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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.

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 tried to find the proteins interacting with hNTKL-BP1.

To identify proteins interacting with hNTKL-BP1, we screened a human fetal liver cDNA library in a yeast two-hybrid assay using hNTKL-BP1 as the bait. Nine positive clones were identified by screening about 5×10^6 transformants. The candidate proteins were identified following isolation of the pACT2 plasmids, restriction mapping, and sequencing. Database searches using the BLAST program at NCBI web site revealed that one positive interacting protein showed 100% identity to the *Homo sapiens* zinc finger protein 363 (ZNF363) mRNA (GenBank Accession No. NM_015436), named *hPirh2*. Mating tests were performed to testify the specific interaction.

To confirm the interaction between hNTKL-BP1 and hPirh2, in vitro binding assays were performed using GST fusion proteins. Myc-hNTKL-BP1 was generated using the rabbit reticulocyte lysates and assessed for their capability to form a stable complex with the GST-hPirh2 protein or GST alone, the negative control. In Fig. 1, hNTKL-BP1 was shown to efficiently bind to GST-hPirh2, but not to GST alone. To further verify whether hNTKL-BP1 interacts with hPirh2 in mammalian cells, in vivo binding assays were performed. The results of the co-immunoprecipitation are shown in Fig. 2. The bands in lane 3 of Figs. 2A and B indicate that Myc-hNTKL-BP1 and pEGFP-hPirh2 could be expressed in cotransformed cells. When anti-GFP antibody was used for immunoprecipitation, Myc-hNTKL-BP1 protein was coprecipitated and detected by Western blot with anti-myc antibody (lane 2 of Fig. 2A). When anti-myc antibody was used for immunoprecipitation, GFP-hPirh2 protein was coprecipitated and detected by Western blot with anti-GFP antibody (lane 2 of Fig. 2B). pEGFP and pCMV-Myc vectors were used as control (lane 1 of Figs. 2A and B). Both results of in vitro and

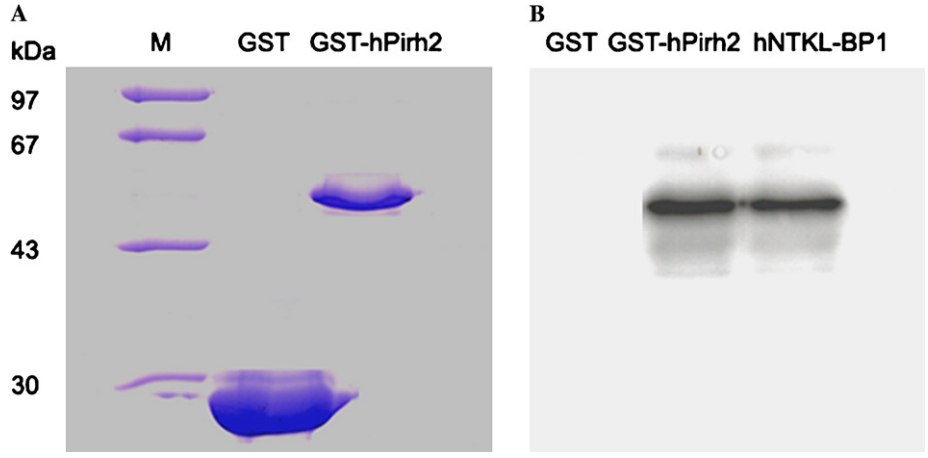

Fig. 1. Interaction of hNTKL-BP1 with hPirh2 in vitro. (A) Fusion proteins of GST-hPirh2 and GST were expressed and purified, respectively. (B) Interactions of hNTKL-BP1 with hPirh2 were analyzed by GST-pull-down assay. hNTKL-BP1 binds to hPirh2, but not to GST alone. The translation product of hNTKL-BP1 in vitro was used as a control.

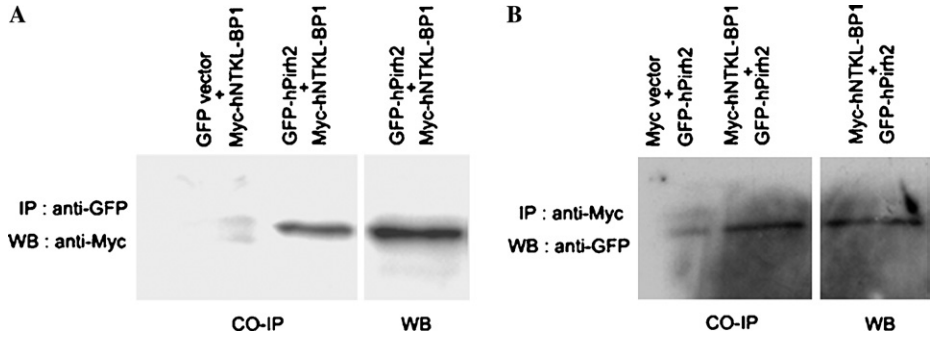

Fig. 2. Interaction of hNTKL-BP1 with hPirh2 in vivo. (A) Western blot was performed with anti-myc antibody. pEGFP vector was used as control (lane 1); pEGFP-hPirh2 was expressed in cotransformed cells (lane 3); and when anti-GFP antibody was used for immunoprecipitation, Myc-hNTKL-BP1 protein was coprecipitated (lane 2). (B) Western blot was performed with anti-GFP antibody. pCMV-Myc vector was used as control (lane 1); Myc-hNTKL-BP1 was expressed in cotransformed cells (lane 3); and when anti-myc antibody was used for immunoprecipitation, GFP-hPirh2 protein was coprecipitated (lane 2).

in vivo assays are in agreement with those of the yeast two-hybrid assay.

To examine whether hNTKL-BP1 colocalizes with hPirh2 in cells, hPirh2 and hNTKL-BP1 were cotransfected into SMMC 7721 cells. The immunofluorescent staining results showed that hNTKL-BP1 was localized in cytoplasm surrounding the nucleus (Fig. 3A) and hPirh2 exhibited a diffuse nuclear and cytoplasm distribution (Fig. 3B). Merged images (Fig. 3D) showed that hNTKL-BP1 and hPirh2 colocalized in cytoplasm surrounding the nucleus. This suggested that the interaction between hNTKL-BP1 and hPirh2 could have occurred in cytoplasm surrounding the nucleus.

To determine the binding sites between hNTKL-BP1 and hPirh2, we constructed two deletion mutants of hNTKL-BP1 containing N-terminal coiled-coil domain and C-terminal coiled-coil domain, respectively, and two deletion mutants of hPirh2 containing a ZnF_NFX domain and a RING finger domain, 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. Both the yeast two-hybrid assays and β-galactosidase experiments (Fig. 4) showed that C-terminus of hNTKL-bp1 and N-terminus of hPirh2 were identified as the regions responsible for the interaction between two proteins. Furthermore, the interaction between C-terminus of hNTKL-BP1 and hPirh2 was strongest. This suggested that the C-fragment of hNTKL-BP1 could substitute the whole hNTKL-BP1 protein in interaction with the hPirh2 protein.

Pirh2 (p53 induced RING-H2 protein) was initially reported as androgen receptor N-terminal interacting protein (ARNIP) [5]. Pirh2 contains 261 amino acids and a RING-H2 (C3H2C3) finger domain, which is defined by a consensus sequence with six cysteines and two histidines that coordinate two zinc ions. With the intact RING-H2 domain, Pirh2 can function as a ubiquitin–protein ligase (E3) in vitro and in vivo. Recently Pirh2 was found to be regulated by p53 and interact with p53 [4]. Pirh2 is involved in an autoregulatory feedback loop that controls p53 function. Pirh2 can facilitate degradation of p53 via ubiquitin–proteasome pathway independently of Mdm2. Individual E3s often control the ubiquitination of multiple substrates [6] so Pirh2 may

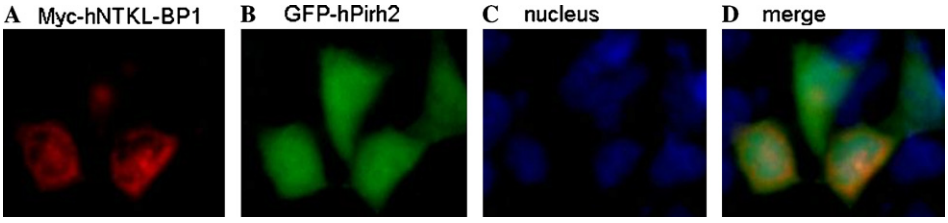

Fig. 3. Colocalization of hNTKL-BP1 and hPirh2. SMMC 7721 cells were transfected with pEGFP-hPirh2 and pCMV-Myc-hNTKL-BP1. (A) Cytoplasm surrounding the nucleus localization of hNTKL-BP1 (red). (B) Diffuse nuclear and cytoplasm distribution of hPirh2 (green). (C) Nucleuses were stained with DAPI (blue). (D) Colocalization (yellow) of hNTKL-BP1 and hPirh2 in cytoplasm surrounding the nucleus. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

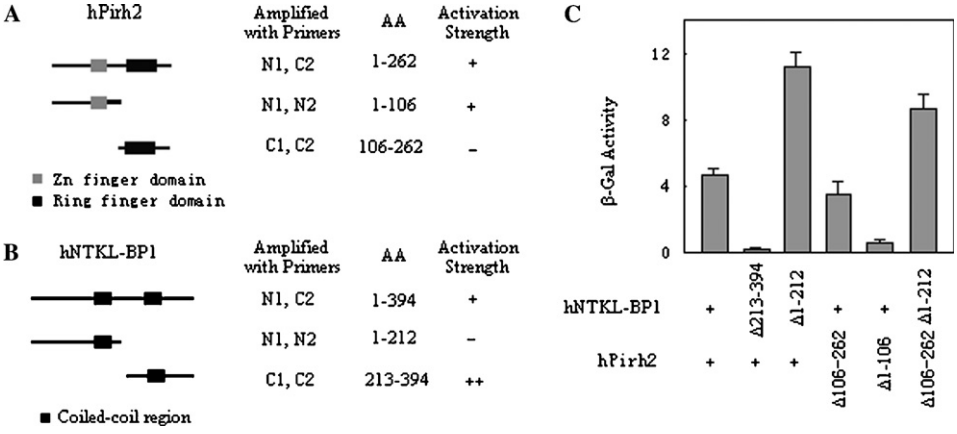

Fig. 4. C-terminus of hNTKL-BP1 and N-terminus of hPirh2 were identified as the regions responsible for the interaction between two proteins. The hNTKL-BP1-C fragment can substitute the full hNTKL-BP1 in interaction with hPirh2. (A) The sequential deletion of hPirh2. (B) The sequential deletion of hNTKL-BP1. (C) Quantitative assays for β-galactosidase. The error bars indicate SEM ($n = 3$).

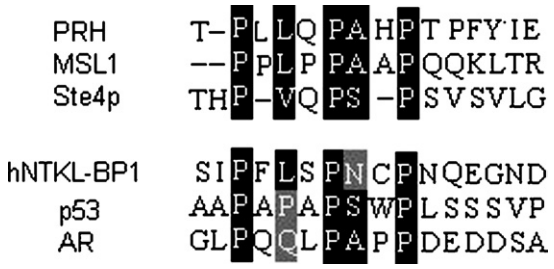

Fig. 5. Alignment of a proline-rich sequence shared by the Pirh2-binding regions of hNTKL-BP1, p53, and AR. This region was observed in PRH, MSL1, and Ste4p to bind the members of RING family [7]. Black shading indicates 100% conservation of amino acid similarity, white on gray does not match the conserved sequence.

target other substrates. Maybe hNTKL-BP1 is one of them. Borden proposes that a proline-rich (P-X-L/V-X-P-A/S-X-P) sequence mediates interaction with RING proteins [7]. hAR (androgen receptor) and p53 also contain a proline-rich sequence that matches this consensus at five out of six positions. Furthermore, this motif is within the region binding to Pirh2 of hAR and P53 (Fig. 5). Interestingly, in the C-terminus of hNTKL-BP1 responsible for the interaction with hPirh2, hNTKL-BP1 protein also contains a proline-rich sequence. This suggests that it may play roles in interaction of hNTKL-BP1 with hPirh2, and hNTKL-BP1 may be a substrate of hPirh2. It stimulates 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. Recently, Tip60 protein was shown to bind to hPirh2 to stabilize the hPirh2 protein levels [10].

Many proteins have been demonstrated to be substrates of ubiquitin ligase. These proteins participate in a variety of cellular functions, including regulation of cyclin-dependent kinase activity, activation of transcription, and signal transduction [8]. The interaction of E3s with specific substrates can be constitutive or regulated by phosphorylation [9]. For example, for SCF^{CDC4} E3, each of the known substrates must be phosphorylated before they can be recognized by SCF^{CDC4} [8]. Bioinformatic assays revealed hNTKL-BP1 has 27 predicted phosphorylation sites. In preliminary experiments, we have observed that hNTKL-BP1 could interact with NTKL (data not shown), which contains a potential protein kinase domain without enzyme activity. Through interaction with hNTKL-BP1, NTKL may play as an inhibitor preventing phosphorylation of hNTKL-BP1. This indicates that the interaction between hNTKL-BP1 and NTKL may affect the interaction between hNTKL-BP1 and hPirh2. The relationship of hNTKL-BP1 with hPirh2 and NTKL deserves further investigation.

Acknowledgments

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