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HIPK2 associates with RanBPM

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Abstract

Using the yeast two-hybrid system, we have identified the Ran-binding protein (RanBPM) as an interaction partner of homeodomain-interacting protein kinase 2 (HIPK2). RanBPM has been described as a centrosomal protein through which Ran regulates the centrosomal function. HIPK2 is mainly a nuclear protein, which among other functions represses transcription mediated by homeodomain containing transcription factors. Here, we show that overexpressed wildtype HIPK2 and a kinase defective mutant of HIPK2 directly interact with RanBPM in the nucleus of mammalian cells. Overexpressed wildtype RanBPM and a kinase defective mutant of HIPK2 co-localise with HIPK2 in defined nuclear structures. A carboxy- and an amino-terminal deletion of HIPK2 do not seem to be able to bind to RanBPM.

Keywords: HIPK2; Nucleus; RanBPM; Yeast two-hybrid system; Overexpression; Confocal laser scanning microscopy

Homeodomain-interacting protein kinases (HIPKs) constitute a novel family of nuclear protein kinases. Three family members have been isolated so far [1]. HIPK2 has been described as a homeodomain-interacting protein kinase, which acts as a co-repressor for homeodomain transcription factors [1], and as an interaction partner with the interferon type I induced MxA protein [2]. HIPK2 is modified by a ubiquitin-like protein, the small ubiquitin-related modifier 1 (SUMO-1) [3]. HIPK2 may play a regulatory role in the repression of transcription by the NK-3 homeodomain protein [4]. Sumoylated HIPK2 localises to the nucleus in a speckled pattern, but the role of this subcellular localisation is unknown [3].

Ras-like nuclear G protein (Ran) is a unique Ras-like small G protein with a nuclear localisation. It plays different roles by interacting with a wide range of proteins in various intracellular locations. Ran shuttles between the nucleus and cytoplasm and thereby mediates active nuclear and cytoplasmic exchange of macromolecules [5,6]. In addition, Ran is involved in microtubule aster formation during mitosis [6]. Ran-binding protein (RanBPM) was identified via yeast two-hybrid screening using Ran as bait [7]. RanBPM has been described to localise to the centrosomes and appears to be involved in microtubule nucleation, which implies that Ran participates in microtubule organisation [7]. A 90 kDa form of RanBPM that does not display the centrosomal expression pattern has been recently discovered [8].

Here, we identified RanBPM as an interaction partner of HIPK2 by using the yeast two-hybrid system. We show that overexpressed wildtype HIPK2 and a kinase defective point mutant of HIPK2 interact and co-localise in the nucleus with overexpressed RanBPM.

Materials and methods

Yeast two-hybrid assay. Yeast two-hybrid analysis was performed using the MATCHMAKER Two-Hybrid System 3 (Clontech) according to manufacturer's instructions. For bait construction, the 5′-region (HIPK2 cDNA positions 117–1698) starting with the ATG codon of the human HIPK2 cDNA (GenBank Accession No. AF208291) [9] was amplified by PCR by using 5′-GAATTCATGGCC CCCGTGTACGAAG as 5′-primer and 5′-G TCGACCATGGTGAC

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AAAGGGATG as 3′-primer. The 3′-region of human HIPK2 (cDNA positions 1699–3296) was amplified by PCR using 5′-GAATTCACCC ATGACACACTTACTC as 5′-primer and 5′-GTCGACCTTCGGTG GCTCCCAGTG as 3′-primer. The PCR fragments were cloned into the *Eco*RI and *Sal*I sites of pGBKT7 (Clontech), respectively. The pretransformed MATCHMAKER human brain cDNA library (Clontech) in yeast strain Y187 was screened for HIPK2 interaction partners. The effect on cell toxicity and transcriptional activation of the baits was tested by transforming the bait constructs into yeast strain AH109 (Clontech). Both bait constructs did not show any toxic effect and autonomous transcriptional activation on the host strain. In each bait mating 9×10^5 colonies were screened. The positive candidate colonies which grew on minimal synthetic dropout medium for yeast (SD)/–Ade–His–Trp–Leu were plated onto SD/–Ade–His–Trp–Leu/X-α-Gal to perform β-Gal colony-lift filter assays. The positive plasmids were isolated, transformed to *Escherichia coli* KC8, and plated on M9/amp/–Leu to selectively rescue only activation domain (AD)/library plasmids. The positive plasmids were co-transformed onto SD/–Ade–His–Trp–Leu plates again.

Sequencing. Plasmid DNA of all positive clones was isolated with the QIAamp DNA Isolation Kit (Qiagen) according to manufacturer's protocol. The inserts of all positive clones were sequenced by dideoxy sequencing using a 373A automated sequencer (Applied Biosystems) by using 5′-CTATTCGATGATGAAGATACCCCACCAAACCC as forward primer and 5'-TCGTAGATACTAAAAAACCCCGCAAG TTCAC as reverse primer. The positive cDNAs coding for RanBPM were sequenced with primers Ran1 5′-GTAAAAATTGTCAGTAA GGG, Ran2 5′-GATTTCCTATCGGAGATCG, Ran3 5′-GTAGTCC AAGTATGAGCCCCAG, Ran4 5′-GTTGTGTGGAGGAAGTCAG GCCG, and Ran5 5′-GACTACCTACATTAGCTATG. Prediction of protein sorting signals and localisation sites was done by using PSORT II (http.//psort.nibb.ac.jp).

β-Gal assay. The 5′ HIPK2-pGBKT7 or 3′ HIPK2-pGBKT7 bait constructs were co-transfected with RanBPM–pACT2 into yeast strain Y187 onto SD/–Trp/–Leu/–His plates according to manufacturer's protocol (Clontech). The β-Gal assay was performed using *o*-nitrophenyl β-D-galactopyranoside (ONPG) (Sigma) as substrate.

Deletion mutatgenesis of HIPK2. The carboxy-terminal deletion of HIPK2 (ΔC) results in a gene product of amino acids from 1 to 527, the amino-terminal deletion of HIPK2 (ΔN) in a gene product of amino acids from 559 to 1198 (Fig. 2B). The corresponding cDNA fragments were cloned into pcDNA3 (Invitrogen) as described [10].

Transient transfection, co-immunoprecipitation, and Western blotting. Human embryonic kidney 293T cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated foetal bovine serum, L-glutamine, and penicillin/streptomycin. The cDNA coding for human RanBPM was subcloned into pCMV-myc (Clontech) via *Sfi*I and *Xho*I. RanBPM–pCMV-myc was cotransfected with Flag-HIKP2–pcDNA3.1 into 293T cells. Transient transfections were performed by the calcium phosphate method using 2 μg of each plasmid DNA as indicated. Prior to transfection, approximately 5×10^5 cells were plated in 60 mm dishes to a confluency of approximately 20%. Cells were harvested at 20–24 h following transfection and lysed in 500 μl lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 0.5% Triton X-100, and 20 μl/ml protease inhibitor cocktail (Sigma)). Fifty μl was taken for direct Western blot analysis and additional 500 μl lysis buffer was added to the original lysate. Immunoprecipitation was done with 2 μg/ml anti-Flag monoclonal antibody M2 (Sigma) at 4 °C for 1 h. Then, 20 μl/ml protein A/G plus–agarose beads (Santa Cruz Biotechnology) was added, incubated, and continuously rotated overnight at 4 °C. Immunoprecipitates were washed four times with 1 ml lysis buffer and resuspended in SDS loading dye. For Western blot analysis, half of the immunoprecipitated proteins (20 μl) were separated on 10–20% gradient gels (Daiichi) and blotted onto nitrocellulose membranes (Amersham-Pharmacia) according to standard protocols. The first antibodies were anti-Flag or anti-c-myc (Clontech) at a 1:1000 dilution, respectively. The secondary antibody was anti-mouse IgG1 coupled to horseradish peroxidase (Santa Cruz Biotechnology) at a dilution of 1:5000. Specific bands were detected using chemiluminescence detection reagents (Amersham-Pharmacia). To test the expression, 20 μl total cell lysates were analysed in parallel by Western blotting.

Immunofluorescence and confocal scanning microscopy. HeLa cells were maintained in RPMI 1640 containing 10% heat-inactivated foetal bovine serum, L-glutamine, and penicillin/streptomycin (all from Gibco Life Technologies). Expression vectors with inserts coding for Flag-HIPK2 and c-myc-RanBPM were transiently transfected into HeLa cells grown on CELLocate coverslips (Eppendorf). The cells were washed with PBS and fixed for 10 min with 4% paraformaldehyde at room temperature. After blocking with blocking solution (10% FCS, 10% BSA, 1% cold water fish gelatine, and 50 mM NH_4Cl), the cells were incubated with a 1:50 dilution of FITC conjugated monoclonal anti-Flag and a 1:100 dilution of the rhodamine-based Cy3 conjugated monoclonal anti-c-myc antibody (all from Sigma) in the case of Flag-HIPK2 and c-myc-RanBPM co-localisation staining. Before use, the anti-c-myc antibody was diluted in human serum and centrifuged at 40,000 rpm for 30 min at 4 °C (Beckman, Optimatm TLX) and supernatant was applied directly.

In parallel, the cells were incubated with a monoclonal anti-α-tubulin (Sigma) and polyclonal anti c-myc-antibodies (Clontech). The secondary antibodies were Cy3 conjugated anti-mouse IgG (Sigma) and a FITC conjugated anti-rabbit IgG (Sigma). The slides were mounted with Mowiol and visualised with a Leica TCS-NT confocal scanning microscope equipped with an argon/krypton laser light. A PlanApo objective with 63× magnification was applied for examination. *z*-Scans of about 0.2 μm were performed to identify co-localisation on a subcellular level. FITC-fluorescence excitation was determined at 488 nm and green fluorescence emission was measured at >515 nm. To selectively measure red, Cy3-specific fluorescence, we excited at 568 nm and emission wavelength was >595 nm. Co-localisation of FITC- and Cy3-labelled proteins was identified by overlay analysis and is indicated as orange, respectively, yellow fluorescence.

Results and discussion

To identify novel proteins interacting with HIPK2, we used the yeast two-hybrid system to screen for proteins that bind to either the carboxy or amino terminus of HIPK2. We screened a human brain cDNA library, since relatively high levels of HIPK2 mRNA are expressed in neuronal tissue [9].

In our screenings, 28 positive cDNAs were isolated by using the 5′ HIPK2 bait and 39 clones by using the 3′ HIPK2 bait. Among these, 29 cDNA clones consisted of so far undescribed sequences; two independently isolated cDNAs coded for human RanBPM (GenBank Accession No. AB008515) [7]. A 2667 bp long cDNA, clone 3-15 (GenBank Accession No. AF306501), was isolated with the 5′ HIPK2 bait and a 826 bp long cDNA, clone 16-1, with the 3′ HIPK2 bait. Both cDNA clones contained the RanBPM ATG start codon and the longer of the two clones (clone 3-15) contained 322 bp additional 3′ untranslated sequences as the described RanBPM cDNA.

To further evaluate the interaction between RanBPM and HIPK2 in yeast, a quantitative β-galactosidase assay was performed. Plasmids containing either the 5′ or the 3′

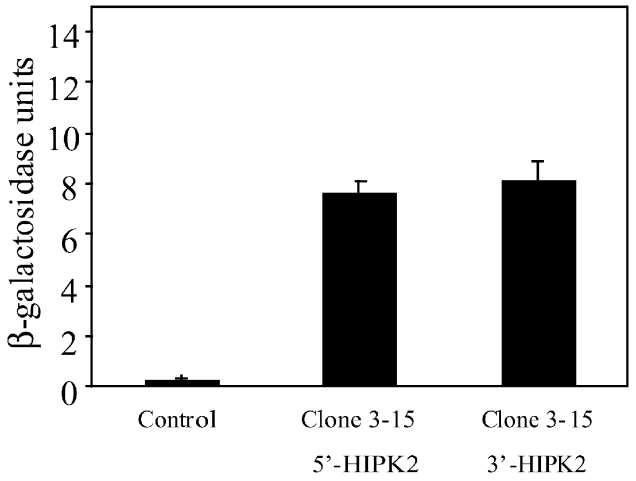

Fig. 1. Association of RanBPM with HIPK2 in yeast. pACT2-RanBPM and the 5′ or 3′ HIPK2-pGBK7 baits were co-transformed into yeast strain Y187 as indicated. From selected positive colonies, β-galactosidase activity was measured. Error bars represent the standard deviation from three independent colonies.

HIPK2 cDNA fragment fused to the cDNA fragment coding for the DNA-binding domain of Gal4 were co-transformed with a plasmid containing the full length RanBPM cDNA fused to the activation domain of Gal4 into yeast strain Y187 (Fig. 1). The colonies expressing either 5′ HIPK2 or 3′ HIPK2 showed a significant increase in β-galactosidase activity in the presence of RanBPM in comparison to the control expressing RanBPM alone (Fig. 1) or HIPK2 alone (data not shown).

We next tested whether RanBPM is able to associate with HIPK2 in mammalian cells. We co-transfected 293T cells with constructs encoding tagged wildtype HIPK2 and RanBPM, respectively (Fig. 2A). After immunoprecipitation of Flag-HIPK2, a clear signal was detected after Western blot analysis of c-myc-RanBPM. This showed that RanBPM can bind to wildtype HIPK2 in mammalian cells when both proteins are overexpressed.

A more detailed co-immunoprecipitation analysis was performed with several mutants of HIPK2 (Fig. 2B). Protein expression of full length Flag-HIPK2 is lower than that of truncated Flag-HIPK2. The kinase defective HIPK2 mutant K221A showed an association with RanBPM after immunoprecipitation similar to that obtained with wildtype HIPK2 (Fig. 2C). Only a faint signal was observed with the HIPK2 ΔN and HIPK2 ΔC deletion mutants under these conditions (Fig. 2C). This suggests that both, amino- and carboxy-termial sequences of HIPK2, are necessary for association with RanBPM in mammalian cells.

HIPK2 interacts with proteins that show a nuclear localisation [2,3]. In addition, we obtained data showing that HIPK2 activates the tumour suppressor protein p53 via downregulation of Mdm2 protein levels [9]. Another member of the HIPK family, HIPK3, interacts with the death receptor CD95 and inhibits CD95-mediated Jun NH_2-terminal kinase activation [11]. HIPK2 may also play a role in death receptor mediated apoptosis, since overexpressed HIPK2 associates with TRADD [12]. However, the intracellular localisation of overexpressed TRADD is not clear at present.

The probability prediction for a nuclear localisation [13] is 89% for RanBPM and 94.1% for human HIPK2. The nearest neighbour algorithm [14] suggests a 56.5% likelihood for RanBPM and a 69.6% likelihood for human HIPK2 to exist as a nuclear protein. The expected nuclear co-localisation of HIPK2 and RanBPM was detected by immunofluorescence after overexpression of both proteins in HeLa cells (Figs. 3A and B). In contrast to HIPK2, RanBPM showed an additional cytoplasmic distribution, which was due to unspecific binding of the anti-c-myc antibody (Fig. 3B). Therefore, in addition to

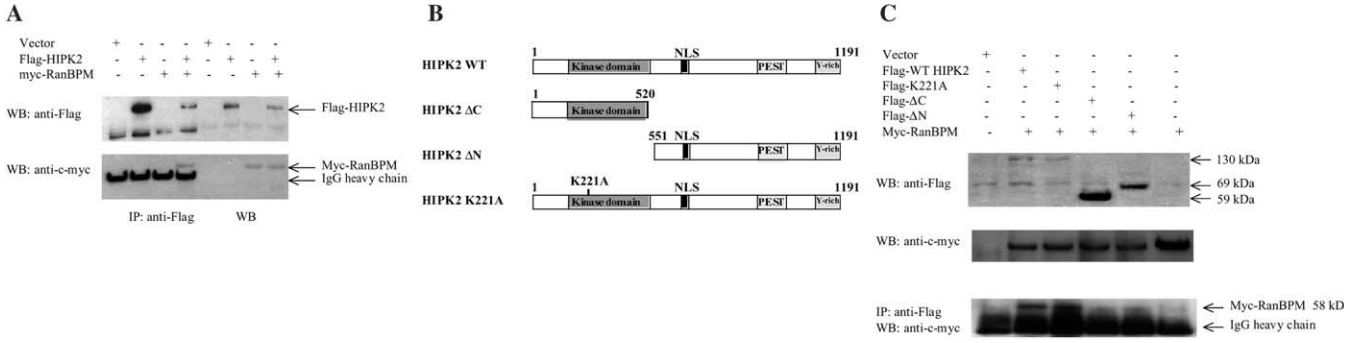

Fig. 2. Association of RanBPM with HIPK2 and mutated HIPK2 in 293T cells. (A) 293T cells were transiently co-transfected with the indicated combinations of expression constructs coding for wildtype Flag-HIPK2 and c-myc-RanBPM or vector. Cells were lysed and immunoprecipitated with an anti-Flag antibody. Western blotting was performed with total lysates (WB) and the immunoprecipitates (IP) with either the anti-Flag antibody (top) or anti-c-myc antibody (bottom). (B) Schematic diagram of wildtype (WT), the carboxy-terminal deletion (ΔC), the amino-terminal deletion (ΔN), and the kinase defective point mutant K221A [10]. Numbers on the top indicate amino acid residues. The kinase domain, the putative nuclear localisation signal (NLS), the PEST region, and the tyrosine (Y)-rich regions are indicated. (C) 293T cells were transiently co-transfected with the indicated combinations of expression constructs coding for wildtype (WT) Flag-HIPK2 or the Flag-HIPK2 mutants and c-myc-RanBPM. The first lane shows vector transfected cells. The assay was done as described in (A). The molecular weight of c-myc-RanBPM is 58 kDa.

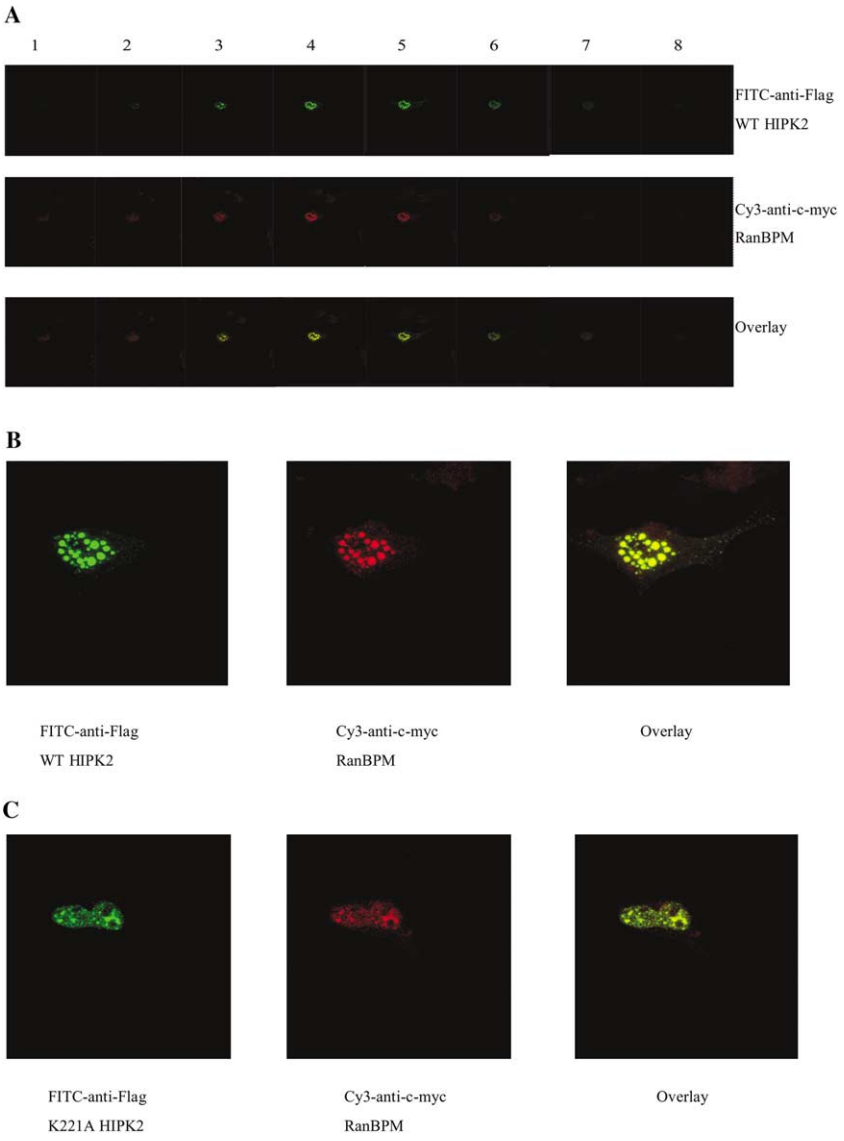

Fig. 3. Intracellular localisation of overexpressed RanBPM and HIPK2. Wildtype Flag-HIPK2 and c-myc-RanBPM were transiently transfected into HeLa cells. Cells were incubated with a monoclonal anti-c-myc antibody coupled with Cy3, which stains c-myc-RanBPM. Cells were then incubated with a monoclonal anti-Flag antibody coupled with FITC, which stains Flag-HIPK2 as indicated. Yellow fluorescence indicates co-aggregation. (A) Eight individual *z*-scans demonstrate expression of HIPK2, RanBPM and co-localisation in the overlay, respectively. (B) The projected image (overlay of eight individual *z*-scans) shows co-localisation in the large nuclear speckles. (C) The kinase defective mutant K221A of HIPK2 also co-localises with RanBPM.

its centrosomal localisation [7], a fraction of RanBPM overexpressed in HeLa cells was clearly localised to the nucleus and associates with HIPK2. The expression of HIPK2 as a highly symmetric speckled pattern in the interphase nucleus co-localised with RanBPM. The co-localisation of anti-c-myc-RanBPM (red) and anti-flag-HIPK2 antibodies (green) in the nuclear speckles was most prominent in individual sections selected from the scanned cell in the *z*-axis (Fig. 3A). This indicated that not all the speckles staining for HIPK2 contain RanBPM protein as well.

Although the nuclear and highly organised speckled HIPK2 pattern was found in all cells transfected with the wildtype gene, the size of the speckles may vary in size and numbers (Figs. 3A and B). This may be either due to experimental overexpression or may hint at a dynamic process related to the cell cycle or other processes involving nuclear organisation.

Overexpression of the kinase defective K221A mutant of HIPK2 in HeLa cells resulted in a more diffuse pattern of the nuclear speckles. The co-expression of RanBPM with the HIPK2 K221A mutant yielded a similar picture (Fig. 3C). The overlay showed that RanBPM associated with the HIPK2 K221A mutant only in the nucleus (Fig. 3C). Most likely, the HIPK2 mutant dictates the nuclear distribution of RanBPM.

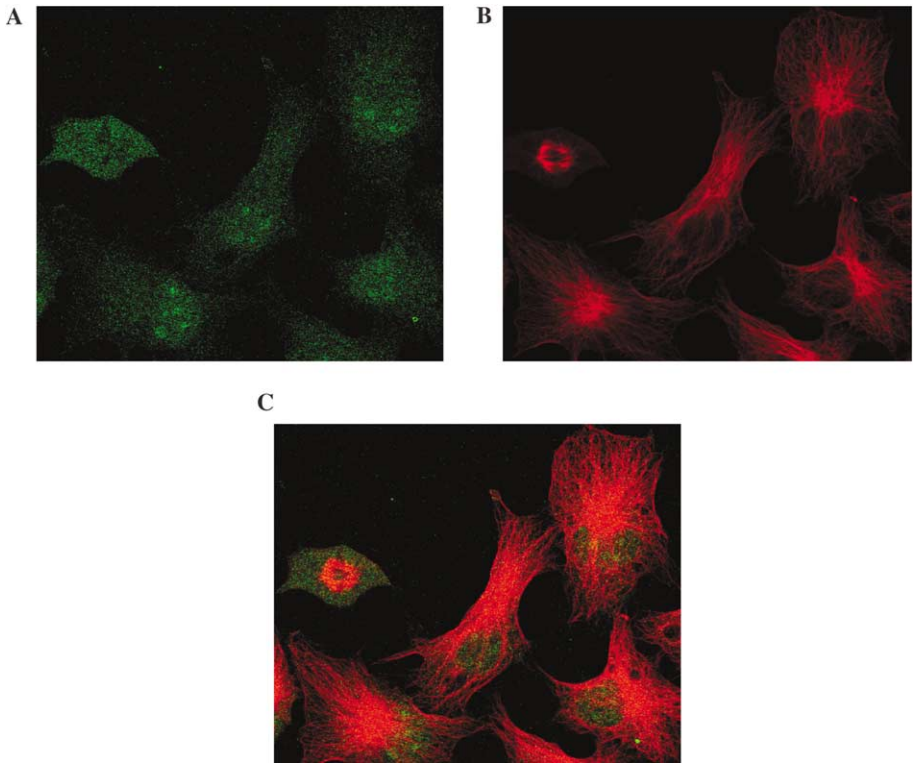

Fig. 4. Intracellular localisation of α-tubulin and overexpressed RanBPM. Wildtype Flag-HIPK2 and c-myc-RanBPM were transiently transfected into HeLa cells. (A) Cells were incubated with a polyclonal anti-c-myc antibody and a FITC conjugated anti-rabbit IgG-antibody as secondary antibody. (B) Cells were then incubated with a monoclonal anti-α-tubulin antibody and a Cy3 conjugated anti-mouse IgG antibody as secondary antibody. (C) Overlay of representative single TIFF files from *z*-scans are shown, co-localisation is not seen.

Next, we used a carboxy-terminal deletion of HIPK2 (ΔC), which does not contain the putative nuclear localisation signal but contains the kinase domain, and an amino-terminal deletion of HIPK2 (ΔN), which does not contain the kinase domain [9], to analyse the regions involved in interaction. A nuclear and cytoplasmic distribution of the HIPK2 ΔC deletion mutant has been demonstrated [3,9]. Here, HIPK2 ΔC was mainly localised in the nucleus, whereas HIPK2 ΔN showed a major cytplasmic localisation. In contrast, another amino-terminal deletion of HIPK2, which contained only part of the putative nuclear localisation signal, still remained mainly in the nucleus [3]. Neither HIPK2 ΔC nor HIPK2 ΔN displayed a co-expression with RanBPM (data not shown), which is in agreement with the fact that RanBPM did not co-immunoprecipitate with the HIPK2 deletion mutants (Fig. 2C). Full length HIPK2 was a prerequisite for the localisation of RanBPM to nuclear speckles.

The question arising about how RanBPM and HIPK2 are translocated into the nucleus cannot be answered at this stage. However, co-staining experiments show that microtubules are not directly involved: neither HIPK2 nor RanBPM co-localises with α-tubulin (Fig. 4).

Along this line, the small GTPase Ran has been described to be associated with γ-tubulin and organisation of chromosomes on the one hand and with the microtubule organising centres on the other [15]. γ-Tubulin organises α- and β-tubulin. We cannot answer the question whether RanBPM is also involved in microtubule organisation at this experimental stage. Microtubules are involved in nuclear transport, as has been recently demonstrated for p53 [16].

The centrosomal localisation of RanBPM has not been confirmed [8]. The 55 kDa protein of RanBPM may be a truncated version of a 90 kDa protein [8]. Until now, the functional significance of binding of HIPK2 to RanBPM remains to be identified. Three-dimensional reconstitution of the nuclear pattern in transfected as well as in naturally HIPK2 and RanBPM expressing cells will contribute to our understanding of nuclear proteins and their influence of gene transcription and protein transport.

Acknowledgments

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