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# Association of DNA-dependent protein kinase with hypoxia inducible factor-1 and its implication in resistance to anticancer drugs in hypoxic tumor cells

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Abbreviations: AP1, activator protein 1; C/EBP-β, cAMP response element-binding protein β; DNA-PK, DNA-dependent protein kinase; Egr1, early growth response gene product 1; HIF-1, hypoxia inducible factor-1; NF-κB, nuclear factor kappa B

## Abstract

**Tumor hypoxia contributes to the progression of a malignant phenotype and resistance to ionizing radiation and anticancer drug therapy. Many of these effects in hypoxic tumor cells are mediated by expression of specific set of genes whose relation to therapy resistance is poorly understood. In this study, we revealed that DNA-dependent protein kinase (DNA-PK), which plays a crucial role in DNA double strand break repair, would be involved in regulation of hypoxia inducible factor-1 (HIF-1). HIF-1β-deficient cells showed constitutively reduced expression and DNA-binding activity of Ku, the regulatory subunit of DNA-PK. Under hypoxic condition, the expression and activity of DNA-PK were markedly induced with a concurrent increase in HIF-1α expression. Our result also demonstrated that DNA-PK could directly interact with HIF-1, and especially DNA-PKcs, the catalytic subunit of DNA-PK, could be involved in phosphorylation of HIF-1α, suggesting the possibility that the enhanced expression of DNA-PK under hypoxic condition might attribute to modulate HIF-1α stabilization. Thus, the correlated regulation of DNA-PK with HIF-1 could contribute to therapy resistance in hypoxic tumor cells, and it provides new evidence for developing therapeutic strategies enhancing the efficacy of cancer therapy in hypoxic tumor cells.**

**Keywords:** DNA-PK; drug resistance; HIF-1; hypoxia Introduction

## Introduction

Hypoxia, a reduction of oxygen tension below the normal level for a tissue, plays an important role in development of tumor cells (Brahimi-Horn *et al*., 2001; Goonewardene *et al*., 2002). Tumor cells undergo genetic and adaptive changes such as increased expressions of hypoxia-inducible factor-1 (HIF-1), NF-κB, AP-1, C/EBP-β and Egr-1, which allow them to survive and even proliferate in a hypoxic environment, and thus a wide range of gene expressions is induced by hypoxia (Koong *et al*., 1994; Yao *et al*., 1994; Yan *et al*., 1997; Yan *et al*., 1999; Harris, 2002; Joung *et al*., 2003).

HIF-1 is a heterodimer that consist of HIF-1α, which is tightly regulated by oxygen concentration, and HIF-1β, which is a constitutively expressed aryl hydrocarbon receptor nuclear translocator (ARNT). In the absence of oxygen, HIF-1α forms an active complex with HIF-1β and binds to hypoxia-response element (HRE), thereby activating the expression of numerous hypoxia-responsible genes.

Tumor hypoxia contributes to the progression of a more malignant phenotype by selecting for cells with a diminished apoptotic potential, and the hypoxic cells are intrinsically more resistant to radiotherapy and chemotherapy based on cell killing effect in well-oxygenated cells (Brown, 2000; Harris, 2002; Wouters *et al*., 2002). In fact, HIF-1α was overexpressed in colon, breast, gastric, lung, skin, ovarian, pancreatic and renal carcinomas and associated with cell proliferation and poor prognosis (Zhong *et al*., 1999), and the elevated level of HIF-1 is closely correlated with radioresistant and aggressive disease in oropharyngeal cancer (Aebersold *et al*., 2001). HIF-1α deficient

cells are more susceptible to anticancer drug and ionizing radiation (Unruh *et al*., 2003), and also P-glycoprotein (P-gp), the multidrug resistance (MDR) gene product, is regulated by HIF-1 (Comerford *et al*., 2002; Wartenberg *et al*., 2003), suggesting that HIF-1 expression could be involved in development of chemoresistance and radioresistance. However, mechanisms underlying therapy resistance of hypoxic cells are multifactorial and not fully understood.

Previously, it has been reported that the expression of DNA-dependent protein kinase (DNA-PK) is associated with resistance against chemotherapeutic agents such as adriamycin and chlorambucil (Christodoulopoulos *et al*., 1998; Muller *et al*., 1998; Shen *et al*., 1998) and participated in the development of MDR (Kim *et al*., 2000; Um *et al*., 2001). Also, the increased expression and activity of DNA-PK are essential for cellular resistance to ionizing radiation in human tumor cells (Kienker *et al*., 2000; Ader *et al*., 2002; Marples *et al*., 2002; Shintani *et al*., 2003; Soubeyrand *et al*., 2003).

The DNA-PK is formed by association of the 460-kDa catalytic subunit (DNA-PKcs) with a DNA binding subunit known as Ku, a heterodimer of 70-kDa (Ku70) and 86-kDa (Ku80), and the enzyme plays a role in repair of DNA double-strand breaks (DSBs) and single strand nick and gap (Gottlieb and Jackson, 1993; Morozov *et al*., 1994; Jeggo, 1997; Jin *et al*., 1997; Kim *et al*., 2002). Defects in DNA-PK subunit have shown to result in a reduced capacity to DNA DSBs repair and consequently a hypersensitivity to radiation (Lees-Miller *et al*., 1995; Gu *et al*., 1997) and etoposide, a DNA-damaging agent (Jin *et al*., 1998).

Recently, it has been shown that DNA repair pathways involved in DNA DSBs repair likely include potential HIF-1 target (Unruh *et al*., 2003), and also the expression of Ku70 or Ku80 is up-regulated by hypoxia in several cells (Ginis and Faller, 2000; Lynch *et al*., 2001), suggesting that DNA-PK might be involved in regulation of HIF-1.

Therefore, in the present report, the modulation of DNA-PK and its role on HIF-1 expression were investigated under hypoxic condition, one of the most basic environmental stresses that cancer cells may experience. Our results showed that HIF-1$\alpha$ expression was positively correlated with the expression of DNA-PK, which could contribute to radioresistance and chemoresistance in hypoxic tumor cells. This is the first report that demonstrates a new role of DNA-PK on HIF-1 regulation.

## Materials and Methods

### Cell Lines and hypoxic conditions

Human hepatoma HepG2 cell line was grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO Invitrogen Corp., Carlsbad, CA) containing 10% (v/v) fetal bovine serum (FBS) (GibcoGIBCO Invitrogen Corp., Carlsbad, CA), streptomycin (100 μg/ml), and penicillin (100 U). Mouse hepatoma HepaC1C7 cells and its HIF-1β–deficient HepaC4 cells (Maxwell *et al*., 1997; Griffiths *et al*., 2002) were cultured in DMEM with 10% (v/v) FBS and antibiotics. Murine embryonic fibroblast MEF and its Ku-deficient $Ku70^{-/-}$ cells immortalized by SV40 transfection were maintained in DMEM supplemented with 10% (v/v) FBS and antibiotics. DNA-PKcs deficient SCID cells and isogenic wild type murine embryonic fibroblast CB-17 cells were maintained in DMEM supplemented with 10% (v/v) FBS and antibiotics.

The cells were subjected to normoxia in a humidified atmosphere containing 5% $CO_2$ level with 20% $O_2$ (v/v) at 37°C. For the hypoxic condition, cells were incubated at 5% $CO_2$ level with 1% $O_2$ (v/v) balanced with $N_2$ gas. Deferoxamine (DFX, Sigma Co.) was treated as mimic hypoxic condition (Wood *et al*., 1996).

### Cell extract preparation and electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from normoxic or hypoxic cells as described previously (Shintani *et al*., 2003). In brief, $3\times10^6$ cells were washed with cold phosphate buffered saline and harvested quickly and resuspended in 300 ml of lysis buffer [10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulphonyl fluoride (PMSF)]. The cells allowed swelling in ice for 10 min. After 0.05% Nonidet P40 was added, the tube was vigorously mixed 3 times for 3 s on a vortex, and centrifuged at 250×g for 10 min to pallet the nuclei. The nuclear pellet was resuspened in 30 ml of ice-cold nuclear extraction buffer (5 mM HEPES, pH 7.9, and 26% glycerol (v/v), 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF) and then incubated on ice for 30 min with intermittent mixing, and centrifuged at 24,000 *g* for 20 min at 4°C. The nuclear extract was either used immediately or stored at -70°C for later use. 10 μg of nuclear extract was incubated with $^{32}$P-labeled double-stranded oligonucleotide, 5'-AGTTGAGGGGACTTTCCCAGGC-3' for Ku binding and $^{32}$P-labeled HRE consensus oligonucleotide, 5'-TCTGTACGTGACCACACTCACCTC-3' (Santa Cruz Biotechnology, Inc., Santa-Cruz, CA) in binding buffer (10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 4% glycerol) containing 50 μg/ml of poly (dI-dC). The DNA-protein complex was separated from free oligonucleotide on 4% non-denaturing polyacrylamide gel using 0.5xTBE buffer (44.4 mM Tris-HCl, pH 8.0, 44.5 mM boric acid, 1

mM EDTA) for 3 h at 120 V. The gels were dried and autoradiographed (Byun *et al*., 2002). For super-shift assay, 1 μg of monoclonal antibody specific to Ku70, HIF-1α, HIF-1β or CBP was incubated with nuclear extracts for 2 h at 4°C prior to the DNA shift assay.

### Western blot analysis

Whole cell lysates or nuclear extract containing an equal amount of protein were separated by SDS-PAGE and blotted to nitrocellulose membrane (Hybond-ECL, Amersham Biosciences, Buckinghamshire, England). The membrane was incubated with antibody as specified, followed by secondary antibody conjugated with horseradish peroxidase. Specific antigen-antibody complexes were detected by enhanced chemiluminescence (Pierce Biotechnology, Inc., Rockford, IL). The following antibodies were used in these studies. The antibodies to HIF-1β, Ku70/80, and CBP were obtained from (Santa Cruz Biotechnology, Inc., Santa-Cruz, CA) and the anti-DNA-PKcs, and anti-HIF1α antibodies were obtained from Neomarker (Lab Vision Corp., Fremont, CA). Secondary antibodies were obtained from Amersham Biosciences, Buckinghamshire, England.

### Immunoprecipitation assay

For immunoprecipitation, nuclear extracts from HepaC1C7 cells treated with DFX or hypoxia for 4 h were incubated with antibody to HIF-1α, HIF-1β, Ku70, Ku80, CBP or DNA-PKcs for overnight at 4°C. Then protein G-Sepharose beads (Sigma-aldrich Corp., ST Louis, MO) were added and constantly mixed for 4 h. The beads were collected by centrifugation for 5 min at 12,000 rpm, 4°C and washed three times with cold extraction buffer. The beads with immunocomplexes were boiled and electrophoresed on 8% SDS-polyacrylamide gels and analyzed by Western blotting.

### Growth inhibition assay

Cells were seeded in 96 well plates at $4\times10^3$ cells/well and treated with 5-, 10 μM DFX for 6 h, before VP-16 was added for 96 h. Growth inhibition was assessed by MTT assay. Briefly, following treatment of the cells, the medium was replaced with fresh medium containing 500 μg/ml MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] solution and the plates were incubated in the dark room for 4 h. The water-insoluble MTT-formazan crystals were dissolved in dimethyl sulfoxide, and reduction of MTT was determined at 570 nm using ELISA reader (Bio-Tec Instruments, Inc., Winooski, VT). The concentration of each anticancer drug, which reduced cell growth rate by 50% after 96 h treatment ($IC_{50}$), was determined from the growth inhibition plots.

### DNA-dependent protein kinase assay

The kinase activity of DNA-PK was determined using the Signa TECT™ DNA-dependent Protein Kinase Assay System from Promega Corp., Madison, WI. In brief, 10 μg of nuclear extract was incubated with activator DNA, a biotinylated p53-derived peptide substrate, and [$\gamma$-$^{32}$P] ATP at 30°C for 5 min. The sample was terminated by adding termination buffer. Each termination reaction sample was spotted onto SAM$^{2TM}$ Biotin Capture Membrane and washed with 2 M NaCl and 2 M NaCl in 1% $H_3PO_4$. The SAM$^{2TM}$ Membrane squares were analyzed using Molecular Imager System (Bio-Rad Laboratories, Inc., Model GS 525, Hercules, CA).

## Results

### Involvement of HIF-1 in Ku regulation

Since previous reports have shown that HIF-1 and NF-κB are corporatively activated under hypoxic condition (Jeong *et al*., 2003), and Ku, regulatory subunits of protein kinase, activity is closely correlated with NF-κB activity (Um *et al*., 2001; Lim *et al*., 2002), Ku and NF-κB activities in mouse hepatoma HepaC1C7 cell and its HIF-1β-deficient HepaC4 cells, which is unable to form a functional HIF-1 complex, were analyzed by EMSA. As shown in Figure 1, HepaC4 cells showed constitutively reduced DNA-binding activities of Ku and NF-κB compared with those of wild type HepaC1C7 cells, and it resulted from reduced basal expressions of Ku70/80 and NF-κB p65. These results suggested that HIF-1 could affect the regulation of Ku.

To determine whether Ku expression and its activity could be modulated under hypoxic condition, Ku expression and its activity of HepG2 cells exposed to hypoxia and HepaC1C7 cells treated with deferoxamine (DFX), the hypoxia mimicking chemical, were examined (Figure 2). The expression and activity of Ku were markedly increased in these cells under hypoxic condition with a concurrent increase in HIF-1α in a time-dependent manner. These results suggest that Ku expression and its activity are up-regulated with a concurrent increase in hypoxia-induced HIF-1α expression.

### Deficiency of DNA-PK component resulted in down-regulation of HIF-1

To elucidate the correlation between the regulation of HIF-1 and Ku, the level of HIF-1 expression was examined in the Ku-deficient Ku70$^{-/-}$ cells and their wild type MEF under hypoxic condition using DFX treatment. As shown in Figure 3, the expression of HIF-1α of MEF cells was strongly induced as early

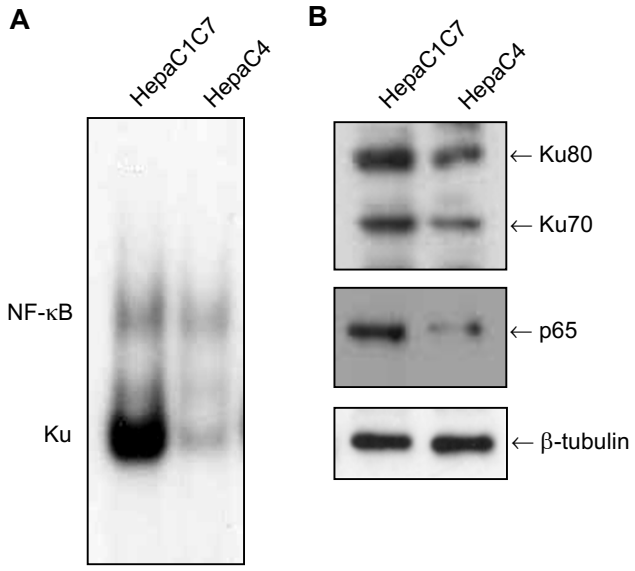


**Figure 1.** Reduced activity and expression of Ku and NF-κB in HIF-1β deficient HepaC4 cells. (A) Nuclear extracts isolated from HepaC4 cells and its wild type HepaC1C7 cells were analyzed for Ku and NF-κB DNA-binding activities by EMSA. (B) Nuclear expression levels of NF-κB p65 and Ku70/80 in HepaC4 and HepaC1C7 cells were evaluated by Western blot analysis. β-tubulin level was shown as reference control.

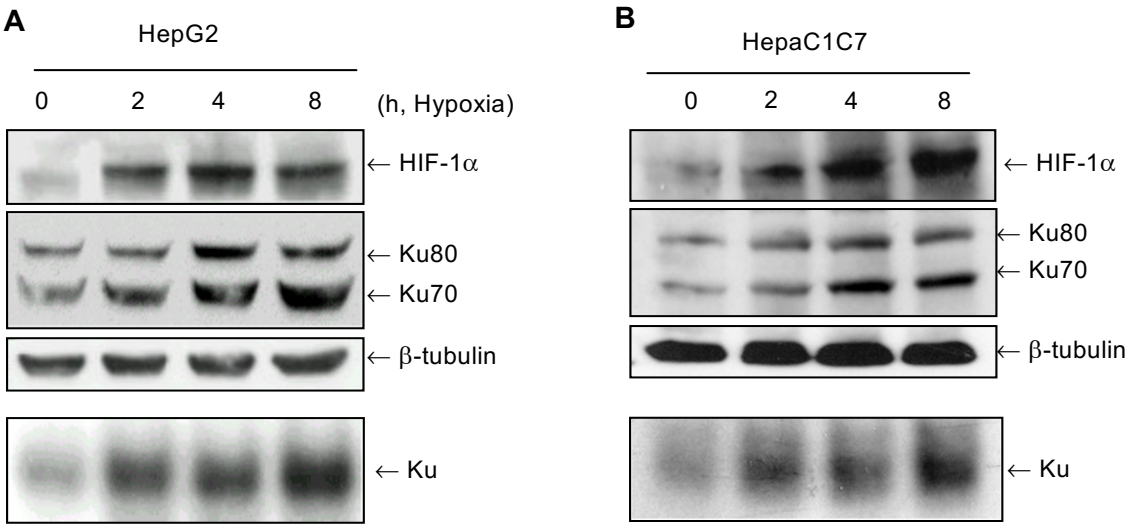


**Figure 2.** Enhanced Ku activity and its expression level under hypoxic condition. HepG2 cells were exposed to hypoxia (94% $N_2$, 5% $CO_2$, 1% $O_2$) for the indicated time. (A) HepaC1C7 cells were treated with 260 μM deferoxamine (DFX) for the indicated time. (B) Western blot analysis was carried out to measure the expression levels of HIF-1α and Ku70/80. β-tubulin was used to show relative expression and loading difference (top and middle panels). Nuclear extracts of these cells were prepared and subjected to EMSA for Ku DNA-binding activity (bottom panel).

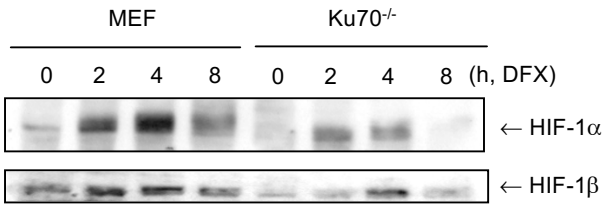


**Figure 3.** Modulation of HIF-1 in Ku-deficient cells. Ku-deficent $Ku70^{-/-}$ cells and their wild type MEF cells were treated with 260 μM DFX for the indicated time. Nuclear extracts were prepared, and Western blot analysis was performed using anti-HIF-1α and anti-HIF-1β.

as at 2 h and maximized at 4 h but that of $Ku70^{-/-}$ cells was markedly reduced in under same hypoxic condition. Also, the expression of HIF-1β in MEF cells was increased during 2-4 h but that in $Ku70^{-/-}$ cells was transiently increased at 4 h under same hypoxic condition. These results suggest that intracellular level of Ku could affect the regulation of HIF-1 expression.

To test the hypothesis that DNA-PKcs may affect on the HIF-1 regulation, the modulation of HIF-1 expression by DNA-PKcs under hypoxic condition was investigated. HepaC1C7 cells were exposed to hy-

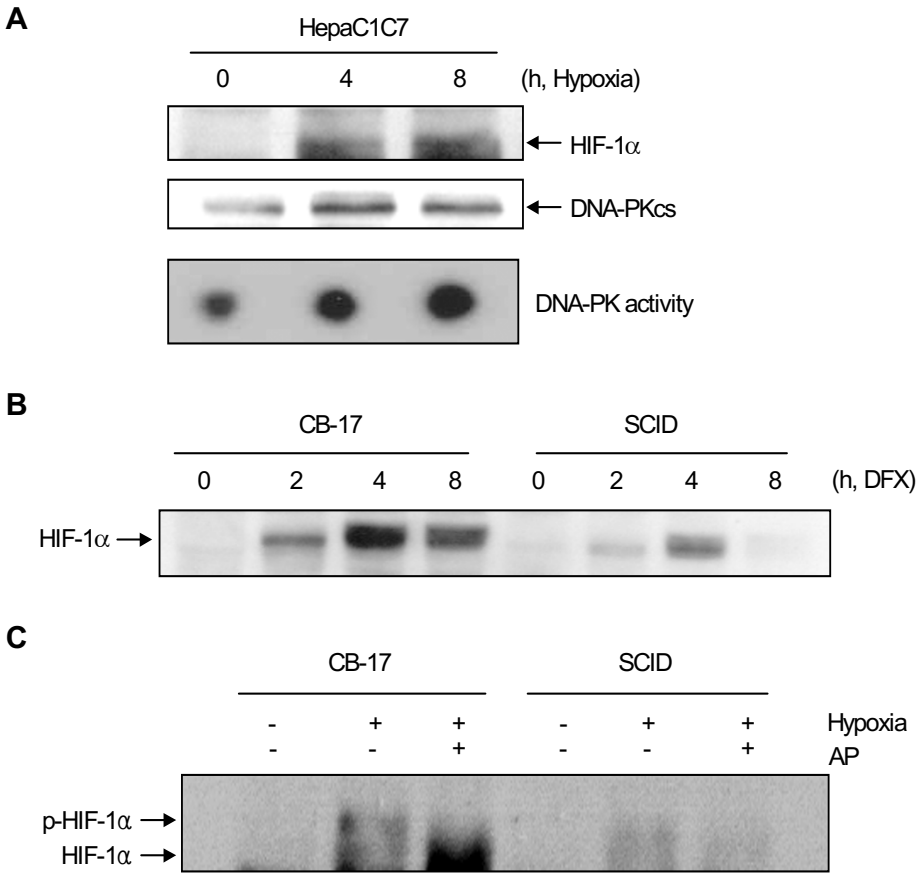


**Figure 4.** Effect of hypoxia on DNA-PKcs and DNA-PK activity, and the modulation of the expression and phosphorylation of HIF-1α by the expression of DNA-PKcs. Nuclear extracts were isolated from HepaC1C7 cells exposed to hypoxia for the indicated time. HIF-1α and DNA-PKcs expression was analyzed by Western blotting (A, top and middle panels), and DNA-PK activity was measured as described in Material and Method (A, bottom panel). CB-17 and SCID cells were treated with 260 μM DFX for the indicated time. Nuclear extracts were isolated and subjected to Western blot analysis to measure HIF-1α expression level (B) Nuclear extracts from CB-17 cells and SCID cells exposed to hypoxia for 4 h were incubated without or with alkaline phosphatase (AP) at 37°C for 2 h, and HIF-1α protein level was analyzed by Western blotting using anti-HIF-1α antibody (C).

poxia, and the changed levels of HIF-1α and DNA-PKcs were determined. As shown in Figure 4A (top and middle panels), the HIF-1α expression was markedly increased consistent with an increase of DNA-PKcs expression under hypoxia. Therefore, to examine whether the up-regulated Ku and DNA-PKcs expression under hypoxia could lead to increase the activity of whole DNA-PK complex, the DNA-PK activity was analyzed. As expected, the DNA-PK activity was significantly increased under hypoxia in a time dependently (Figure 4A, bottom panel), suggesting that DNA-PK activity could be correlated with HIF-1α expression.

To confirm the involvement of DNA-PKcs in HIF-1 regulation, the expression of HIF-1α of DNA-PKcs-deficient SCID cells was compared with that of parental CB-17 cells. The expression of HIF-1α was examined in SCID and CB-17 cells treated with DFX for the indicated time (Figure 4B). Under hypoxic condition, the HIF-1α expression of SCID cells was markedly down-regulated as compared to that of CB-17 cells, whose HIF-1α expression was rapidly induced at 2 h, reaching a peak at 4 h, suggesting that the DNA-PKcs may be positively correlated with HIF-1 regulation. Curiously, in this data, the mobility of HIF-1α migration was slower in CB-17 cells than in SCID cells. It has been demonstrated that the migration of phosphorylated protein can slow down on SDS-polyacrylamide gels as compared to unphosphorylated protein (Richard *et al*., 1999). Thus, to examine whether slowly moving HIF-1α protein of CB-17 cells could be resulted from phosphorylation, the nuclear extracts from CB-17 cells and SCID cells exposed to hypoxia for 4 h were treated with alkaline

phosphatase. As shown in Figure 4C, slowly moving HIF-1α could become unphosphorylated form in CB-17 cells by phosphatase, whereas no remarkable difference of HIF-1α by phosphatase was found in SCID cells. These results suggest the possibility that DNA-PK could be participated in the phosphorylation of HIF-1α and activate HIF-1α under hypoxia.

**Interaction between HIF-1and DNA-PK components**

To test the direct interaction between HIF-1 and DNA-PK components, HepaC1C7 cells were treated with DFX, and the nuclear extracts were immunoprecipitated with antibody against Ku70 or Ku80. The co-immunoprecipitated proteins were resolved on a polyacrylamide gel and proteins were detected by immunoblotting using anti-HIF-1α antibody. HIF-1α was detected in the immunoprecipitated proteins with anti-Ku70 or anti-Ku80 antibody (Figure 5A, left two panels). Since it is mandatory to form heterocomplex with HIF-1α and HIF-1β for HIF-1 function, we carried out immunoprecipitations with antibodies against Ku80 and immunoblotted with an antibody against HIF-1β to examine whether HIF-1β was present in Ku immunoprecipitates. HIF-1β was detected in the immunoprecipitated proteins with anti-Ku80 antibody (Figure 5A, middle panel). Conversely, the nuclear extracts of HepaC1C7 cells treated with DFX were immunoprecipitated with antibody against HIF-1α. Ku70 and Ku80 were detected in the immunoprecipitated proteins with anti-HIF-1α antibody (Figure 5A, right panel), suggesting that the Ku component of DNA-PK could interacted with HIF-1 components.

We next examined whether DNA-PKcs could interact with HIF-1α under hypoxic condition. The nuclear extracts of HepG2 cells exposed to hypoxia for 4 h were incubated with anti-DNA-PKcs antibody. HIF-1α was detected in the immunoprecipitated proteins with anti-DNA-PKcs antibody (Figure 5B). These results indicate that DNA-PK complex could physically interact with HIF-1 complex including HIF-1α and HIF-1β.

To confirm further the association of HIF-1 and Ku complex, the supershift assay using anti-Ku70 antibody was performed with nuclear extracts from HepaC1C7 cells treated with DFX. As shown in Figure 6A, DFX-induced DNA binding activity of HIF-1 was supershifted by anti-Ku70 antibody (lane 5) and anti-CBP antibody, which was used as a positive control (lane 3) due to binding with HIF-1 during transcriptional activation. Conversely, the Ku DNA binding activity induced by hypoxia was supershifted by antibody to HIF-1α, HIF-1β or CBP (Figure 6B. lane 3, 4, 5 and 6). These results provide strong evidence that Ku contributes formation of HIF-1 binding complex.

**HIF-1β-deficiency affects cellular susceptibility to chemotherapeutic agent and the mimic of hypoxia**

From the result shown above, HIF-1 expression can be up-regulated with a concurrent increase in DNA-

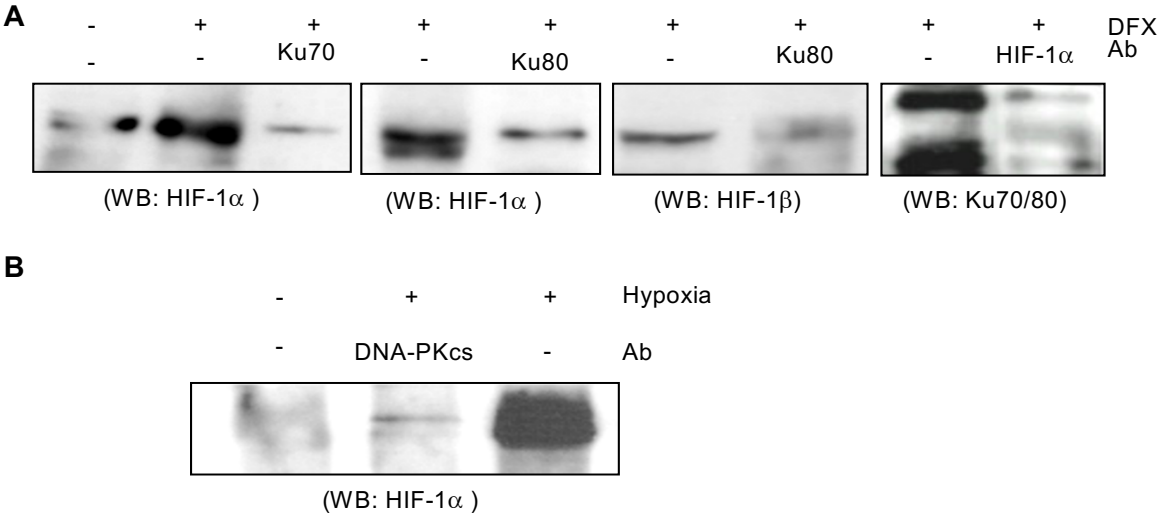


**Figure 5.** Interaction between HIF-1 and DNA-PK complex. HepaC1C7 cells were treated with 260 μM DFX for 4 h and the nuclear extracts were immunoprecipitated with anti-Ku70, anti-Ku80 or HIF-1α antibody. The co-immunoprecipitated proteins were resolved on a polyacrylamide gel and proteins were detected by Western blotting using anti-HIF-1α, anti-HIF-1β or anti-Ku70/80 antibody (A) HepG2 cells were exposed to hypoxia for 4 h, and the nuclear extracts were immunoprecipitated with anti-DNA-PKcs antibody. The immunoprecipitates were resolved in SDS-PAGE, and Western blotting was carried out using anti-HIF-1α antibody (B).

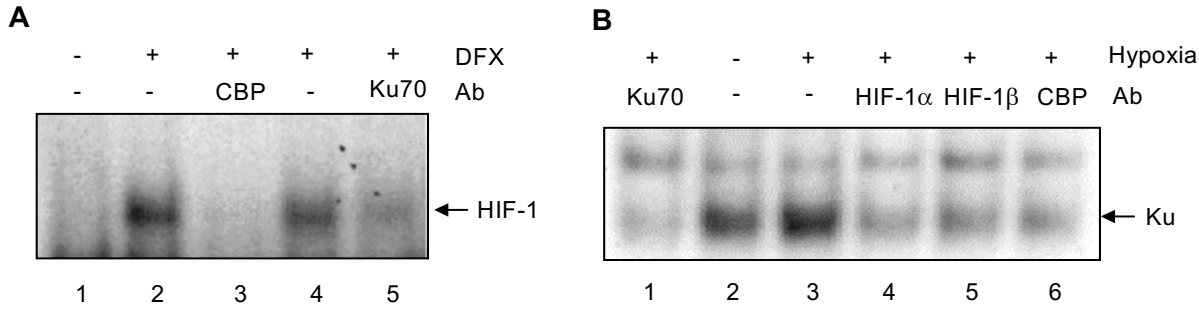


**Figure 6.** Analysis of interaction between HIF-1 and Ku by supershift assay. The nuclear extracts from HepaC1C7 cells treated with 260 μM DFX for 4 h (A, lane 2-5) or untreated cells (A, lane 1) were isolated. For supershift assay, the nuclear extracts were preincubated with anit-Ku70 antibody (A, lane 5) or anti-CBP antibody as a positive control (A, lane 3) and subjected to EMSA. Also, the cells were exposed to hypoxia (B, lane1 and 3-6) for 4 h or normoxia (B, lane 2), and the nuclear extracts were prepared for supershift assay using anti-HIF-1α (B lane 4), anti-HIF1β (B, lane 5), anti-CBP (B, lane 6) or anti-Ku70 antibody as a positive control (B, lane 1).

PK expression and thus the expressions of HIF-1 and Ku appears to be involved in development of chemoresistance, we now examined whether the chemosensitivity and its modulation by DFX could differentially display in HIF-1β-deficient HepaC4 cells, which is unable to form functional HIF-1 and concurrently have reduced Ku activity, and parental HepaC1C7 cells. As shown in Table 1, HepaC4 cells were more significantly susceptible to etoposide (VP-16) than HepaC1C7 cells. In contrast, the treatment of DFX (5- and 10 μM) with VP-16 of HepaC1C7 cells resulted in induction of approximately 7- and 9-fold resistance to VP-16, respectively, whereas the VP-16 sensitivity of HepaC4 cells was not changed significantly by DFX treatment. These results showed that an increased HIF-1 activity may be responsible for resistance to anticancer drug under hypoxic condition.

**Table 1.** Modulation of chemosensitivity by DFX in HIF-1β deficient HepaC4 and wild type HepaC1C7 cells.

| Cells | Drugs | $IC_{50}$ | Resistance induction (fold) |
|---|---|---|---|
| HepaC1C7 | VP-16 (nM) | 45 | |
| | +5 μM DFX | 250 | 5.6 |
| | +10 μM DFX | 396 | 8.8 |
| Hepa1C4 | VP-16 (nM) | 18 | |
| | +5 μM DFX | 18 | 1.0 |
| | +10 μM DFX | 32 | 1.8 |

Each cell line ($4\times10^3$ cells/well) was treated with VP-16 for 96 h in the presence or absence of 5-, 10 μM DFX pretreatment for 6 h. Growth inhibition assay was performed by MTT method and $IC_{50}$ was determined by growth inhibition plot. Values (folds of resistance induction) indicate the ratio of $IC_{50}$ for VP-16 alone to the $IC_{50}$ for VP-16 in the presence of DFX. Values are the average of two independent experiments and triplicate determinants in each experiments. VP-16, etoposide; DFX, deferoxamine.

## Discussion

HIF-1 is an attractive molecular target for development of novel cancer therapeutics. In human cancer cells, intratumoral hypoxia and genetic alterations affecting signal transduction pathways lead to increased HIF-1 activity. In this study, we revealed the involvement of the DNA-dependent protein kinase (DNA-PK) pathway in the regulation of HIF-1, and it could be implicated in resistance to cancer therapy of hypoxic tumor cells.

DNA-PK is a critical enzyme in DNA DSBs repair, and defects in DNA-PK subunits have been shown to result in hypersensitivity to ionizing radiation and etoposide that lead to DNA DSBs (Lees-Miller *et al*., 1995; Gu *et al*., 1997; Jin *et al*., 1998). In addition, it has been reported that Ku- or DNA-PKcs deficient cells are sensitive to anticancer drug including non-DNA damaging agents (Kim *et al*., 1999; Um *et al*., 2003).

In the present study, HIF-1β deficient HepaC4 cells which are unable to form a functional HIF-1 complex (Wood *et al*., 1996; Maxwell *et al*., 1997; Griffiths *et al*., 2002) showed constitutively reduced Ku and NF-κB activities and enhanced chemosensitivity compared to parental HepaC1C7 cells, which showed the induction of drug resistance against etoposide (VP-16) under hypoxic condition, suggesting DNA-PK as well as NF-κB could be involved in HIF-1 regulation. These results were in agreement with previous reports that NF-κB is activated by hypoxia (Koong *et al*., 1994) and regulate HIF-1 mediated gene expression (Figueroa *et al*., 2002; Jeong *et al*., 2003; Jung *et al*., 2003). Also, our results showed that the increased expression and activity of DNA-PK were accompanied with an increase in HIF-1α expression

under hypoxic condition. It has been reported that hypoxia could induce chromosomal breaks at fragile sites (Coquelle *et al*., 1998) and HIF-1α dependent repair of DNA double strand break (Unruh *et al*., 2003), suggesting the possibility that a DNA-PK could be activated in response to hypoxia. In fact, it has been shown that Ku could be involved in response to hypoxia (Ginis and Faller, 2000; Lynch *et al*., 2001).

In the current study, Ku- and DNA-PKcs deficient sublines showed significantly down-regulated HIF-1α expression level under hypoxic condition, suggesting a possibility that DNA-PK could function as a positive regulator of the HIF-1 expression. Our results also revealed that DNA-PK complex could directly interact with HIF-1 and suggest the possibility that DNA-PKcs could be involved in phosphorylation of HIF-1. DNA-PK has been known to interact and phophorylate certain transcription factors (Anderson, 1993; Bannister *et al*., 1993; Jackson *et al*., 1993; Araki *et al*., 1999). Although the phosphorylation domain of HIF-1 by DNA-PK is remained to be defined, several studies have reported that functionally active HIF-1 is necessary to be phosphorylated, which is important for HIF-1 mediated gene expression and the regulation of cell survival or death under hypoxia (Wang *et al*., 1995; Salceda *et al*., 1997; Minet *et al*., 2001; Semenza, 2002). For example, p42/p44 mitogen-activated protein kinase phosphorylate HIF-1α, enhancing the transcriptional activity of HIF-1 (Sang *et al*., 2003), and HIF-1α stabilization under hypoxia is dependent on a phosphatidylinositol 3-kinase (PI3K) /Akt pathway (Minet *et al*, 2001). The present results suggest the possibility that the hypoxia-induced activation of DNA-PK, which belongs to the PI3K family, is associated with phosphorylation of HIF-1α, and it leads to HIF-1α stabilization.

On the other hand, HIF-1 has been shown to mediate hypoxia-induced P-glycoprotein expression as a pathway for resistance of some tumors to chemotherapeutics (Comerford *et al*., 2002; Wartenberg *et al*., 2003) and enhanced expression of DNA-PK participates in development of multidrug resistance (MDR), which protect cancer cells from variety of drugs with different structure and function (Kim *et al*., 2000; Um *et al*., 2001). Therefore it is likely that stabilization of HIF-1α by DNA-PK could be one of the multitude mechanisms in resistance to chemotherapy of hypoxic tumor cells. We revealed that expression levels of HIF-1 and DNA-PK may be mutually controlled through direct interaction. This might have a role as a negative factor for tumor therapy in hypoxic tumor cells. To elucidate the interrelated regulation between HIF-1 and DNA-PK, further investigation will be required.

Taken together, present study suggests that correlated activation of DNA-PK and HIF-1 contributes to development of chemoresistance in hypoxic tumor cells. These findings provide new therapeutic strategies in hypoxic tumor cells, which are resistant to cancer therapy such as ionizing radiation and chemotherapy.

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