PTEN modulates insulin-like growth factor II (IGF-II)-mediated signaling; the protein phosphatase activity of PTEN downregulates IGF-II expression in hepatoma cells

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Abstract **The PTEN gene (phosphatase and tensin homologous on chromosome 10) is frequently mutated or deleted in a number of malignancies including human hepatocellular carcinoma (HCC). We reported previously that the hepatitis B virus X (HBx) protein, known to be a causative agent in the formation of HCC, activates insulin-like growth factor II (IGF-II) expression through Sp1 phosphorylation by protein kinase C (PKC) or mitogen-activated protein kinase (MAPK) signaling. In this report we demonstrate that the PTEN effect on HBx induced IGF-II activation in a hepatoma cell line. Expression of PTEN and IGF-II was inversely related in different hepatoma cell lines. PTEN expression induced decreased Sp1 DNA binding by dephosphorylating Sp1 and interfered with transcriptional transactivation of IGF-II by HBx in hepatoma cells. The protein phosphatase activity was involved in PTEN downregulation of IGF-II transcription through downregulation of MAPK, MAPK kinase phosphorylation and PKC translocation. Our data suggest that PTEN blocks Sp1 phosphorylation in response to HBx, by inactivating PKC, MAPK and MAPK kinase which eventually downregulate IGF-II expression, during the formation of HCC.**

Key words: Hepatitis B virus X protein; Insulin-like growth factor II; PTEN; Sp1 phosphorylation

1. Introduction

PTEN (phosphatase and tensin homologous on chromosome 10, also called MMAC1 or TEP1) is a recently cloned tumor suppressor gene [1], which has dual specificity, dephosphorylating both lipid [2] and protein substrates [3]. The loss of PTEN expression has been detected in many cancers, including glioblastomas, breast and prostate carcinomas and several syndromes with multiple tumor incidence [4]. In addition, germ-line mutations in the PTEN gene have been associated with Cowden syndrome and related diseases in which patients suffer from a significantly increased risk of certain tumors, including breast and thyroid cancers [5,6]. In further support of the involvement of human PTEN mutations in tumorigenesis, mice that are heterozygous for a PTEN deletion also display frequent hyperplastic/dysplastic changes in numerous tissues [7]. PTEN encodes a dual-specificity phosphatase that recognizes lipid substrate, the phosphatidylinositol 3-kinase (PI3K) products PI-3,4P2 and PI-3,4,5P3 (PIP3) and protein substrates. It can dephosphorylate tyrosine-, serine- and threonine-phosphorylated peptides in vitro [3,8]. A number of studies have also clearly demonstrated that PTEN is a phosphatidylinositol 3-phosphatase and dephosphorylates PIP3, a product of PI3K, which is required for the phosphorylation and activation of protein kinase (PK) B/Akt [9], a survival factor that protects various cell types against apoptosis. Consistent with the role of PTEN in the PI3K/Akt signaling pathway, several lines of evidence suggest that PTEN negatively regulates cell survival [10,11]. Several proteins including Akt, PKA and PKC, which phosphorylate and activate $p70^{S6k}$, as well as mitogen-activated protein kinase (MAPK) kinase (MEK)/MAPK could potentially transduce mitogenic signals mediated by PI3K, but so far, Akt is the only one that has been found to be affected by PTEN [12,13]. We recently demonstrated that transient or stable transfection of the hepatoma cell line HepG2 with a hepatitis B virus X protein (HBx) expression vector resulted in an increase in insulin-like growth factor II (IGF-II) expression [14]. Elevated levels of IGF-II expression were shown to play a role in the neoplastic transformation of various cell types and were also found in hepatocellular carcinomas, indicating that autocrine production of IGF-II may also play a role in the deregulation of hepatic cell proliferation [14]. In our previous work, we also reported that HBx transactivates the IGF-II p4 promoter through the two Sp1 binding sites by PKC- and MAPK-mediated Sp1 phosphorylation, resulting in increased binding of the phosphorylated form of Sp1 to its target DNA [15]. In this study, we demonstrate that the protein phosphatase activity of PTEN is involved in Sp1 dephosphorylation by MAPK, MEK and PKC inactivation which is eventually involved in downregulation of IGF-II expression in hepatoma cell lines.

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Abbreviations: PTEN, phosphatase and tensin homologous on chromosome 10; IGF-II, insulin-like growth factor II; PI3K, phosphoinositol 3 kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; HBx, hepatitis B virus X-protein

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2. Materials and methods

2.1. Cell culture and plasmids

HepG2 cells were grown and maintained in a humidified 5% CO_2/95% air atmosphere in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (JBI, Daegu, Korea) and 1% penicillin/streptomycin solution. Antibiotics and culture media were obtained from JBI. Schneider line 2 (SL2) cells were maintained in Schneider *Drosophila* medium supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin solution. SL2 cells were grown and maintained at 22°C. The HBx expression vector for eukaryotic cell expression was constructed using pMAMneo (Clontech, Palo Alto, CA, USA) by inserting full-length HBx cDNA. The PTEN wild type (wt) and mutant eukaryotic expression vectors (PTEN C124S, PTEN G129E) under the control of cytomegalovirus promoter were a kind gift from Dr. Ji Sung Kil, Kyung Hee University, Seoul, Korea.

2.2. Transient transfection and CAT assay

DNA transfection was carried out using the calcium phosphate precipitation method. The cells were transiently transfected with a plasmid construct (pIG0) containing 540 bp of the fourth promoter region of the human IGF-II gene ligated upstream of the CAT reporter in pGEM [14]. After 16 h, the cells were washed with phosphate-buffered saline (PBS) and fed again with fresh serum-containing medium, then the plates were incubated for an additional 48 h. The cells were then harvested and extracts were assayed for CAT activity according to Gorman et al.'s method [16].

2.3. RNA extraction and Northern blot analysis

Total cellular RNA was extracted with Trizol (Life Technologies, Grand Island, NY, USA) according to the procedure of Chomczinski and Sacchi [17]. The RNA (15 μg) was denatured in 5% formaldehyde and electrophoresed on 1% agarose gels and transferred to Hybond-N^+ nylon membranes (Amersham Pharmacia, Piscataway, NJ, USA). Hybridization was carried out following the established protocol using a radiolabeled 600 bp *Eco*RI/*Pst*I fragment of the human IGF-II gene [14].

2.4. Nuclear extract preparation and electrophoretic mobility shift assay (EMSA)

Nuclear extracts (5 μg) from cells were prepared and analyzed by EMSA as previously described [15]. DNA binding reactions were carried out in binding reaction mixture containing a ^{32}P-labeled double-stranded oligonucleotide probe corresponding to the Sp1 consensus sequence at 22°C then resolved on a 4% non-denaturing polyacrylamide gel.

2.5. Schneider cell transfection and in vivo phosphorylation of Sp1

SL2 cells were plated onto 100 mm plastic Petri dishes at a density of 10^7 cells per plate 20–24 h before transfection. The cells were transfected with a Sp1 expression vector (pSp1) and PTEN expression vector (pPTEN) or HBx expression vector (pMAM-HX) using the calcium phosphate precipitation method. The transfected cell cultures were left undisturbed for 48 h before harvesting. In vivo phosphorylation and immunoprecipitations were performed as described by Lee et al. [18]. For the binding reaction, cell extracts were adjusted to 250 mM NaCl using TENT buffer (5 mM Tris–HCl (pH 7.5), 5 mM EDTA (pH 8.0), 100 mM NaCl, 1% (v/v) Triton X-100) containing 100 mM NaCl. ^{32}P-labeled Sp1 was then immunoprecipitated with polyclonal Sp1 antibody conjugated with agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA), resolved on 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and autoradiographed.

2.6. Protein extraction and immunoblotting

Protein extraction and immunoblotting were performed precisely as described [15]. The anti-PTEN monoclonal antibody 6H2·1, which was raised against the C-terminus of the PTEN [18], and polyclonal anti-MAPK, anti-phospho-MAPK and anti-PKCα (New England Biolabs, Hercules, CA, USA) were used at a 1:1000 dilution. Polyclonal anti-MEK, anti-phospho-MEK and IGF-II were purchased from Santa Cruz and used at a 1:1000 dilution. Anti-actin antibody (Sigma, St. Louis, MO, USA) was used at a 1:250 dilution as an internal control.

2.7. PKC translocation assay

HepG2 cells (5×10^6) were transiently transfected with pCMV, PTEN wt, C124S, G129E and HBx expression vectors using the calcium phosphate method. The transfected cells were harvested and washed three times with PBS, and their extracts were prepared. The cells were resuspended in 500 μl of buffer A (20 mM Tris–HCl (pH 7.5), 0.5 mM EGTA, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml of leupeptin, 10 μg/ml of aprotinin), sonicated by seven brief bursts with a Branson sonifier with a microtip setting of 1, and centrifuged at $100\,000\times g$ for 1 h at 4°C. The supernatant and pellet, corresponding to the soluble cytoplasmic fraction and insoluble particulate membrane fraction, respectively, were adjusted to 1% SDS and 2% β-mercaptoethanol. The extracts were analyzed by Western blot with rabbit anti-PKCα (New England Biolabs).

3. Results

3.1. PTEN suppresses IGF-II expression

To analyze PTEN expression in different hepatoma cell lines, we examined the expression of the PTEN protein by Western blotting. Fig. 1A shows that the abundance of PTEN varied among different cell lines, with HepG2 and PLC/PRF/5 having relatively low levels of the PTEN protein. Since PTEN has been characterized as a tumor suppressor

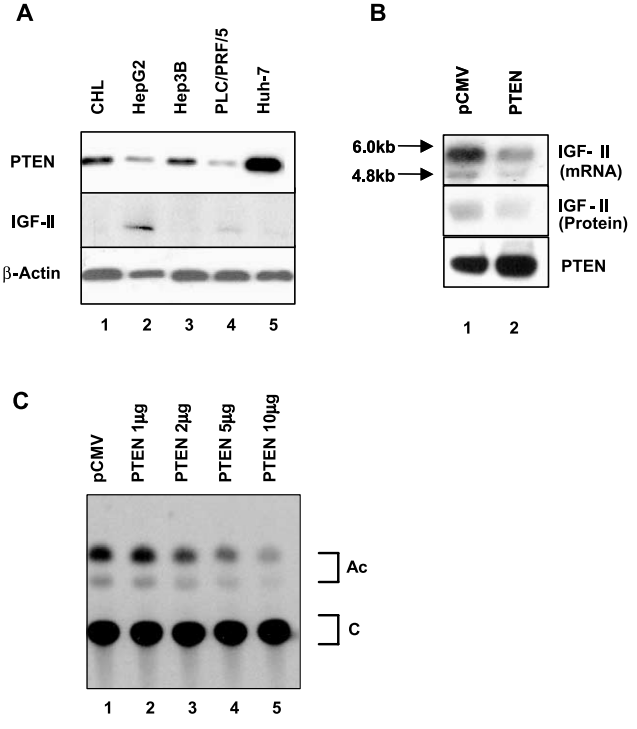

Fig. 1. PTEN suppresses IGF-II expression. A: Expression of PTEN and IGF-II in different hepatoma cell lines. The levels of PTEN and IGF-II protein in Chang liver, HepG2, Hep3B, PLC/PRF/5 and Huh 7 cells were determined by Western blot analysis (4 μg/lane) using an anti-PTEN monoclonal antibody and a polyclonal IGF-II antibody. An anti-actin antibody was used as an internal control. B: PTEN downregulated the expression of IGF-II mRNA and protein. The levels of IGF-II mRNA and protein in HepG2 cells were determined by Northern blotting (10 μg/lane) and Western blot analysis (4 μg/lane). The amounts of PTEN were determined in control (pCMV) and PTEN-transfected HepG2 cells by Western blot analysis. C: Dose dependence of PTEN on transcriptional repression of IGF-II p4 promoter. IGF-IIp4-CAT was co-transfected into HepG2 cells with either a control vector (pCMV) or varying concentrations of a PTEN expression vector (pPTEN).

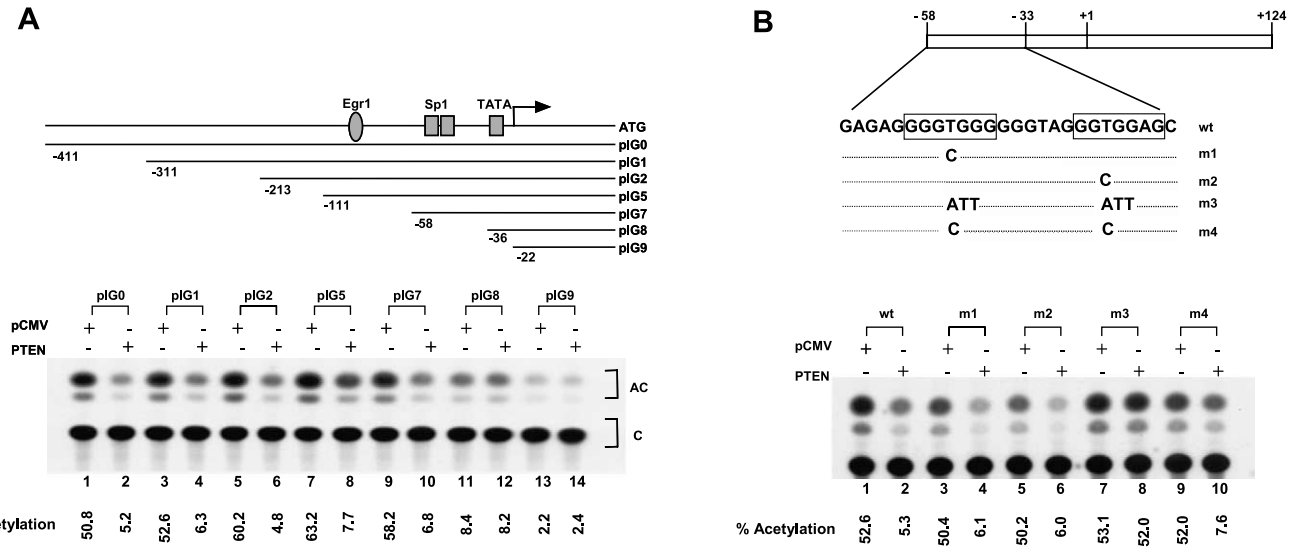

Fig. 2. PTEN downregulates IGF-II transcription through Sp1 sites. A: Mapping of the PTEN-responsive element in the p4 promoter of the IGF-II gene. The diagram shows the p4 promoter of IGF-II (−411–+124) fused to the CAT gene and the 5′ deletion mutants (pIG1, −311–+124; pIG2, −213–+124; pIG5, −111–+124, pIG6, −72–+124; pIG7, −58–+124; pIG8, −36–+124; pIG9, −22–+124). The different IGF-II–CAT plasmids were co-transfected into HepG2 cells with either a control vector (pCMV) or a PTEN expression vector (pPTEN). B: Evidence that PTEN regulates IGF-II expression through the two Sp1 binding sites. a: Diagram of the mutations introduced into the two Sp1 binding sites (GGGTGG) contained within construct pIG7. The T in either the 5′ or 3′ Sp1 binding site or both sites was changed to C in m1, m2, and m4, respectively. Both Sp1 binding sites were mutated by inserting three base changes in each Sp1 binding site in mutant m3. b: Effect of PTEN on transcription of the Sp1 binding site mutant and wt in HepG2. The parental plasmid pIG7 and the four mutants were co-transfected into HepG2 cells with either a control vector (pCMV) or a PTEN expression vector (pPTEN).

and elevated levels of IGF-II are present in a variety of human tumors where it functions as an autocrine or paracrine growth stimulator, we first analyzed PTEN protein levels against the level of IGF-II in a panel of hepatoma cell lines by Western blotting. Interestingly, the levels of IGF-II were inversely correlated with those of PTEN (Fig. 1A). HepG2 had the highest levels of IGF-II in accordance with its lowest PTEN levels. To further investigate whether PTEN inhibits IGF-II expression in HepG2 cells, we overexpressed PTEN and examined the status of IGF-II expression. Transcripts derived from promoter 3 (6.0 kb) and promoter 4 (4.8 kb) were detected. The transiently PTEN-expressing HepG2 cell line showed a decrease in the expression of IGF-II transcripts (Fig. 1B). These results show that PTEN downregulates the IGF-II transcript from promoters 3 and 4. Western blot analysis using an IGF-II antibody revealed that the protein levels of IGF-II correlated with the abundance of mRNA in PTEN-expressing HepG2 cells (Fig. 1B). To examine the downregulation of IGF-II by PTEN on the IGF-II promoter 4 in these cells, 10 μg of the wt IGF-II promoter–CAT construct (pIG0) was co-transfected with 5 μg of the control expression vector or a human PTEN expression vector into HepG2 cells (Fig. 1C). The CAT activity was diminished by PTEN in a concentration-dependent manner in PTEN-transfected HepG2 cells. These results show that PTEN downregulates the IGF-II transcription in the hepatoma cell line, resulting in the downregulation of the mRNA transcript and the expression of this protein.

3.2. *Mapping of the PTEN-responsive element in the p4 promoter of IGF-II*

To determine the PTEN-responsive element sequences in promoter 4 of IGF-II, 5′ deletions of the promoter were constructed. The plasmids were co-transfected with either a control (pCMV) or the human PTEN expression vector (pPTEN) into HepG2 cells. The levels of CAT activity were determined 48 h after transfection. The activities of constructs pIG0 (−411), pIG1 (−311), pIG2 (−213), pIG5 (−111), pIG7 (−58), pIG8 (−36), pIG9 (−22) showed a 9.8-, 8.3-, 10.3-, 7.8-, 8.5-, 1.1- and 1.1-fold decrease, respectively, by PTEN (Fig. 2A). Since the sequences between −58 and −36, which showed PTEN responsiveness to the IGF-II p4 promoter, contain two Sp1 binding sites, we investigated the importance of these binding sites for PTEN activity. To examine the role of the Sp1 binding sites in PTEN-mediated transcriptional regulation, both Sp1 binding sites were inactivated by changing the three internal base pairs of each site (Fig. 2Ba, m3). Since both of the Sp1 binding sequences (GGGTGG) showed variations of the Sp1 consensus binding sequences (GGGCGG), a single T (m1, m2) and both Ts (m4) of the variant Sp1 sites were changed into C, in order to test the ability of the Sp1 consensus binding (GGGCGG) sequences to confer PTEN-mediated transcriptional regulation (Fig. 2Ba).

The effect of PTEN on the Sp1 binding site mutant was examined by co-transfection of the plasmid into HepG2 cells with and without a PTEN expression vector (Fig. 2Bb). Wt and mutant construct m1, m2, and m4 activities were decreased 9.8-, 8.2-, 8.3- and 6.8-fold, respectively, by PTEN, whereas m3 was decreased only 1.1-fold. Thus, the change of GGGTGG to the consensus Sp1 binding site GGGCGG had no effect on the ability of Sp1 to activate transcription, whereas inactivation of both Sp1 sites abolished Sp1 activation. These results suggest that PTEN can downregulate transcription of the IGF-II p4 promoter through the Sp1 binding sites. The GGGTGG Sp1 binding sites in the IGF-II p4 promoter and the consensus Sp1 binding site GGGCGG conferred equal responsiveness to PTEN.

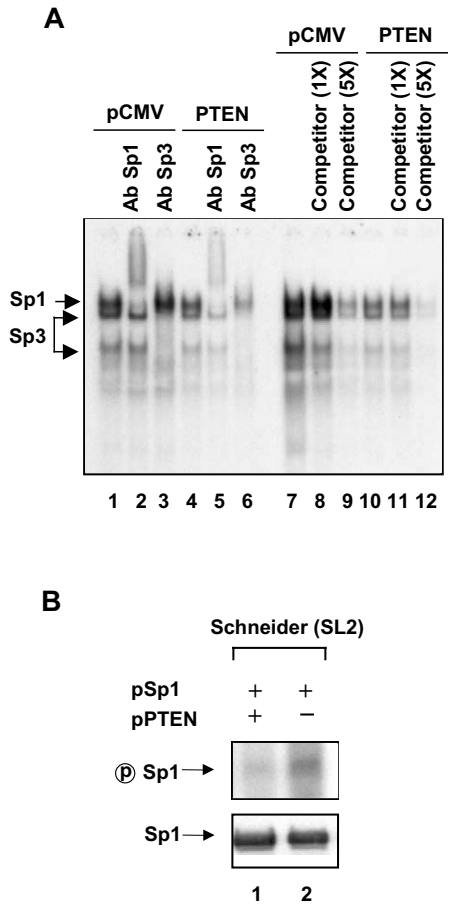

Fig. 3. Decrease of DNA binding and in vivo phosphorylation of Sp1 by PTEN. A: Decrease of Sp1 binding to the Sp1 consensus sequence by PTEN. Nuclear extracts obtained from pCMV (control) and pPTEN-transfected HepG2 cells were preincubated at 37°C with a Sp1 consensus sequence DNA probe and were tested for Sp1 binding activity in a gel retardation assay. One μg of a Sp1 and Sp3 antibody was used for supershift gel retardation experiments (lanes 2, 3, 5 and 6). Increasing amounts of cold Sp1 probe were used for the competition experiment using pCMV or pPTEN cell-transfected nuclear extracts (lanes 8, 9, 11 and 12). B: PTEN down-regulates Sp1 phosphorylation. pSp1-transfected (lane 2) or pSp1, pPTEN double-transfected (lane 1) Schneider cells were incubated with 1 mCi/ml [^{32}P]*ortho*-phosphate in phosphate-free medium for 1 h. After harvesting, the collected cells were lysed with IP buffer and the radiolabeled Sp1 was immunoprecipitated with a polyclonal Sp1 antibody (Santa Cruz) and resolved by 8% SDS–PAGE. Phosphorylated Sp1 is indicated by (P)Sp1. As a control, immunoblotting was performed with a polyclonal antibody raised against recombinant Sp1. Each lane contains 20 μg of protein (Sp1).

3.3. *Decrease of DNA binding of Sp1 and in vivo phosphorylation of Sp1 by PTEN*

Modulation of Sp1-directed transcription by PTEN is a complex phenomenon which depends on the signals that modulate the intrinsic transcriptional activity or the DNA binding activity of the transcription factor Sp1. We tested the ability of PTEN to modulate the Sp1 DNA binding activity. The nuclear extracts from control (pCMV) and PTEN-expressing HepG2 cells were checked for binding activity to Sp1 consensus sequences of IGF-II promoter 4 (Fig. 3A). The Sp1 probe binding activity of nuclear extracts from HepG2 cells transfected with PTEN (Fig. 3A, lanes 4–6 and 10–12) was compared to that of control vector-transfected HepG2 cells (Fig. 3A, lanes 1–3 and 7–9). In transiently PTEN-transfected HepG2 cells, the Sp1 binding to its consensus DNA probe decreased compared to the control vector-transfected HepG2 cells (Fig. 3A, lanes 4 and 10). In the presence of an Sp1 antibody, the slowest migrating complex is supershifted in all cell lines (Fig. 3A, lanes 2 and 5). To determine if any of the retarded bands shown in Fig. 3A represent Sp3 as well as Sp1, gel shift analyses were performed in the presence of a specific anti-Sp3 antibody. When the anti-Sp3 antibody was present in the binding reaction, the slowest migrating complex was unaltered whereas the other two complexes were shifted (Fig. 3A, lanes 3 and 6). These results demonstrate that Sp1 and Sp3 can both bind to the Sp1 consensus sequence in the IGF-II promoter. Competition experiments with increased amounts of unlabelled Sp1 oligonucleotide showed a gradual decrease of the retarded bands including the Sp1 band (Fig. 3A, lanes 8, 9, 11 and 12). These results suggest that a common set of nuclear factors can interact with the Sp1 consensus sequence within IGF-II promoter 4. Since PTEN inhibits Sp1 binding to Sp1 consensus sequences in HepG2 cells, we investigated whether PTEN affected Sp1 phosphorylation, which might be a cause of the decrease of Sp1 binding to its consensus DNA sequences. Schneider cells which lack Sp1 were transfected with a Sp1 expression vector (pSp1) and a PTEN expression vector (pPTEN) and in vivo labeled with [^{32}P]*ortho*-phosphate. The cells were lysed, nuclear extracts were prepared and Sp1 was precipitated with a Sp1 antibody. After running on an 8% SDS–PAGE gel, the gel was autoradiographed. The results in Fig. 3B show that PTEN dephosphorylates Sp1 (lane 1) while nuclear extracts of Schneider cells which were transfected with pSp1 showed basal levels of the phosphorylated band (lane 2). These results showed that the PTEN protein induces dephosphorylation of Sp1, in vivo. In order to check whether a decrease in the amount of Sp1 occurred in PTEN-expressing HepG2 cells, immunoblotting experiments using nuclear extracts and Sp1 polyclonal antibodies were done (Fig. 3B). No difference in the amounts of Sp1 in the extracts obtained from the two different cell lines was detected. Thus, at least under our experimental conditions, PTEN does not decrease the actual amount of Sp1 in the cells. These results confirm that in the presence of a similar amount of Sp1, PTEN induces posttranscriptional modifications that render the Sp1 complex less efficient in its DNA binding ability.

3.4. *Inverse relationship of PTEN and HBx in IGF-II promoter activation*

We have previously shown that HBx transactivates IGF-II promoter 4 through two Sp1 binding sites in HepG2 cells [14,15]. To examine whether PTEN has an effect on HBx transactivation, HBx and PTEN were transiently coexpressed in HepG2 cells and the transactivating activities of HBx and PTEN on the IGF-II p4 promoter were examined using the CAT assay. As shown in Fig. 4, HBx restores the PTEN-induced decrease in the IGF-II promoter activity in a dose-dependent manner (Fig. 4A), while the HBx transactivation activity on the IGF-II p4 promoter was repressed by PTEN in a dose-dependent manner (Fig. 4B). These results suggest that HBx has a functional inverse relationship in vivo with the PTEN function and might have an effect on the IGF-II p4 promoter through the same pathway.

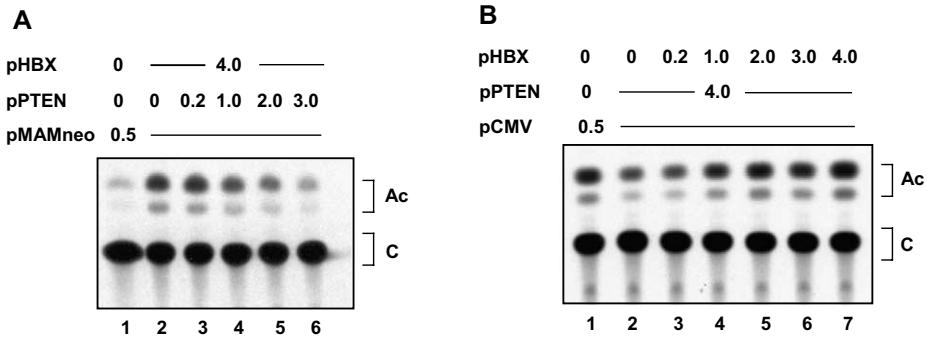

Fig. 4. Inverse relation of PTEN and HBx in IGF-II promoter activation. A: PTEN interferes with the transactivation activity of HBx in HepG2 cells. HepG2 cells were transiently co-transfected with 3 μg of IGF-IIp4-CAT, 4 μg of HBx expression vector (pMAM-HX) and 0, 0.2, 1.0, 2.0, and 3.0 of a PTEN expression vector (pPTEN) and analyzed for CAT activity. B: HBx restores the PTEN-induced decrease of IGF-II promoter activity. HepG2 cells were transiently co-transfected with 3 μg of IGF-IIp4-CAT, 4 μg of a PTEN expression vector (pPTEN) and 0, 0.2, 1.0, 2.0, 3.0 and 4.0 μg of pMAM-HX and analyzed for CAT activity.

3.5. *The protein phosphatase activity is involved in PTEN downregulation of IGF-II signaling*

Having shown that the overexpression of PTEN downregulates IGF-II expression in hepatoma cell lines, we next investigated whether inhibition of IGF-II expression was due to either the protein or phospholipid phosphatase activity of PTEN. For this purpose, we compared the effect of wt PTEN, G129E mutant PTEN and C124S mutant PTEN on the activity of the IGF-II p4 promoter. The C124S mutation results in a phosphatase-dead protein, with neither lipid nor protein phosphatase activity. The G129E mutation results in only a loss of lipid phosphatase activity with retention of protein phosphatase activity. If the downregulation of IGF-II expression in PTEN-expressing HepG2 cells is only mediated through its lipid phosphatase activity, which is known to counter the PI3K signaling pathway, one would expect that the G129E mutant might have a similar effect on IGF-II expression to that of the C124S mutant. As shown in Fig. 5A, the IGF-II p4 promoter activity was not modified in the cells overexpressing the C124S mutant, but decreased in the cells overexpressing wt PTEN and the G129E mutant, both of which have intact protein phosphatase activity (Fig. 5A). These results suggest that the protein phosphatase rather than the lipid phosphatase activity is important for downregulation of IGF-II expression. We next investigated the ability of PTEN in the regulation of the activities of MEK and MAPK, which is known to be involved in the HBx-mediated increase of IGF-II expression [15]. For this purpose, we analyzed the phosphorylation status of MEK or MAPK in a hepatoma cell line expressing wt PTEN, C124S or G129E mutant by Western blotting using antibodies which interact specifically with the phosphorylated form of MEK or MAPK (Fig. 5B). Induction of wt PTEN or G129E in HepG2 cells resulted in a reduction in the phosphorylation of MEK and MAPK compared to controls (lanes 2 and 4). Conversely, overexpression of the C124S lipid phosphatase-dead mutant resulted in an increase of the phosphorylation of MEK and MAPK compared to control cells. The reduction in the phosphorylation of MEK and MAPK caused by overexpression of PTEN was not the result of a reduction in the absolute amounts of the MEK or MAPK proteins (Fig. 5B). In addition, PTEN also decreases HBx-mediated phosphorylation of MEK and MAPK in this cell line (Fig. 5B, lanes 5 and 6). We next investigated the ability of PTEN, in the regulation of the activity of PKC, which is also known to be involved in the HBx-mediated increase in IGF-II expression [15]. For this purpose, we examined the subcellular distribution of PKC in hepatoma cell lines expressing wt PTEN, C124S or G129E

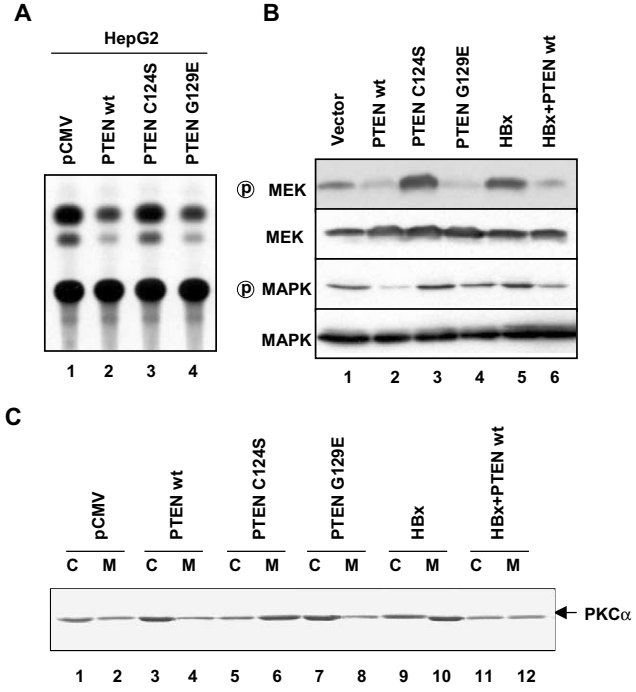

Fig. 5. The protein phosphatase activity is involved in PTEN downregulation of IGF-II signaling. A: The effect of wt and PTEN mutants G129E and C124S on IGF-II transcription. HepG2 cells were transiently co-transfected with 3 μg of IGF-IIp4-CAT and 4 μg of PTEN wt, C124S, G129E expression vectors and analyzed for CAT activity. PCMV vector-transfected HepG2 cells were used as a control. B: The effect of wt and PTEN mutants G129E and C124S on MEK and MAPK phosphorylation. 20 μg protein of cell lysates from pCMN, PTEN wt, C124S, G129E, HBx- and HBx+PTEN-transfected cells were resolved on 8% SDS–PAGE and Western blotted with polyclonal anti-MEK, anti-phospho-MEK, anti-MAPK and anti-phospho-MAPK antibodies as indicated in Section 2. C: The effect of wt and PTEN mutants G129E and C124S on PKC translocation. 20 μg protein of cytosolic or membrane fractions from pCMV, PTEN wt, C124S, G129E, HBx- and HBx+PTEN-transfected cells were resolved on 8% SDS–PAGE and Western blotted with a polyclonal anti-PKC antibody.

mutants by Western blotting (Fig. 5C). Induction of the wt PTEN and G129E mutant in HepG2 cells resulted in a decrease in membrane-bound PKC compared to controls (Fig. 5C, lanes 3, 4, 7 and 8). Conversely, overexpression of the C124S phosphatase-dead mutant resulted in an increase in membrane-bound PKC (Fig. 5C, lanes 5 and 6) compared to the PTEN wt or G129E mutant. In addition, PTEN also decreased HBx-mediated translocation of PKC in this cell line (Fig. 5C, lanes 9–12).

4. Discussion

It is well established that HBx plays an essential role in IGF-II activation during the formation of HCC [14,15]. Here, we demonstrate that overexpression of PTEN in hepatoma cells inhibits IGF-II expression and cell growth by blocking MAPK, MEK and PKC activation in a PTEN protein phosphatase activity-dependent manner. Several lines of evidence from this study also demonstrate that PTEN inhibits the phosphorylation of Sp1, resulting in a decrease in Sp1 binding to the Sp1 consensus sequence in the IGF-II p4 promoter. Overexpression of PTEN induced the dephosphorylation of MAPK and MEK and inhibited PKC translocation which resulted in Sp1 transcription factor dephosphorylation. Wt PTEN led to the suppression of IGF-II transcription, MEK and MAPK phosphorylation and PKC translocation. In contrast, expression of the phosphatase-dead mutant, C124S, did not suppress IGF-II transcription, MEK and MAPK phosphorylation and PKC translocation while the G129E mutant, which showed a loss of lipid phosphatase activity but retained the protein phosphatase activity, exerted the same effect as wt PTEN. Therefore, PTEN exerts an IGF-II suppression effect through its protein phosphatase activity. Our results, in contrast to those of a recent report that shows that both the protein and lipid phosphatase activities are induced in PTEN-mediated growth suppression [19], indicate that only the protein phosphatase activity is needed in IGF-II inactivation. In their experiment, the lipid phosphatase branch of PTEN upregulated p27 by blocking PI3K signaling, whereas the protein phosphatase branch downregulated cyclin D1, demonstrating bifurcation of PTEN's lipid and protein phosphatase signaling which ultimately converges at the G1/S transition to block cell cycle progression [19]. A recent report by Went et al. [20] demonstrated that PTEN blocks MAPK phosphorylation in response to insulin stimulation by inhibiting the phosphorylation of insulin receptor substrate (IRS)-1 and IRS-1/Grb2/SOS complex formation, which leads to downregulation of cyclin D1, inhibition of cell cycle progression and suppression of cell growth. Although we assume that the Akt signaling branch of PTEN is not involved in the observed downstream effect, based on the failure of the lipid phosphatase PTEN mutant to exert the same effect as the protein phosphatase mutant at the transcriptional level, the Akt phosphorylation status in HepG2 cells and subsequent experiments on Akt signaling which might involve the activation of PTEN in downregulation of IGF-II expression need to be verified. Our results together with previous results [14,15] which describe IGF-II activation by PKC- and MAPK-dependent Sp1 phosphorylation clearly demonstrate that PTEN blocks Sp1 phosphorylation in response to HBx by inactivating PKC, MAPK and MEK which eventually downregulates IGF-II expression thus inhibiting cell growth during the formation of HCC.

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References

[1] Steck, P.A., Pershouse, M.A., Jasser, S.A., Yung, W.K., Lin, H., Ligon, A.H., Langford, L.A., Baumgard, M.L., Hattier, T. and Davis, T. (1997) Nat. Genet. 15, 356–362.
[2] Furnari, F.B., Huang, H.J. and Cavenee, W.K. (1998) Cancer Res. 58, 5002–5008.
[3] Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S.I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R. and Bigner, S.H. (1997) Science 275, 1943–1947.
[4] Steck, P.A., Pershouse, M.A., jasser, S.A., Yung, W.K., Lin, H., Ligon, A.H., Langford, L.A., Baumgard, M.L., Hattier, T. and Davis, T. (1997) Nat. Genet. 15, 356–362.
[5] Eng, C. and Peacocke, M. (1998) Nat. Genet. 19, 223.
[6] Eng, C. (1998) Int. J. Oncol. 12, 223.
[7] Liaw, D., Marsh, D.J., Li, J., Dahia, P.L., Wang, S.I., Zheng, Z., Bose, S., Call, K.M., Tsou, H.C., Peacocke, M., Eng, C. and Parsons, R. (1997) Nat. Genet. 16, 64–67.
[8] Furnari, F.B., Lin, H., Hang, H.S. and Cavenee, W.K. (1998) Cancer Res. 58, 5002–5008.
[9] Li, J., Simpson, L., Takahashi, M., Miliaresis, C., Myers, M.P., Tonks, N. and Parsons, R. (1998) Cancer Res. 58, 5667–5672.
[10] Davies, M.A., Lu, Y., Sano, T., Fang, X., Tang, P., LaPushin, R., Koul, D., Bookstein, R., Stokoe, D. and Yung, W.K. et al. (1998) Cancer Res. 58, 5285–5290.
[11] Stambolic, B., Suzuki, A., de la Pomapa, J.L., Brothers, G.M., Mirtsos, C., Sasaki, T., Ruland, J., Penninger, J.M., Siderovski, D.P. and Mak, T.W. (1998) Cell 95, 29–39.
[12] Carpenter, C.L. and Cantley, L.C. (1996) Biochim. Biophys. Acta 1288, M11–16.
[13] Bondeva, T., Pirola, L., Bulgarelli-Leva, G., Rubio, I., Wetzker, R. and Wymann, M.P. (1998) Science 282, 293–296.
[14] Lee, Y.I., Lee, S., Lee, Y.I., Bong, Y.S., Hyun, S.W., Yoo, Y.D., Kim, S.J., Kim, Y.W. and Poo, H.R. (1998) Oncogene 16, 2367–2380.
[15] Park, S.M., Lee, J.H., Shin, J.H. and Lee, Y.I. (2001) Biochem. Biophys. Res. Commun. 283, 303–307.
[16] Chomczinski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[17] Gorman, C.M., Moffat, L.F. and Howard, B.H. (1982) Mol. Cell. Biol. 2, 1044–1051.
[18] Perren, A., Weng, L.P., Boag, A.H., Ziebold, U., Thakore, K., Dahia, P.L., Komminoth, P., Lees, J.A., Mulligan, L.M., Mutter, G.L. and Eng, C. (1999) Am. J. Pathol. 155, 1253–1260.
[19] Weng, L.P., Brown, J.L. and Eng, C. (2001) Hum. Mol. Genet. 10, 599–604.
[20] Weng, L.P., Smith, W.M., Brown, J.L. and Eng, C. (2001) Hum. Mol. Genet. 10, 605–616.