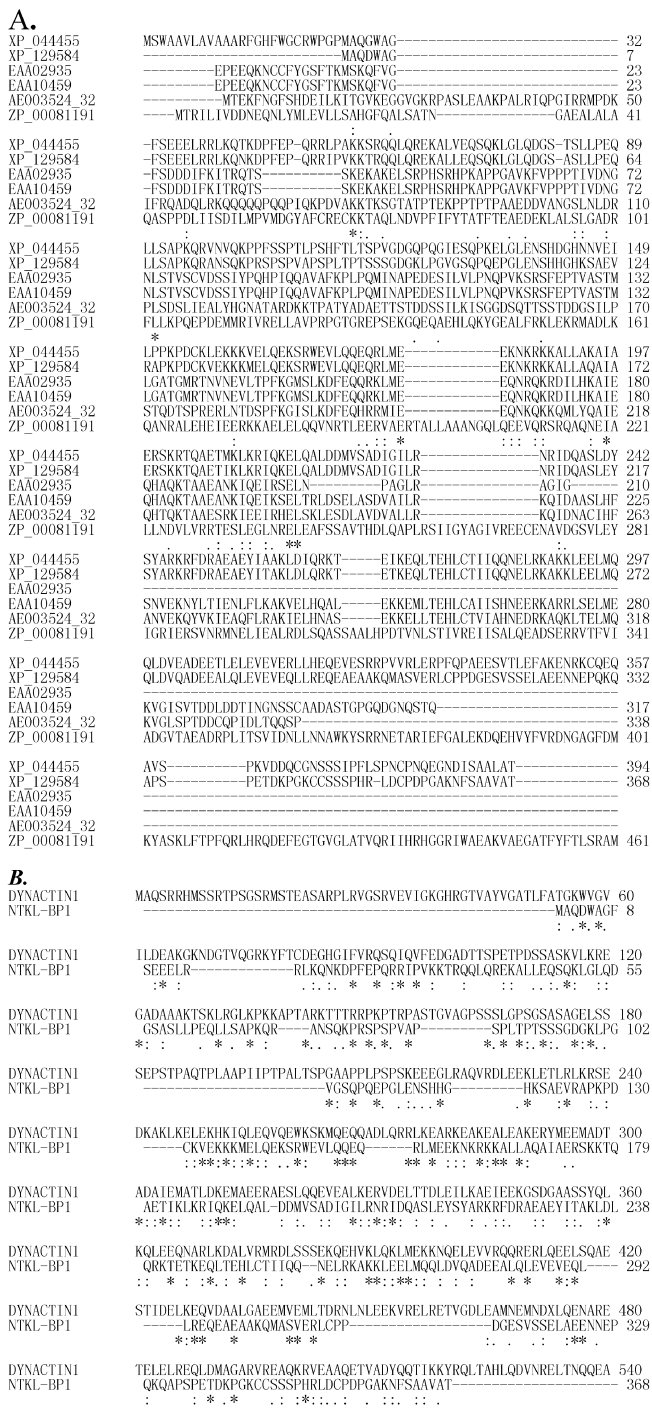

Fig. 2A, B Alignment of NTKL-BP1 deduced amino acid sequence. Amino acids that are identical (*), conservatively substituted (:), and semi-conservatively substituted (.) are shown. Gaps (–) have been introduced to maximize alignment. **A** Alignment of the amino acid sequences of NTKL-BP1 and its homologues: human (XP_044455), mouse (XP_129584), *A. thaliana* (EAA02935), *A. gambiae* (EAA10459), *D. melanogaster* (AE003524_32), *G. metallireducens* (ZP_00081191) **B** Homology comparison of NTKL-BP1 and mouse dynactin 1

Tissue expression patterns of *NTKL-BP1*

Northern analysis showed the *NTKL-BP1* gene has two variants of transcript, 4.7 kb and 2.5 kb. Each transcript has different expression levels in different tissues. The expression level of the longer *NTKL-BP1* in the liver was quite low (Fig. 3).

NTKL can interact with the NTKL-BP1 in vivo

The results of co-immunoprecipitation experiment were shown in Fig. 4. The bands in *lane 1* of Fig. 4A, B indicated that Myc-NTKL (blot with anti-myc antibody) and the HA-NTKL-BP1 (blot with anti HA antibody) could be expressed in the co-transfected COS-7 cells. When the rabbit anti-HA polyclonal antibody was used, the precipitated proteins contained two components, HA-NTKL-BP1 and Myc-NTKL (Fig. 4A, B *lane 2*). When the mouse anti-Myc antibody was used, the precipitated proteins contained again two components, Myc-NTKL and HA-NTKL-BP1 (Fig. 4A, B *lane 3*). This result revealed that NTKL could interact with the NTKL-BP1 within mammalian cells.

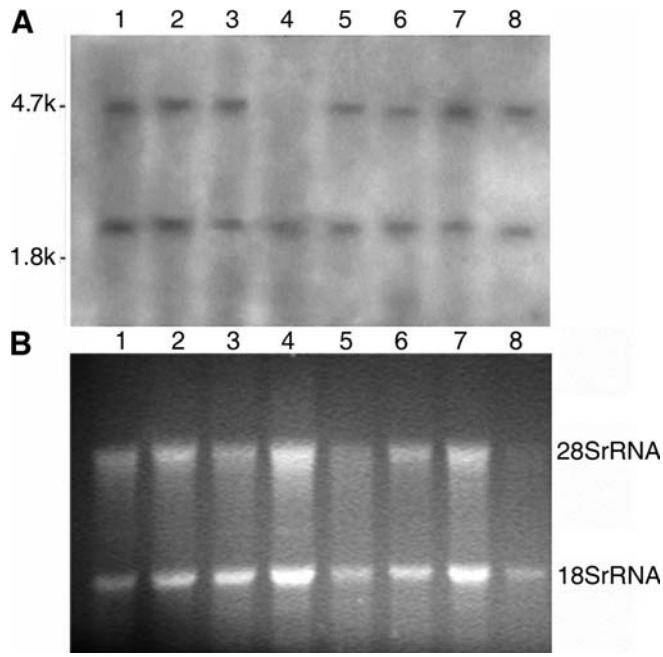

Fig. 3A, B Expression of NTKL-BP1 mRNA in different mouse normal tissues. **A** Northern blot analysis of *NTKL-BP1* mRNA in eight normal mouse tissues. *Lane 1* small intestine, *lane 2* kidney, *lane 3* skeletal muscle, *lane 4* liver, *lane 5* lung, *lane 6* spleen, *lane 7* brain (whole), *lane 8* heart. **B** Control; the rRNAs are indicated

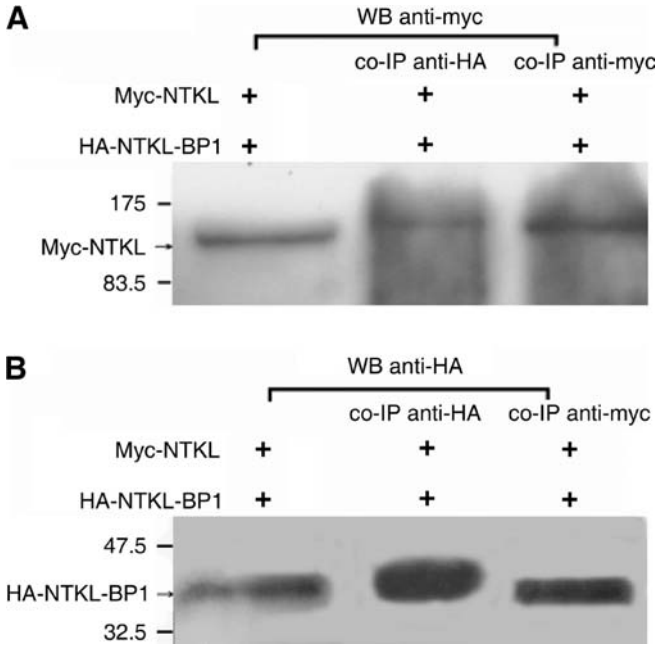

Fig. 4A, B Co-immunoprecipitation of NTKL and NTKL-BP1 in COS-7 cells transfected with pCMV-Myc-NTKL and pcDNA3.1/HA2-NTKL-BP1. **A** Detected using mouse anti-myc monoclonal antibody. Expression of NTKL in cells (*lane 1*), co-immunoprecipitation of NTKL with anti-HA antibody (*lane 2*), and immunoprecipitation of NTKL with anti-myc antibody (*lane 3*). **B** Detected using rabbit anti-HA antibody. Expression of NTKL-BP1 in cells (*lane 1*), immunoprecipitation of NTKL-BP1 with anti-HA antibody from cells co-transfected with the *NTKL* and *NTKL-BP1* expression plasmids (*lane 2*), and co-immunoprecipitation of NTKL-BP1 with anti-myc antibody (*lane 3*)

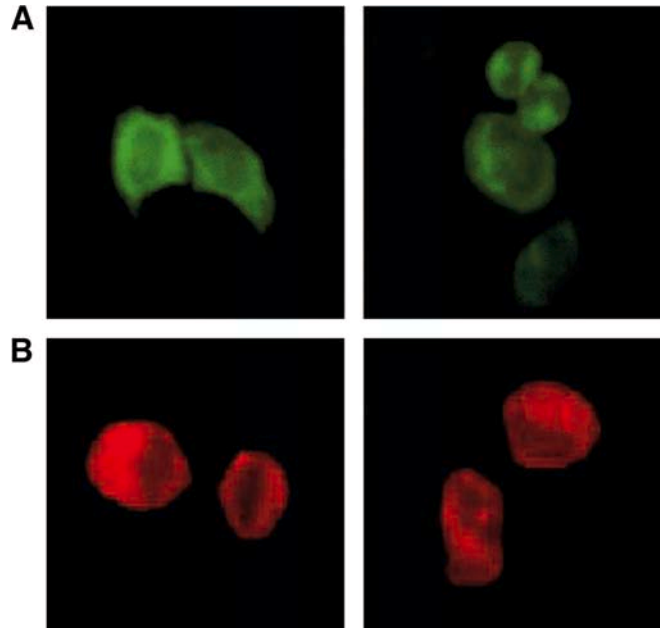

Fig. 5A, B Subcellular localization of NTKL, NTKL-BP1 in SMMC-7721 cells. **A** Cells were transfected with Myc-NTKL protein showing cytoplasm localization (*green*) (400×). **B** Cells were transfected with HA-NTKL-BP1 also showing cytoplasm localization (*red*) (400×)

Subcellular localization of NTKL and NTKL-BP1 in SMMC-7721

Immunofluorescent staining of SMMC-7721 cells transfected by Myc-tagged *NTKL* with anti-myc antibody showed that the NTKL was localized in the cytoplasm (Fig. 5A). Immunofluorescent staining of the cells transfected by HA-tagged *NTKL-BP1* with the rabbit anti-HA polyclonal antibody showed the HA-NTKL-BP1 localized in cytoplasm surrounding the nucleus (Fig. 5B).

Discussion

In this study, we isolated a novel gene, *NTKL-BP1*, through a yeast two-hybrid method, followed by cloning the full-length cDNA sequence using PCR amplification from a mouse fetal library and PCR. The result of Northern blotting showed that two isoforms were expressed in mouse multiple tissues. We obtained the shorter one, which contains 2,537 bp and encodes a 368-amino-acid protein. Homologous analysis using the amino acid sequence as a query to search the GenBank database revealed that a homologue of NTKL-BP1 existed across organisms from the plant to animal kingdoms: *A. thaliana, D. melanogaster, G. metallireducens; A. gambiae,* mouse and human. The results showed that the novel gene, *NTKL-BP1*, is highly conserved. The NTKL-BP1 protein could interact with the mitosis-association protein NTKL. With immunofluorescence staining, the two proteins were detected in the cell cytoplasm. The NTKL-BP1 protein was predicted to contain one bipartite nuclear localization signal, but the results of subcellular localisation showed that it was located in the cytoplasm surrounding the nucleus.

NTKL is a member of a large family found in a broad range of eukaryotes (Liu et al. 2000). Proteins similar to it were also found in *D. melanogaster, C. elegans* and *A. thaliana*. These proteins have protein kinase-like domains in the N-terminal region (Liu et al. 2000; Kato et al. 2002). The human NTKL protein has two variants; variant 2 was identified to be independent of microtubule polymerization (Kato et al. 2002). The NTKL protein, although possessing an N-terminal kinase-like domain, probably has no kinase activity (Liu et al. 2000; Kato et al. 2002). Moreover, the multimer formation of NTKL was found in mammalian cells and confirmed in vitro (Kato et al. 2002). No other proteins interacting with it were reported before.

The mitosis-associated proteins were involved in many aspects. Mitotic cell division usually follows strict rules of spindle formation and the subsequent segregation of condensed chromosomes to ensure faithful transmission of entire genomes to daughter cells (Rieder et al. 2001). The localization of an increasing number of protein kinases to the centrosome has revealed the importance of protein phosphorylation in controlling

many of these transitions (Mayor et al. 1999). Reversible phosphorylation of proteins by kinases and phosphatases plays a key regulatory role in several eukaryotic cellular functions, including control of the involvement of cyclin-dependent kinases (CDKs) and cyclins in regulation of cell cycle progression (Sommi et al. 2002; Fry et al. 2001). Some of these associated genes are overexpressed in human carcinoma, suggesting a possible involvement of abnormal regulation of centrosomal kinases in carcinogenesis and tumour progression (Villerbu et al. 2002; Frey et al. 2001). The NTKL protein may be involved in the mitosis process through regulating phosphorylation or dephosphorylation of other mitosis-related proteins.

In the GenBank database, we found that several novel eukaryotic proteins were structurally related to the NTKL-BP1 protein (Fig. 2). The NTKL-BP1 protein was also similar to dynacin 1 and the Nip100 protein of yeast. The mouse dynactin 1 is a homologue of the *Drosophila* p150-Glued (LOC226570) protein, which is one component of the microtubule-associated complex that includes the cytoplasmic linker protein (CLIP)-170, EB1 and cytoplasmic dynein (Allan 1994). With the method of live-cell imaging, it was found that $p150^{Glued}$ could target to the plus ends of growing microtubules (Boylan et al. 2000). Its phosphorylation influences plus-end binding specificity (Huang et al. 1999). The cytoplasmic dynein intermediate chain and $p150^{Glued}$ of the dynein-dynactin complex undergo coordinated phosphorylation changes at two G2/M transitions (Vaughan et al. 2002). The phosphorylation may positively regulate mitotic processes, such as spindle assembly or orientation, or negatively regulate interphase processes (Huang et al. 1999; Vaughan 2002). The gene *nip100* encodes the yeast homologue of dynactin complex protein $p150^{Glued}$. The nip100Δ strains are viable but undergo a significant number of failed mitoses in which the mitotic spindle does not properly partition into the daughter cell. The result suggests that the yeast dynactin complex is responsible for spindle translocation in late anaphase (Kahana et al. 1998). Many centrosome-associated proteins transported from centrosome to microtubule are dependent on the complex, including dynein and the dynactin.

The protein NTKL-BP1 contains BRCT (BRCA1 C-terminal) domain. The protein BRCA1 is found within many DNA damage repair and cell cycle checkpoint proteins. The 53BP1 protein is also a vertebrate BRCT motif protein, originally described as directly interacting with p53 and recently shown to be implicated in the early response to DNA damage (Deng and Brodie 2000; Deng 2002; Derbyshire 2002; Joo 2002).

Previous studies suggested that the pair of interacting proteins might have some function in mitosis progression, as a regular factor or a checkpoint protein. The NTKL protein with three protein kinase domains was predicted to have no kinase activity, but as one inhibitor of other kinases regulating some binding proteins by phosphorylation and dephosphorylation (Kato et al. 2002). The NTKL-BP1 contains 23 potential phosphorylation sites (Ser: 16, Thr: 5, Tyr: 2). The NTKL may be responsible for phosphorylation of these serine/threonine sites, or as an inhibitor preventing phosphorylation of NTKL-BP1 through binding to it, which needs to be validated with more experiments. Both NTKL and NTKL-BP1 are highly conserved proteins during evolution. Clarification is needed of their roles in both the mitotic process and microtubule change in cell cycle.

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