

RKIP Downregulation Induces the HBx-Mediated Raf-1 Mitochondrial Translocation

Kim, Sun Young¹, Sung Goo Park¹, Hyeyun Jung¹, Seung-Wook Chi¹, Dae Yeul Yu², Sang Chul Lee¹, and Kwang-Hee Bae^{1*}

¹Medical Proteomics Research Center, KRIBB, Daejeon 305-806, Korea

²Ageing Research Center, KRIBB, Daejeon 305-806, Korea

Received: December 20, 2010 / Revised: February 18, 2011 / Accepted: February 22, 2011

The Raf-1 kinase inhibitory protein (RKIP) can regulate multiple key signaling pathways. Specifically, RKIP binds to Raf-1 kinase and inhibits the Ras-Raf-1-MEK1/2-ERK1/2 pathway. Additionally, Raf-1 has been shown to translocate to mitochondria and thereby protect cells from stress-mediated apoptosis. Recently, HBx was found to stimulate the mitochondrial translocation of Raf-1, contributing to the anti-apoptotic effect. We found that RKIP was downregulated during HBx-mediated hepatocarcinogenesis. In this study, we show that RKIP bound to Raf-1 and consequently inhibited the translocation of Raf-1 into mitochondria. This promoted the apoptosis of cells treated with apoptotic stimulus. Thus, the downregulation of RKIP increased the level of free Raf-1 and thereby elevated the mitochondrial translocation of Raf-1 during HBx-mediated hepatocarcinogenesis. The elevated Raf-1 mitochondrial translocation induced the increased anti-apoptotic effect and subsequently promoted HBx-mediated hepatocarcinogenesis.

Keywords: Hepatitis B virus X, hepatocarcinogenesis, Raf-1, RKIP

Hepatitis B virus X (HBx), a multifunctional protein, has effects on gene transcription, genotoxic stress responses, protein degradation, cell cycle checkpoint, cell proliferation, and apoptosis. Additionally, it transactivates several transcription factors and is essential for hepatitis B virus (HBV) replication [10]. Malignant transformation has been reported in specific cell lines transfected with the HBx gene and in the corresponding transgenic mouse model [8, 13]. Thus, this protein appears to play a key role in the neoplastic transformation of hepatocytes in HBV-

infected liver. However, the details of the mechanisms whereby HBx induces hepatocellular carcinoma (HCC) require elucidation. Previously, we reported the expression profiling of liver tissue from HBx-transgenic mice with early-stage HCC [9]. Through this proteomic approach, we identified 22 proteins that may be involved in HBx-mediated HCC, particularly at the early stages. Among these, Raf-1 kinase inhibitory protein (RKIP) was found as a downregulated protein during all stages of hepatocarcinogenesis.

RKIP is a cellular inhibitor protein of the MAP kinase cascade that binds and blocks the phosphorylation of regulatory sites on Raf-1, thereby repressing Raf-1 activation and downstream signal transduction. RKIP is a member of the ubiquitously expressed and evolutionarily conserved phosphatidylethanolamine binding protein (PEBP) family and functions as a suppressor of cancer metastasis [4, 5]. After stimulation, RKIP is phosphorylated at S153 by protein kinase C (PKC), inducing its dissociation from Raf-1 [3, 11]. In addition, it was known that HBx protein triggers activation of the Raf-1/MEK kinase cascade, which is essential for HBV gene expression. Recent reports show that RKIP protein and mRNA are downregulated in HCC [6, 12, 14]. In our experiments, RKIP expression was also considerably lower at all stages of HCC (Fig. 1), implying that its downregulation is involved in HBx-mediated hepatocellular carcinogenesis. Tumor cells acquire resistance to apoptotic stimuli and it has been demonstrated that conventional therapies exert their cytotoxic activities primarily by inducing apoptosis in the cells.

To investigate the functional role of RKIP downregulation during HBx-mediated hepatocarcinogenesis, we first constructed a Huh-7 cell line expressing HBx using a retroviral infection system (Fig. 2A). Using Western blot analysis, we confirmed the expression of HBx in the Huh-7 human hepatoma cell line (Fig. 2B). After that, pcDNA-RKIP was transfected into the Huh7 cell line expressing

*Corresponding author

Phone: +82-42-860-4268; Fax: +82-42-869-4593;
E-mail: khbae@kribb.re.kr

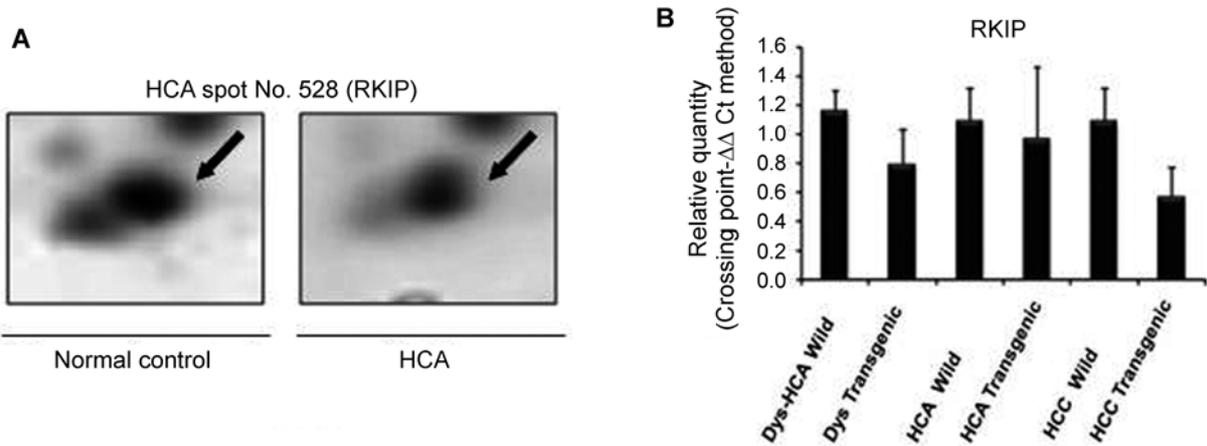


Fig. 1. RKIP is downregulated during HBx-mediated hepatocarcinogenesis. **A.** Enlargement of area containing RKIP on the 2-DE gel. **B.** Real-time PCR analysis of RKIP. Total RNA samples were isolated from the liver tissues of wild-type and transgenic mice using TRIzol (Invitrogen), as described previously [9, 15]. Data were normalized to the intensity for GAPDH. The values are means ± SD (n=3). RKIP was downregulated at all stages of HCC.

HBx. The pcDNA-RKIP S153V was also used to monitor the effect of binding to Raf-1. As shown in Fig. 2C, RKIP was successfully expressed. Next, we examined whether the RKIP expression level changes by HBx expression. The result clearly showed that the expression level of endogenous RKIP was significantly reduced in HBx-transfected Huh-7 cells, compared with Huh-7 control cells (Fig. 2D).

Because HBx can stimulate the mitochondrial translocation of Raf-1 and mitochondrially localized Raf-1 protects cells from apoptotic stresses [2], we performed the cell viability assay after apoptotic stimulation with doxorubicin. Doxorubicin

(adriamycin) is a widely used chemotherapeutic drug that targets topoisomerase II in mammalian cells and causes severe double-stranded breaks in DNA [1, 7]. As shown in Fig. 3A, the overexpression of RKIP induced the decreased viability against doxorubicin, compared with mock control cells. RKIP S153V mutant overexpression showed the more significant reduced viability (Fig. 3A). The analysis of PARP cleavage by activated caspases also demonstrated that overexpression of RKIP or RKIP S153V gave rise to more activation of caspases after the apoptotic stimulus (Fig. 3B). The mutant RKIP (S153V) stabilizes the binding

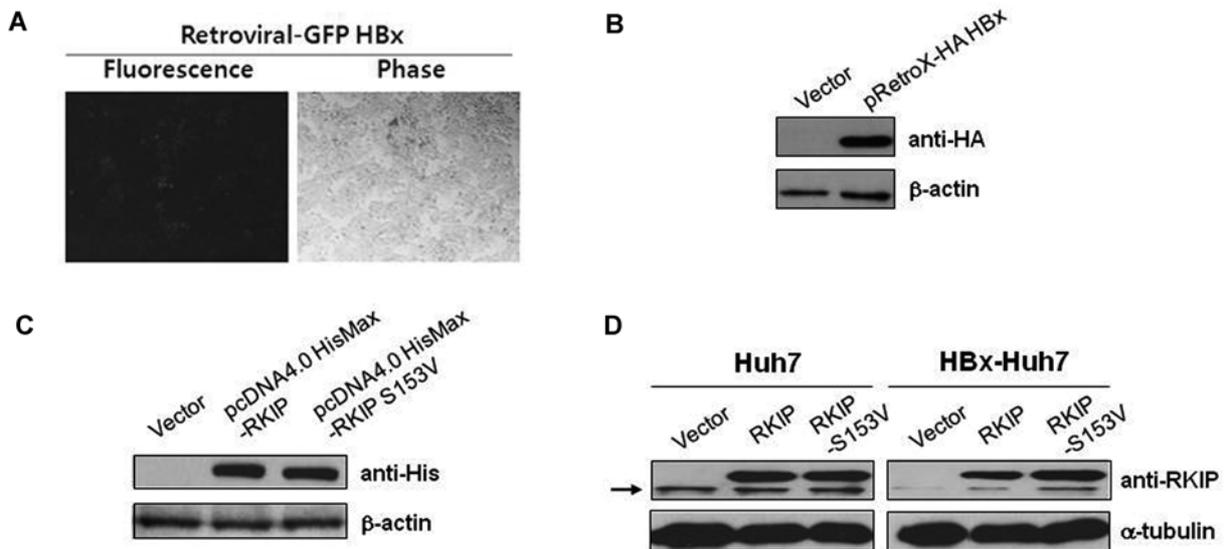


Fig. 2. Construction of Huh-7 cells expressing both HBx and RKIP. **A.** Expression of HBx was monitored directly by fluorescence microscopy after enrichment using a FACS sorter. We infected Huh-7 cells with the DNA encoding HBx using a retroviral system (HBx-IRES-GFP). **B.** Expression of HBx was confirmed by Western blot analysis. **C.** The Western blot analysis was performed to confirm the overexpression of RKIP. **D.** The Western blot analysis to check if the HBx influences the expression level of endogenous RKIP. The endogenous RKIP is indicated by the arrow.

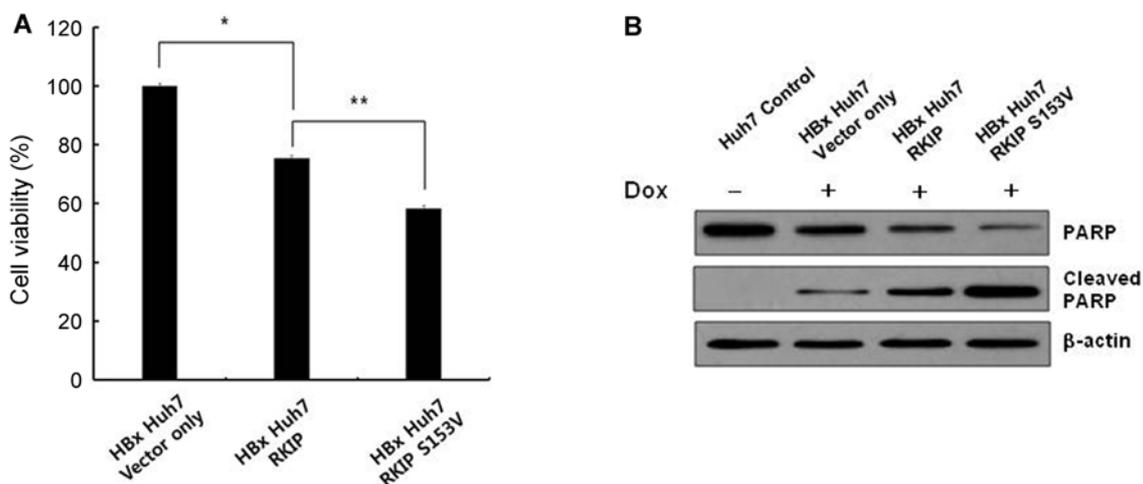


Fig. 3. Cell viability and PARP cleavage assays of Huh-7 cells expressing HBx and RKIP after treatment with doxorubicin.

A. Cell viability assay of Huh-7 cells expressing HBx and RKIP after treatment with doxorubicin. Cells were seeded in 6-well plates, and cultured in DMEM. Then, cells were treated with doxorubicin (final concentration: 0.25 μ M) for 2 day and the cell viability was measured using CCK-8 assay. *, **: $P < 0.05$. **B.** Western blot analysis of PARP cleavage in Huh-7 cells expressing HBx and RKIP after treatment with doxorubicin.

with Raf-1 and inhibits MAPK signaling [3]. Thus, we treated the MEK inhibitor U0126 to block the effect of the MAPK pathway by RKIP on cell viability. The treatment with U0126 had no effect on cell viability upon doxorubicin treatment (data not shown), implying no correlation between the MAPK pathway and cell viability in Huh-7 cells expressing HBx.

Since HBx interacts and binds with Raf-1 and subsequently translocates together into mitochondria [2], we examined the effect of RKIP overexpression on translocation of Raf-1. The results clearly demonstrated that RKIP overexpression prevents the translocation of Raf-1 into mitochondria (Fig. 4). Additionally, a more decreased level of Raf-1 was detected in the case of RKIP S153V mutant, implying the effect of RKIP-Raf-1 interaction on Raf-1 translocation into mitochondria.

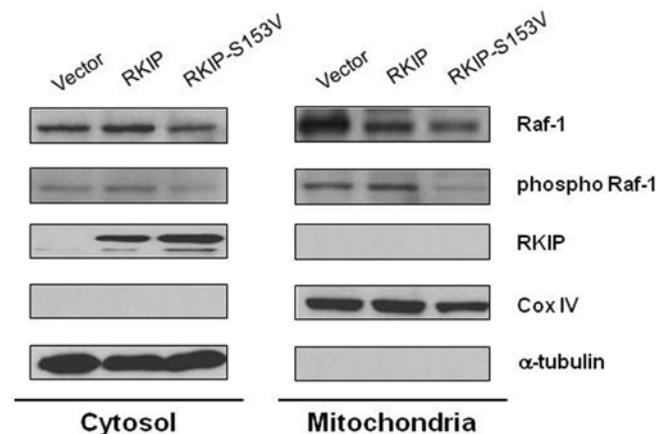


Fig. 4. Subcellular fractionation analysis for detecting Raf-1 translocation.

Cox IV and α -tubulin were used as mitochondrial and cytosolic loading controls, respectively.

Taken together, RKIP binds to Raf-1 and consequently inhibits the translocation of Raf-1 into mitochondria. This promotes the apoptosis of cells treated with apoptotic stimulus. As mentioned above, the function of RKIP is not limited to the Raf/MEK/ERK pathway. Therefore, we did not rule out the possibility that another mechanism(s) also has an effect on cell viability by RKIP.

In conjunction with earlier reports, the present results suggest that the downregulation of RKIP during HBx-mediated hepatocarcinogenesis induces the translocation of Raf-1 into mitochondria by increase of the free Raf-1 level in the cytosol, and subsequently the enhanced mitochondrial Raf-1 imparts the anti-apoptotic effect on cells. These data provide the possibility of RKIP as a potential therapeutic target for HCC diagnosis and novel drug development for treatment of the disease.

Acknowledgments

We thank Drs Do Hee Lee, Sayeon Cho, and Byoung Chul Park for careful reading of the manuscript and helpful advice. This work was supported by the KRIBB Open Innovation Program and the National Research Foundation of Korea Grant funded by the Korean Government (No. 2010-0008754 and No. 2010-0020305).

REFERENCES

- Alexia, C., G. Fallot, M. Lasfer, G. Schweizer-Groyer, and A. Groyer. 2004. An evaluation of the role of insulin-like growth factors (IGF) and of type-I IGF receptor signaling in hepatocarcinogenesis and in the resistance of the hepatocarcinoma

- cells against drug-induced apoptosis. *Biochem. Pharmacol.* **68**: 1003–1015.
- Chen, J. and A. Siddiqui. 2007. Hepatitis B virus X protein stimulates the mitochondrial translocation of Raf-1 *via* oxidative stress. *J. Virol.* **81**: 6757–6760.
 - Corbit, K. C., N. Trakul, E. M. Eves, B. Diaz, M. Marshall, and M. R. Rosner. 2003. Activation of Raf-1 signaling by protein kinase C through a mechanism involving Raf kinase inhibitory protein. *J. Biol. Chem.* **278**: 13061–13068.
 - Granovsky, A. E. and M. R. Rosner. 2008. Raf kinase inhibitory protein: A signal transduction modulator and metastasis suppressor. *Cell Res.* **18**: 452–457.
 - Klysik, J., S. J. Theroux, J. M. Sedivy, J. S. Moffit, and K. Boekelheide. 2008. Signaling crossroads: The function of Raf kinase inhibitory protein in cancer, the central nervous system and reproduction. *Cell Signal.* **20**: 1–9.
 - Lee, H. C., B. Tian, J. M. Sedivy, J. R. Wands, and M. Kim. 2006. Loss of Raf kinase inhibitor protein promotes cell proliferation and migration of human hepatoma cells. *Gastroenterology.* **131**: 1208–1217.
 - Lee, T. K., T. C. Lau, and I. O. Ng. 2002. Doxorubicin-induced apoptosis and chemosensitivity in hepatoma cell lines. *Cancer Chemother. Pharmacol.* **49**: 78–86.
 - Lee, Y. I., J. M. Hwang, J. H. Im, Y. I. Lee, N. S. Kim, D. G. Kim, *et al.* 2004. Human hepatitis B virus-X protein alters mitochondrial function and physiology in human liver cells. *J. Biol. Chem.* **279**: 15460–15471.
 - Kim, S.-Y., P. Y. Lee, H.-J. Shin, D. H. Kim, S. Kang, H.-B. Moon, *et al.* 2009. Proteomic analysis of liver tissue from HBx-transgenic mice at early stages of hepatocarcinogenesis. *Proteomics* **9**: 5056–5066.
 - Neuveut, C., Y. Wei, and M. A. Buendia. 2010. Mechanisms of HBV-related hepatocarcinogenesis. *J. Hepatol.* **52**: 594–604.
 - Odabaei, G., D. Chatterjee, A. R. Jazirehi, L. Goodglick, K. Yeung, and B. Bonavida. 2004. Raf-1 kinase inhibitor protein: Structure, function, regulation of cell signaling, and pivotal role in apoptosis. *Adv. Cancer Res.* **91**: 169–200.
 - Schuieler, M. M., F. Bataille, T. S. Weiss, C. Hellerbrand, and A. K. Bosserhoff. 2006. Raf kinase inhibitor protein is downregulated in hepatocellular carcinoma. *Oncol. Rep.* **16**: 451–456.
 - Tang, H., N. Oishi, S. Kaneko, and S. Murakami. 2006. Molecular functions and biological roles of hepatitis B virus X protein. *Cancer Sci.* **97**: 977–983.
 - Xu, Y.-F., Y. Yi, S.-J. Qiu, Q. Gao, Y.-W. Li, C.-X. Dai, *et al.* 2010. PEBP1 downregulation is associated to poor prognosis in HCC related to hepatitis B infection. *J. Hepatol.* **53**: 872–879.
 - Jeon, Y.-J., D.-H. Kim, H. Jung, S. J. Chung, S.-W. Chi, S. Cho, *et al.* 2010. Annexin A4 interacts with the NF- κ B p50 subunit and modulates NF- κ B transcriptional activity in a Ca²⁺-dependent manner. *Cell Mol. Life Sci.* **67**: 2271–2281.