PML Is Required for Homeodomain-interacting Protein Kinase 2 (HIPK2)-mediated p53 Phosphorylation and Cell Cycle Arrest but Is Dispensable for the Formation of HIPK Domains

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Abstract

Here we demonstrate that endogenous human homeodomain-interacting protein kinase (HIPK) 2 and the highly homologous kinase HIPK3 are found in a novel subnuclear domain, the HIPK domains. These are distinct from other subnuclear structures such as Cajal bodies and nucleoli and show only a partial colocalization with promyelocytic leukemia (PML) nuclear bodies (PML-NBs). A kinase inactive HIPK2 point mutant is localized in the nucleoplasm. The occurrence of HIPK domains in PML–/– fibroblasts reveals their independence from the PML protein. HIPK2 can be almost completely recruited to PML-NBs by the PML isoform PML IV, but not by PML-III. PML IV-mediated recruitment of HIPK2 does not rely on its kinase function and also occurs in PML–/– fibroblasts, showing that this PML isoform is sufficient for recruitment of HIPK2. Whereas the architecture of HIPK domains is PML independent, HIPK2-mediated enhancement of p53-dependent transcription, p53 serine 46 phosphorylation and the antiproliferative function of HIPK2 strictly rely on the presence of PML.

Introduction

The cell nucleus is highly organized and contains several substructures lacking delineating membranes. These nuclear compartments include the nucleoli, PML-NBs (also named nuclear domain 10 or PML oncogenic domain), the splicing factor compartments, Cajal bodies, and a rapidly growing family of additional dot-like subnuclear structures (1). The Cajal bodies are involved in the biogenesis of small nuclear ribonucleoproteins, and their structural integrity depends on the presence of coilin (2, 3). PML-NBs contain several critical regulators of cell proliferation, apoptosis, and genome stability, including the tumor suppressor PML. PML-NBs are disrupted in APL cells, whereas transiently recruited proteins include p53, CBP, and the serine/threonine kinase HIPK2 (9, 13, 14). An important function of HIPK2 is the inducible phosphorylation of p53 at the NH2-terminal serine 46, which contributes to p53-dependent gene expression and, thereby, cell cycle arrest and apoptosis (13, 14). HIPK2 is evolutionary conserved, contains an NH2-terminal kinase domain and localizes to nuclear speckles, raising the question whether (a) HIPK domains form an independent subnuclear compartment and (b) whether HIPK2-mediated p53 phosphorylation and cell cycle arrest depend on PML-NBs.

Materials and Methods

Expression Vectors and Antibodies. Antibodies recognizing Flag (M2), p21Waf (F-5), and PML (PG-M3) were obtained from Santa Cruz Inc., and the phospho-p53-serine 46 antibody was obtained from Cell Signaling Technology. Antibodies detecting the nucleolar marker NOH61 was a kind gift of Dr. M. Schmitt-Zachmann [German Cancer Research Center (DKFZ), Heidelberg, Germany], the polyclonal antiHIPK2 antibody was described previously (14). GFP-HIPK2 fusion proteins were constructed by standard PCR techniques, expression vectors for PML-IV, PML-III (15), Flag-HIPK2, Flag-HIPK3 K221A, p53, GFP-p53, the pG13-luc reporter plasmid (14), Flag-HIPK3 (16) and the pEF-Puro plasmid (17) were described previously.

Cell Culture and Transient Transfections. Human U2OS osteosarcoma cells, H1299 cells, and MEFs were grown in DMEM supplemented with 10% (v/v) FCS and 1% (v/v) penicillin/streptomycin in a humidified incubator at 37°C and 5% CO2. Cells were transfected using the Superfect reagent (Qiagen Inc.) or Metafectene (Biontex Inc.) according to the manufacturer’s instructions.

Western Blot Analysis. Cells were harvested, and cell pellets were lysed in NP40 lysis buffer [20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM phenylmethylsulfonylfluoride, 10 mM NaF, 0.5 mM sodium vanadate, leupeptin (10 μg/ml), aprotinin (10 μg/ml), 1% (v/v) NP40, and 10% (v/v) glycerol]. Cell debris was removed by centrifugation, and equal amounts of protein contained in the supernatants were analyzed by reducing SDS-PAGE and Western blotting as described previously (14).

Immunofluorescence. Cells were grown in 12-well plates on coverslips and transfected with 10–300 ng of expression vector, were washed once with PBS, and were fixed for 5 min at −20°C with methanol/acetone (1:1). Staining with primary and secondary antibodies was done essentially as described previously (14).

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5 The abbreviations used are: PML-NB, promyelocytic leukemia nuclear body; PML, promyelocytic leukemia; HIPK, human homeodomain-interacting protein kinase; APL, acute promyelocytic leukemia; MEF, mouse embryonic fibroblast; RAR, retinoic acid receptor; GFP, green fluorescent protein; CBP, cAMP-response-element-binding protein.
**Colony Assays.** Cells were transfected with the indicated vectors and pEF-Puro. A sample of the cells was tested to ensure comparable protein expression. Two days after transfection, puromycin (1 μg/ml) was added, and surviving cells were further grown to colonies, which were stained with crystal violet.

**Results and Discussion**

To compare the intranuclear localization of human HIPK2 with that of other subnuclear compartments, U2OS cells were transfected with a GFP-tagged HIPK2 expression plasmid and analyzed by indirect immunofluorescence for costaining with the nucleolus marker protein NOH61 (18). These experiments revealed the majority of HIPK2 in subnuclear speckles and only a minor fraction in the nucleoplasm, but no colocalization with the nucleolus helicase NOH61 (Fig. 1A). When a similar experimental approach was taken to compare localization of HIPK2 with that of the Cajal body marker protein coilin (Fig. 1B), no overlapping localization of both compartments was found. A control experiment ensured partial colocalization of GFP-HIPK2 with PML (Fig. 1C). Additional experiments comparing HIPK2 and the highly homologous kinase HIPK3 revealed extensively overlapping localization (Fig. 1D) of both kinases in a domain that we will refer to as the HIPK domain.

The PML protein occurs in at least seven different isoforms, which differ in their COOH-terminal portions. All of the isoforms can target p53 into PML-NBs (19), but only PML-IV is able to regulate p53 activity (15, 20) and to induce premature senescence (19). To test whether distinct PML isoforms differ in their ability to recruit HIPK2 to PML-NBs, either PML-IV or PML-III were expressed in U2OS cells. Indirect immunofluorescence showed that PML-IV completely recruited endogenous HIPK2 to PML-NBs (Fig. 2A), whereas PML-III expression failed to assemble HIPK2 in these subnuclear structures (Fig. 2B). To map the HIPK2 region required for PML-IV-mediated recruitment, various GFP-tagged HIPK2 mutants were expressed either alone or together with PML-IV in U2OS cells. A kinase-deficient point mutant of HIPK2 (GFP-HIPK2 K221A) did not localize to nuclear speckles or nucleoli and was found in the nucleoplasm (Fig. 2C). PML-IV recruited most of the kinase-deficient kinase to PML-NBs (Fig. 2D), showing that the kinase function is not required for PML-NB recruitment, but contributes to its localization to HIPK domains. Deletion of the COOH-terminal 670 amino acids of human HIPK2 (HIPK2 ΔC) resulted in a nucleoplasmic staining of the kinase (Fig. 2E), which is in accordance with results obtained for the murine form of HIPK2 (21). Expression of PML-IV failed to quantitatively recruit HIPK2 ΔC into PML-NBs (Fig. 2F), showing that efficient recruitment relies on the COOH-terminal portion of the kinase. Because HIPK2 and PML share some interaction partners such as CBP and p53 (22), it will be interesting to see whether PML-NB recruitment of HIPK2 involves indirect or direct interactions between HIPK2 and PML.
The fusion of PML to the RAR or the deletion of the PML gene results in the disruption of PML-NBs and a microspeckled distribution of PML-NB resident proteins (22). To investigate whether the NB localization of HIPK2 depends on the presence of PML, its localization was examined in PML\(^{-/-}\) MEFs. The typical HIPK domains still occurred in the absence of PML (Fig. 3A), showing that the formation of these domains is independent of the PML protein and, thus, defines HIPK domains as a novel nuclear domain. Expression of PML-IV in PML\(^{-/-}\) MEFs allowed the assembly of HIPK2 in PML-IV-NBs (Fig. 3B). These experiments demonstrate that PML-IV alone is sufficient for the recruitment of HIPK2 and highlight the importance of this PML splicing variant for HIPK2.

To test whether the recruitment of HIPK2 to PML-NBs affects HIPK2-mediated increase of p21\(^{WAF1}\) expression, we transfected U2OS
HIPK2-mediated p53 functions require PML-NBs. A, U2OS cells were transfected to express the indicated combinations of expression vectors encoding HIPK2 and PML-IV. Total cell lysates were analyzed by Western blotting for the expression of p21\textsuperscript{Waf}\textsuperscript{+} (top panel) and Flag-tagged HIPK2, PML, and β-actin (bottom panel). B, PML\textsuperscript{+} or PML\textsuperscript{−}\textsuperscript{−} MEFs were cotransfected with a luciferase reporter construct controlled by the p21\textsuperscript{Waf} promoter and an HIPK2 expression vector as shown. Transactivation by the empty expression vector was arbitrarily set as 1, error bars, SDs. C, PML\textsuperscript{−}\textsuperscript{−} or PML\textsuperscript{−}\textsuperscript{+} MEFs were transfected with expression vectors for HIPK2, p53, or PML-IV at the indicated combinations. Cell lysates were analyzed by Western blotting for the phosphorylation of serine 46 using phospho-specific antibodies and for the occurrence of the indicated proteins. D, PML\textsuperscript{−}\textsuperscript{−} and PML\textsuperscript{−}\textsuperscript{+} MEFs transfected to express GFP-p53 were treated for 8 h with Adriamycin (0.5 μg/ml), and protein extracts were analyzed by Western blotting as shown. E, the indicated cells were transfected with the plasmids for HIPK2, HIPK2 K221A, or PML-IV, together with a plasmid encoding the puromycin resistance gene. Transfected cells were selected with puromycin and surviving cell colonies were stained with crystal violet.

Fig. 3. HIPK domain formation is independent from PML. A, PML\textsuperscript{−}\textsuperscript{−} MEFs were transfected to express GFP-HIPK2 and were analyzed for distribution of HIPK2 (green). B, PML\textsuperscript{+} MEFs expressing GFP-HIPK2 and PML-IV were analyzed for the distribution of HIPK2 (green) and PML-IV (red), overlapping localization is displayed in yellow.

Fig. 4. HIPK2-mediated p53 functions require PML-NBs. A, PML\textsuperscript{−}\textsuperscript{−} MEFs were transfected to express GFP-HIPK2 and were analyzed for distribution of HIPK2 (green). B, PML\textsuperscript{−}\textsuperscript{−} MEFs expressing GFP-HIPK2 and PML-IV were analyzed for the distribution of HIPK2 (green) and PML-IV (red), overlapping localization is displayed in yellow.

An analysis of p21\textsuperscript{Waf} expression revealed that PML-IV-mediated recruitment of HIPK2 to PML-NBs did not affect p21\textsuperscript{Waf} expression (Fig. 4A). This gain-of-function approach showed that HIPK2 recruitment is not sufficient for transcriptional activation. To address this question by a loss-of-function approach, we transfected PML\textsuperscript{−}\textsuperscript{+} and PML\textsuperscript{−}\textsuperscript{−} MEFs with a p21\textsuperscript{Waf} reporter gene and an expression plasmid encoding HIPK2. The stimulatory effect of HIPK2 on induced transcription of p21\textsuperscript{Waf} occurred only in PML\textsuperscript{−}\textsuperscript{+} cells, but not in MEFs lacking PML (Fig. 4B). Similar results were obtained with experiments testing reporter genes controlled by the promoter for the p53 target gene Bax or by multimers of p53-binding sites (data not shown), revealing that HIPK2 recruitment to PML-NBs is necessary but not sufficient for transcriptional activation. Expression of PML-IV was shown to increase p53 serine 46 phosphorylation (19), but because serine 46 might also be phosphorylated by various kinases including ataxia-telangiectasia-mutated (ATM; Ref. 23), it remained unclear whether HIPK2-mediated p53 serine 46 phosphorylation occurs in PML-NBs or in the nucleoplasm. To address this question, the wild-type or kinase-deficient form of HIPK2 was coexpressed in MEFs from wild-type or PML\textsuperscript{−}\textsuperscript{−} mice along with human p53, because serine 46 is not contained in murine p53. Western blotting experiments using phospho-specific antibodies showed that HIPK2-mediated phosphorylation of serine 46 occurred only in PML\textsuperscript{−}\textsuperscript{+} MEFs, whereas no phosphorylation was seen in PML\textsuperscript{−}\textsuperscript{−} cells (Fig. 4C). Coexpression of PML-IV in PML\textsuperscript{−}\textsuperscript{−} MEFs allowed only a partial restoration of HIPK2-mediated serine 46 phosphorylation in PML\textsuperscript{−}\textsuperscript{−} MEFs, thus also revealing an important contribution of the other PML isoforms. DNA-damage-induced p53 serine 46 phosphorylation was tested by Adriamycin-treatment of MEFs transfected to express moderate amounts of GFP-tagged human p53 (Fig. 4D). These experiments showed that Adriamycin-induced p53 serine 46 phosphorylation, which is largely dependent on HIPK2 as revealed by knock-down experiments (data not shown), occurred only in the presence of PML. The role of PML for HIPK2-mediated biological functions such as regulation of cell proliferation was investigated by colony formation assays. In PML\textsuperscript{−}\textsuperscript{−} MEFs, HIPK2 prevented cell growth depending on its kinase activity (Fig. 4E). In contrast, PML\textsuperscript{−}\textsuperscript{−} cells were completely protected from the antiproliferative activity of HIPK2, thus supporting the relevance of PML-NBs for HIPK2 function. Accordingly, PML\textsuperscript{−}\textsuperscript{−} MEFs show an impaired induction of bona fide p53 target genes such as the proapoptotic Bax.
and the inhibitor of cell cycle, p21Waf (20). In summary, these data support the concept that PML-NBs form a platform for protein modifications, because not only HIPK2-mediated p53 phosphorylation but also radiation-induced acetylation of p53 is impaired in PML-/− cells (20).

Much of the controversy concerning PML-NB function may be explained by recent findings showing their heterogeneity. The analysis of their dynamic properties within living cells has revealed the stationary, slow-moving, and metabolic-energy-dependent dynamics of PML-NBs (24). Because only the subset of PML-NBs containing nuclear DNA helicase II displays transcriptional activity (25), it will be interesting to learn whether HIPK2 is associated with this transcriptionally active subset or whether HIPK2/p53-mediated transcription occurs outside of PML-NBs. There is recent evidence that overexpression of hamster HIPK2 (26) or human HIPK1 (27) results in relocalization or structural changes of PML-NB proteins, but the functional impact of these events for HIPK2 function remains to be elucidated. Our results also imply that the antiproliferative HIPK2/p53 cell death pathway is nonfunctional in APL cells expressing PML-RAR fusion proteins. Successful treatment with agents targeting the p53 pathway will require prior reformation of PML-NBs, e.g., by pharmacological doses of all-trans retinoic acid.

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