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A new human gene hNTKL-BP1 interacts with hPirh2

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Abstract

NTKL (N-terminal kinase-like protein) encodes an evolutionarily conserved kinase-like protein and is mapped around chromosomal breakpoints found in several carcinomas, suggesting that NTKL dysfunction may be involved in carcinogenesis. Recently, we identified a novel mouse gene, mNTKL-BP1 (NTKL-binding protein 1), encoding a protein interacting with NTKL. For further study, a new human gene, hNTKL-BP1, which is highly homologous with mNTKL-BP1, was used as bait in yeast two-hybrid system. hPirh2 (human p53-induced RING-H2 protein) was identified as hNTKL-BP1 interacting protein. The specific interaction of two proteins was confirmed by pull-down assay in vitro and co-immunoprecipitation in vivo. Moreover, an immunofluorescent staining assay showed that hNTKL-BP1 colocalizes with hPirh2 in SMMC 7721 cells. It will stimulate further investigation into whether hNTKL-BP1 is the substrate of hPirh2 or interaction of hNTKL-BP1 with hPirh2 enhances or represses the ubiquitin–protein ligase activity of hPirh2.

Keywords: hNTKL-BP1; hPirh2; Yeast two-hybrid; Pull-down; Co-immunoprecipitation; Colocalize

NTKL (N-terminal kinase-like protein, GenBank Accession No. BE675048) was initially cloned from a mouse adipocyte cell line cDNA library [1]. It encodes a protein containing a potential protein kinase domain at N-terminus. Human NTKL gene, which is ubiquitous in human tissues [2], is located on 11q13 and mapped around chromosomal breakpoints found in several carcinomas, suggesting that NTKL dysfunction may be involved in carcinogenesis. Two splicing variants of *NTKL* were found [2]. Two variants were found in cytoplasm during interphase. But variant 2 was localized to the centrosomes during mitosis [2]. This cell-cycle-dependent centrosomal localization suggests that NTKL might have mitosis-related function. Although NTKL protein showed similarity to protein kinase, the first subdomain and several conserved residues characteristic of protein kinase are absent and the enzyme activity assay showed no protein kinase activity [2]. This suggests that NTKL might exert kinase-related function such as an inhibitor of other kinases recognizing and binding some mitosis-related proteins and regulating the phosphorylation or dephosphorylation of the binding proteins.

A novel mouse protein, mNTKL-BP1 (mouse NTKL-binding protein 1, GenBank Accession No. XM_129584), was identified as NTKL interacting protein and colocalized with NTKL in cytoplasm [3]. mNTKL-BP1 is an evolutionally conserved protein, existing in different organisms from plants to animals. The mNTKL-BP1 protein was observed in many normal mouse tissues and localized in cytoplasm. Bioinformatic analysis revealed that mNTKL-BP1 protein has a predicted bipartite nuclear localization signal, a BRCT (breast carboxy-terminal domain), two coiled-coil domains and 23, predicted phosphorylation sites. Through interaction with mNTKL-BP1, NTKL may play as an inhibitor preventing phosphorylation of mNTKL-BP1.

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The mNTKL-BP1 may also be involved in mitosis-related cellular function by binding NTKL.

Human NTKL-BP1 (GenBank Accession No. NM_152281) is highly homologous with mNTKL-BP1. For further study of the function of NTKL-BP1, we used yeast two-hybrid screening in a human fetal liver cDNA library to find hNTKL-BP1-interacting protein. One of the identified proteins was hPirh2 (GenBank Accession No. NM_015436), a human p53-induced RING-H2 protein with ubiquitin–protein ligase activity [4]. The interaction between the two proteins indicates that hNTKL-BP1 may be a substrate of hPirh2 or hNTKL-BP1 regulates hPirh2 enzyme activity by binding it.

Materials and methods

Yeast two-hybrid screening. The MATCHMAKER Two-Hybrid 3 (Clontech) was used. The full-length *hNTKL-BP1* was cloned into vector pGBKT7 and the plasmid was transformed into the yeast strain AH109 followed by screening a pACT2 human fetal liver cDNA library (Clontech). Yeast transformants were selected according to the manufacturer's instruction. The total 5×10^6 of transformed were screened and nine positive clones were picked up. Mating tests were performed to confirm the specific interaction. The prey plasmids were isolated and sequenced. Homology was searched with the BLAST algorithm through NCBI web site at http://www.ncbi.nlm.nih.gov.

Construction of truncate plasmids and β-galactosidase assay. hNTKL-BP1ΔN (hNTKL-BP1$_{213–394}$), hNTKL-BP1Δ C (hNTKL-BP1$_{1–212}$), hPirh2Δ N (hPirh2$_{106–261}$), and hPirh2Δ C (hPirh2$_{1–106}$) were generated by PCR using special primers in Table 1, respectively. The truncated fragments of hNTKL-BP1 were cloned into pGBKT7 vector, cotransformed with pGADT7-hPirh2 into yeast strain AH109. And the truncated fragments of hPirh2 were cloned into pGADT7 vector, cotransformed with pGBKT7-hNTKL-BP1 into yeast strain AH109. The expressions of reporter genes were detected.

Liquid β-galactosidase activity assays were performed to quantify the strength of interaction for protein pairs using *o*-nitrophenyl β-D-galactopyranoside (ONPG) as a substrate according to the manufacturer's instructions (Clontech).

In vitro binding assays. The full-length *hPirh2* was cloned into pGEX4-1 vector (Amersham-Pharmacia Biotech) and GST-hPirh2 fusion protein was expressed by inducing using 0.04 mM IPTG in *Escherichia coli* strain BL21. The p53 (72–390 aa) was cloned into the vector pGBKT7. Then, the expressed products were purified with glutathione–Sepharose 4B beads (Amersham-Pharmacia Biotech). The Myc-hPirh2 fusion protein was generated by the TNT-coupled reticulocyte lysate system (Amersham-Pharmacia Biotech) following the manufacturer's instruction and detected by Western blot with anti-Myc antibody (Invitrogen). GST-hPirh2 bound to glutathione–Sepharose 4B beads was incubated with 5 μl in vitro translated Myc-hPirh2 protein in 200 μl NETN buffer (50 mM Tris–HCl, pH 7.5, 200 mM NaCl, 2 mM EDTA, 0.1% NP-40, and 1 mM PMSF) for 3 h at 4 °C. The beads were washed four times by H buffer (20 mM Hepes, 50 mM KCl, 20% glycerol, 0.1% Nonidet P-40, and 0.007% β-mercaptoethanol). Then, the pellets were boiled in 20 μl loading buffer, subjected to 15% SDS–PAGE, transferred to PVDF membrane, and immunoblotted with mouse anti-Myc antibody.

Co-immunoprecipitation. SMMC 7721 (human epithelial-like liver cancer) cells were cotransfected with pEGFP-hPirh2 and pCMV-Myc-hNTKL-BP1. After 48 h incubation, cells were harvested and washed twice in cold PBS, and lysed with lysis buffer (Rochester, IN). The lysate supernatant was incubated with protein A/G–agarose (Santa Cruz Biotechnology) for 1 h at 4 °C followed by immunoprecipitation with anti-Myc or anti-GFP antibody (Santa Cruz Biotechnology) at 4 °C overnight. The pellets were washed three times with lysis buffer. The precipitated proteins were eluted from the beads with loading buffer and separated on 15% SDS–PAGE. Proteins were transferred to PVDF membrane and immunoblotted with anti-GFP or anti-Myc antibody (Invitrogen). Bands were visualized by the enhanced chemoluminescence system (Pierce).

Immunofluorescent staining. SMMC 7721 cells were cotransfected with pEGFP-hPirh2 and pCMV-Myc-hNTKL-BP1. Forty-eight hours later, cells were washed twice in cold PBS and fixed with 4% paraformaldehyde (pH 7.4) for 30 min, permeabilized with 0.2% Triton X-100 in PBS for 30 min, and blocked with 3% BSA in PBS for 30 min. Samples were reacted with anti-myc antibody in PBS for 30 min at 37 °C and rinsed with PBS three times. Then, cells were stained with Alexa 594 conjugated goat anti-rabbit IgG and 4′,6-diamidino-2-phenylindole (DAPI) for 30 min at 37 °C, and rinsed with PBS five times. Fluorescent image analyses were performed on an Axioskop 2 universal microscope with an ISIS system (Carl Zeiss).

Results and discussion

We previously reported that mNTKL-BP1 can interact with NTKL and co-localize with NTKL in cytoplasm [3]. NTKL protein contains a potential protein kinase domain at N-terminus and might exert kinase-related function such as an inhibitor of other kinases recognizing and binding some mitosis-related proteins and regulating the phosphorylation or dephosphorylation of the binding proteins. Through interaction with mNTKL-BP1, NTKL may play as an inhibitor preventing phosphorylation of mNTKL-BP1. mNTKL-BP1

Table 1
Sequence of primers used in this work

Gene	No. of primers	Orientation	Sequence
hNTKL-BP1	N1	F	5′ACCCATATGAGCTGGGCAGCAGTGTTGGC3′
	N2	R	5′TCTGGATCCGCTTTAGTTTCATGGTC3′
	C1	F	5′AGCGGATCCAGAAGGAGTTGCAGGC3′
	C2	R	5′CAGCTGCAGATGTGGCCAAAGCAGCTGAAATGTC3′
hPirh2	N1	F	5′CCGGAATTCATGGCGGCGACGGCCCG3′
	N2	R	5′CCGCTCGAGCCACAGTTTTCACAGTGATACTGC3′
	C1	F	5′CCGGAATTCGGAATTTGTAGGATTGGTCCAAAG 3′
	C2	R	5′CCGCTCGAGATCCAGTGAAATTCTACGTCCTCC3′

Nucleotide sequences underlined are restriction sites.