TIP30 Directly Binds p53 Tumor Suppressor Protein *In Vitro*

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TIP30 (30 kDa HIV-1 TAT-interacting protein), also called HTATIP2 or CC3, is a tumor suppressor protein that acts as an angiogenesis inhibitor. TIP30 blocks nuclear import of the mRNA-binding protein HuR, and thereby promotes the cytoplasmic accumulation of HuR by binding to importin- β , which is known to facilitate the cytoplasm-to-nuclear transport of HuR. Accumulation of HuR in the cytoplasm, in turn, enhances the expression of the transcription factor p53, a tumor suppressor that plays an essential role in preserving genome stability and inhibiting cancer growth. In addition to such a post-transcriptional mechanism via which TIP30 increases the p53 level, it has been proposed that TIP30 may regulate p53 protein at the protein level by directly binding to it. In order to investigate the possibility of direct interaction between p53 and TIP30, we have used on three functional regions in p53 and examined their interactions with TIP30 using GST pull-down assay and surface plasmon resonance technique. The results show that that TIP30 binds to the DNA-binding domain and the C-terminal domain of p53.

INTRODUCTION

The protein TIP30 (30 kDa HIV-1 TAT-interacting protein) was first identified in 1997 by a differential display analysis comparing mRNAs from highly metastatic human variant small-cell lung carcinoma (v-SCLC) with those from less metastatic classic small-cell lung carcinoma (c-SCLC) cell lines (Shtivelman, 1997). In the following year human TIP30 was shown to specifically enhance human immunodeficiency virus-1 (HIV-1) Tat-activated transcription (Xiao et al., 1998). The amino acid sequence of human TIP30 was later confirmed to be identical to that of human CC3 (Xiao et al., 1998). TIP30, which exhibits decreased expression in some cancer cells (Li et al., 2009; NicAmhlaoibh and Shtivelman, 2001; Shtivelman, 1997; Tong et al., 2009; Zhao et al., 2006), is a tumor suppressor with pro-apoptotic activity (El Omari et al., 2005; Xiao et al., 2000; Zhao

et al., 2007) as well as with anti-metastatic (Li et al., 2009; Shtivelman, 1997; Tong et al., 2009; Xiao et al., 2000; Zhao et al., 2007) and angiogenesis-inhibiting (NicAmhlaoibh and Shtivelman, 2001) properties. TIP30 binds importin- β , which facilitates the cytoplasm-to-nuclear transport of proteins (El Omari et al., 2005; King and Shtivelman, 2004; Zhao et al., 2008), including HuR (Guttinger et al., 2004; Wang et al., 2004). The cytoplasmic HuR enhances the UV irradiation-induced expression and transcriptional activity of the tumor suppressor p53 by binding to the 3' untranslated region of its mRNA and enhancing its translation (Mazan-Mamczarz et al., 2003).

The p53 tumor suppressor is a hub protein in the human cancer signaling network that is inactivated in approximately half of all human cancers. In response to stress signals, p53 is activated and induces the transcription of a number of genes involved in cell cycle arrest and apoptosis (Hupp et al., 2000; Vogelstein et al., 2000), including the Bcl-2 family member Bax (Chipuk et al., 2004). Using a TIP30 mutant that did not interact with importin- β , Zhao et al. (2008) showed that TIP30 competed with HuR for complex formation with importin- β . As a result, the nuclear import of HuR was inhibited with its accumulation in the cytoplasm. Thus, by binding to importin- β , TIP30 was able to increase the levels of cytoplasmic HuR, and p53 expression. Under oxidative stress conditions, the ectopic expression of TIP30 induces Bax-dependent apoptosis in a cancer cell line that lacks the TIP30 gene (Zhao et al., 2008). In addition, Shi et al. (2005) reported that TIP30 is involved in the apoptosis pathway partly by cooperating with p53 protein. However, the mechanism of such interactions remains unknown.

The proposed interaction between TIP30 and p53 is somewhat analogous to that of mdm2 and p53 since mdm2 modulates p53 expression/activity through interactions at both protein and mRNA levels; mdm2 not only stimulates p53 mRNA translation by binding p53 mRNA (Naski et al., 2009), but also physically interacts with the N-terminal transcriptional activation domain (TAD) of p53 protein (Chen et al., 1993; Chi et al., 2005) that leads to p53 polyubiquitination and degradation of p53 (Li et al., 2003). STRING (<http://string.embl.de/>) is a data-

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Received September 5, 2012; revised October 8, 2012; accepted October 9, 2012; published online November 15, 2012

Keywords: GST pull-down assay, p53, protein-protein interaction, surface plasmon resonance, TIP30

base of known and predicted protein interactions (Szkarczyk et al., 2011), which allows one to predict potential protein-protein interactions. The interaction between p53 and mdm2 can be predicted reliably by this algorithm, and interestingly, STRING also predicts that TIP30 and p53 should be able to interact with each other at the protein level. In order to investigate this potential interaction between TIP30 and p53, we have carried out GST pull-down assays and surface plasmon resonance (SPR) binding analyses for the two proteins. We found that TIP30 protein directly bound the p53 DNA-binding domain (DBD) and p53 C-terminal domain (CTD) *in vitro*. These results suggest that TIP30 plays an important role as a regulatory factor that controls the subcellular localization and function of p53.

MATERIALS AND METHODS

Construction of TIP30 and p53 expression plasmids

Human TIP30 (GenBank ID: BC002439) was a gift from the 21C Human Gene Bank, Genome Research Center, KRIBB, Korea. TIP30 and DNA sequences encoding full-length p53 (FL; residues 1-393), the p53 N-terminal domain (NTD; residues 1-93), the p53 DNA-binding domain (DBD; residues 94-312), and the p53 C-terminal domain (CTD; residues 301-393) were amplified by PCR using oligonucleotide primers, as shown in Table 1 and Fig. 1A. For His-tagged TIP30, the PCR product was digested with *Nde*I and *Bam*HI enzymes and was inserted into the corresponding sites of the pET15b vector containing an N-terminal His tag (Novagen). GST-tagged p53 constructs for use in GST pull-down assays were prepared by digesting PCR products with *Bam*HI and *Eco*RI and inserting into the corresponding sites in the pGEX-2T vector (GE Healthcare). For SPR experiments, PCR products were digested with *Nde*I and *Bam*HI and were inserted into the corresponding sites in the pET15b vector (Novagen).

Purification of full-length and truncated forms of GST-p53 protein

For GST pull-down assays, p53 FL and the truncated forms of p53 were expressed as GST-fusion proteins in *Escherichia coli* strain BL21(DE3) (Novagen). The cell pellets were resuspended in phosphate-buffered saline (PBS) containing 1 mM phenylmethanesulfonyl-fluoride (PMSF), and were then sonicated using an Ultra Cell TM (Sonics and Materials). The lysate was centrifuged at 15,000 rpm for 30 min at 4°C and the supernatant was collected. GST-tagged proteins in the supernatant were bound to glutathione Sepharose beads.

GST pull-down assay

The interactions between TIP30 and various domains of p53 protein (p53 FL, p53 NTD, p53 DBD, and p53 CTD) were investigated first by GST pull-down assay using His-tagged TIP30 and GST-tagged domains of p53. GST protein alone served as a negative control. In interaction experiments, 5 µg of purified His-tagged TIP30 protein and 2 µg of GST-tagged p53 domains were mixed in pull-down assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5 mM EDTA, 3% glycerol, 0.5% NP-40, 1 mM DTT, 0.5 mM PMSF). Following the addition of 10 µl glutathione Sepharose beads, the mixture was incubated for 30 min at room temperature on a rotator. The glutathione Sepharose beads were washed four times with pull-down assay buffer. After washing, 10 µl of 5X SDS-PAGE gel loading buffer was added to the tube and the mixture was heated at 100°C for 10 min. For immunoblotting, the reaction mixtures were resolved by sodium dodecyl-sulfate polyacrylamide gel electrophoresis

(SDS-PAGE) on 15% gels, and were then transferred to nitrocellulose membranes (BioTrace NT; Pall Corporation). Membranes were incubated in TBST buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20) containing 10% nonfat skim milk for 30 min at room temperature on a shaker, followed by incubation with primary antibodies for 1 h at room temperature on a shaker. The primary antibodies used were rabbit anti-His (1:3,000 dilution; Santa Cruz Biotechnology) and goat anti-GST (1:3,000 dilution; GE Healthcare). Membranes were washed three times with TBST (5 min per wash) and were then probed with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature on a shaker. Immunoreactive proteins were detected using the SuperSignal West Pico chemiluminescent substrate (Pierce).

Expression and purification of recombinant His-tagged proteins

In contrast to the GST pull-down assay where only TIP30 was His-tagged, all the proteins used for SPR experiments were tagged with "His" using pET15b vector. These His-tagged proteins are easily purified and detected by using Ni-Sepharose beads and His-antibodies. For production of His-tagged proteins, TIP30, p53 DBD, and p53 CTD pET15b constructs were expressed in the *E. coli* strain BL21(DE3) (Novagen). Overnight cultures in LB (20 ml with 50 µg/ml ampicillin) were used to inoculate 2 L of LB media containing 50 µg/ml ampicillin. Cells were grown at 37°C until reaching an OD₆₀₀ of 0.6, after which protein expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The temperature was adjusted to 20°C and expression was allowed to continue overnight. The cells were collected by centrifugation. The cell pellet was resuspended in lysis buffer (20 mM sodium phosphate, pH 7.4, 0.5 M NaCl, 5 mM imidazole) and was lysed using an Ultra Cell TM (Sonics and Materials). The lysate was centrifuged at 15,000 rpm for 30 min at 4°C. The supernatant was collected and was filtered through a 0.45-µm syringe filter (SFCA membrane; Corning). The supernatant containing the His-tagged protein was loaded onto a Ni-Sepharose column and was eluted with elution buffer (20 mM sodium phosphate, pH 7.4, 0.5 M NaCl, 0.5 M imidazole). The His-tagged protein was dialyzed against binding buffer (20 mM sodium phosphate, pH 6.0, 20 mM KCl, 0.4 mM PMSF, 5 mM 2-mercaptoethanol) and was then applied to an SP-Sepharose column (1.5 × 10 cm; GE Healthcare) equilibrated with binding buffer. The column was eluted with a linear gradient of 0 to 1 M NaCl in the same buffer. The His-tagged protein was further purified by a Sephacryl S-200 column (2.5 × 120 cm; GE Healthcare) equilibrated with 25 mM sodium phosphate (pH 6.0), 150 mM NaCl, 5 mM 2-mercaptoethanol, and 1 mM PMSF. The purified His-tagged protein was stored at -80°C until use. The integrity of the purified proteins was confirmed by SDS-PAGE and Coomassie Brilliant Blue staining.

Biacore biosensor analysis

The interaction of p53 DBD and p53 CTD with TIP30 were analyzed using a Biacore 3000 SPR system (GE Healthcare) using His-tagged proteins (Fig. 4). The rationale for employing His-tagged protein instead of GST-tagged proteins for SPR experiments is as follows. Since a "GST tag" consists of 211 amino acid residues its large size could affect the interaction of the GST-tagged ligand proteins and the analyte proteins. On the other hand, the "His-tag" consists of only 6 amino acid residues. Therefore, it is unlikely that there should be any interactions between the His-tags themselves which may disturb the

Table 1. PCR primers used for fusion protein construct. Enzyme sites for cloning are underlined.

Name	Forward	Reverse	Vector
GST-p53 NTD	CGCGGATCCATGGAGGAGCCGCAGTC	GCCGAATTCTCACAGGGGCCAGGAGGGG	pGEX-2T
GST-p53 DBD	CGCGGATCCATCATCTTCTGTCCCTTC	GCCGAATTCTCAGGTGTTGTTGGGCAGTG	
GST-p53 CTD	CGGGATCCCAGGGAGCACTAAG	CCGGAATTCCTAGTCTGAGTCAGGCCCTT	
GST-p53 FL	CGCGGATCCATGGAGGAGCCGCAGTC	CCGGAATTCCTAGTCTGAGTCAGGCCCTT	
His-p53 DBD	GCGAATTCATATGTCATCTTCTGTCCCTTCCC AGAAAACC	CGGGATCCCTACGTGTTGTTGGGCAGTGCTGCGT TAG	pET15b
His-p53 CTD	GCGAATTCATATGCCAGGGAGCACTAAG	CGCGGATCCCTAGTCTGAGTCAGGCCCTTC	
His-TIP30	GCGAATTCATATGCCGAAACAGAAG	GCGGGATCCCTCATGGCTTGAGAGAG

Fig. 1. (A) Schematic representation of various truncated p53 constructs and TIP30. p53 constructs were fused in frame to the 3' end of the GST gene in the pGEX-2T vector. His-tagged constructs of TIP30 and p53 DBD or CTD were fused in frame at the 3' end of the His gene in the pET15b vector. (B) GST-p53 constructs expressed in *E. coli* culture extracts were bound to glutathione Sepharose beads after lysis, and were separated by SDS-PAGE on 15% gels. (C) His-tagged p53 DBD expressed in *E. coli* culture was purified using an FPLC system and was resolved by SDS-PAGE on 15% gels. (D) His-tagged p53 CTD was purified by the identical methods used for the p53 DBD construct and

was resolved by SDS-PAGE on 20% gels. Protein bands in gels were visualized by Coomassie Brilliant Blue staining.

interaction between ligand and analyte proteins. TIP30 (100 μ g/ml) in 25 mM sodium acetate (pH 4.5) was immobilized on a CM5 sensor chip using an amine coupling kit (GE Healthcare). p53 DBD and p53 CTD in HBS-EP buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% Surfactant P20) at a concentration of 0.02 to 1 μ M, were passed over the surface of the sensor chip at a flow rate of 30 μ l/min at 25°C. The strengths of the interactions between ligand (TIP30) and analyte (p53 domains) were determined by monitoring the rates of association and dissociation reactions recorded in a sensorgram. Kinetic analyses were performed by global fitting of the binding data with a 1:1 Langmuir binding model using BIAevaluation version 4.1.

Transfection and luciferase assay

HEK293T cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. 5×10^5 HEK293T cells were transiently transfected with p21-Luc, p53-GFP, TIP30-V5-6xHis, and a *Renilla* luciferase control vector (Promega), using a lipofectamine 2000 (Invitrogen) as the manufacturer's instruction on the 12 well plates. At 24 h after transfection, cells were lysed and the luciferase activity was determined with a dual luciferase reporter gene assay kit

(Promega). Briefly, cell lysates were mixed with the provided luciferase assay substrate, and firefly luminescence was measured using a VICTOR3 1420 multilabel counter (Wallac, Perkin-Elmer, USA). The firefly luciferase activity of each sample was normalized to the *Renilla* activity.

RESULTS AND DISCUSSION

Expression of TIP30 and p53 in *E. coli*

TIP30 and various p53 constructs were expressed as GST-fusion and His-tagged recombinant proteins and were purified as described in "Materials and Methods". p53 proteins were expressed using the bacterial expression vectors pET15b or pGEX-2T (Fig. 1). Human p53 protein is a homotetramer in which an individual monomer consists of 393 amino acid residues. As shown Fig. 1, we divided p53 into three functional regions: (I) p53 NTD, containing the transcriptional activation domain (residues 1-61) and proline rich domain (residues 62-93); (II) p53 DBD, responsible for sequence-specific DNA binding; and (III) p53 CTD, consisting of a tetramerization (residues 320-356) and a regulatory domain (residues 363-393) (Bell et al., 2002; Joerger and Fersht, 2007).

Purified proteins were resolved by SDS-PAGE and were

Fig. 2. SDS-PAGE and MALDI-TOF MASS spectrum of His-TIP30. (A) TIP30 protein was purified as a His-tagged recombinant protein from an *E. coli* expression system. The integrity of purified proteins, visualized by Coomassie Brilliant Blue staining, was confirmed by SDS-PAGE on 15% gels. (B) A MALDI-TOF mass spectrum of His-TIP30.

Fig. 3. A GST pull-down assay showing the complex formation between TIP30 and various domains of the p53 protein. Complexes were resolved by SDS-PAGE on 15% gels, and Western blots were probed with a mouse anti-His antibody (top panel). The participation of p53 protein was probed using a goat anti-GST antibody (bottom panel). TIP30 formed a complex with a full-length p53 (FL; lane 2), the p53 DBD (lane 4) and the p53 CTD (lane 5), but not with the p53 NTD (lane 3). TIP30 did not interact with GST protein alone, which was included as a control (lane 1).

visualized using Coomassie Brilliant Blue staining. As shown in Fig. 1, the resulting bands corresponded to proteins of 26 kDa (GST), 69.7 kDa (GST-p53 FL), 36.2 kDa (GST-p53 NTD), 50.7 kDa (GST-p53 DBD), 36.5 kDa (GST-p53 CTD), 26.8 kDa (His-p53 DBD), and 12.7 kDa (His-p53 CTD), confirming the purity of recombinant GST- and His-tagged p53 proteins. In Fig. 1B, the apparent molecular weight of GST-p53 NTD was greater than the true value, reflecting an interesting property of intrinsically unfolded proteins (Csizmek et al., 2006). His-tagged TIP30

Fig. 4. BIAcore sensorgrams showing interactions between two domains of the p53 and TIP30 protein immobilized on a CM5 sensor chip. (A) Responses of TIP30 protein obtained by injecting the p53 DBD at concentrations of 0.125, 0.25, 0.50, and 1.0 μ M (highest sensorgram). (B) Responses of TIP30 protein obtained by injecting the p53 CTD at concentrations of 0.02, 0.04, 0.08, 0.12, 0.25, and 0.5 μ M protein (highest sensorgram). Raw binding data were analyzed using BIAevaluation version 4.1 (GE Healthcare) by fitting to a 1:1 Langmuir binding model.

TIP30 protein was purified using a Fast Protein Liquid Chromatography (FPLC) system (Fig. 2A), and its molecular mass was

Fig. 5. Down-regulation p21 gene expression by TIP30. HEK-293T cells were co-transfected with p21 promoter-luciferase plasmid, p53-GFP expression plasmid, and indicated concentrations of TIP30-V5-6xHis expression plasmid. Total DNA was equaled by the addition of the empty expression vector. The relative transactivation values were obtained from both with p53-GFP and without TIP30-V5-6xHis taken as 100%. Results are taken from two independent experiments.

determined by MALDI-TOF mass spectrometry (Fig. 2B). For GST pull-down assays, GST-tagged recombinant p53 domains were bound to glutathione Sepharose beads without a purification step (Fig. 1B). For SPR analysis, His-tagged p53 DBD and CTD were purified using a FPLC system (Figs. 1C and 1D).

Binding of TIP30 to p53 DBD or CTD

First, we investigated the interaction of His-tagged TIP30 and GST-tagged p53 fusion proteins by GST pull-down assay. The reaction mixture containing His-tagged TIP30 protein and GST protein alone was used as a control (Fig. 3, lane 1). We found a band corresponding to GST protein (bottom panel) but no band corresponding to His-tagged TIP30 protein (top panel), which indicated that TIP30 did not interact with GST protein. Reaction mixtures containing His-tagged TIP30 and p53 FL (lane 2), p53 DBD (lane 4), or p53 CTD (lane 5) showed clear bands corresponding to TIP30 protein. However, reaction mixtures containing TIP30 and p53 NTD showed no TIP30 (lane 3). These results indicate that TIP30 interacts with full-length p53 as well as p53 DBD and CTD, but not with p53 NTD.

To further verify the results of the GST pull-down assay, we used SPR spectroscopy, a powerful technique for analyzing protein-protein interactions, to measure the binding affinity between TIP30 and p53. Kinetic analyses performed by a global fitting of the binding data with a 1:1 Langmuir binding model, revealed that TIP30 was physically bound to p53 DBD and to p53 CTD, with K_d values of 86.5 and 149 nM, respectively (Fig. 4), indicating that TIP30 interacts more strongly with p53 DBD than with p53 CTD.

Using GST pull-down assays and surface plasmon resonance (SPR) binding analyses we have demonstrated for the first time that TIP30 protein directly interacts with the p53 DNA-binding domain (DBD) and p53 C-terminal domain (CTD) *in vitro*, in addition to regulating p53 at the mRNA level through HuR. These results suggest that TIP30 plays an important role as a regulatory factor that controls the subcellular localization and function of p53. Our results on the direct binding between TIP30 and p53 suggest two possibilities. First, it is possible that

TIP30 may act as a cofactor for p53 in the transcriptional regulation of the Bax gene (Xiao et al., 1998), encoding a well-known apoptotic factor. This hypothesis could be tested by investigating the effects of TIP30 on the *in vitro* transcriptional activity of p53. Such a cofactor function of TIP30 has previously been reported in relation to Tat protein regulation of HIV-1 mRNA transcription (Xiao et al., 1998). Second, TIP30 may act as a regulator of importin activity. It is known that TIP30 interacts with importin (El Omari et al., 2005; King and Shivelman, 2004; Zhao et al., 2008) and importins have been shown to translocate p53 protein across the nuclear membrane by interacting with the NLS of p53 (Komlodi-Pasztor et al., 2009; Li et al., 2007). Since TIP30 protein interacts with both importin and p53, we can envision a complex in which TIP30 acts as a bridge between p53 and importin, facilitating nuclear translocation of p53. These possibilities are not mutually exclusive, and either could potentially explain the pro-apoptotic activity of TIP30. Future investigations utilizing the smaller TIP30-binding regions within p53 DBD and p53 CTD and those involving the smaller p53-binding regions in TIP30 with p53 should provide knowledge on a more detailed mechanism of binding between TIP30-p53.

Transcriptional repression of the p21 promoter by TIP30 and p53 interaction

To assess the regulatory impact of TIP30 proteins on p53 activity, we introduced p53-dependent p21 transactivation without DNA damage. For this purpose, the p21 promoter-luciferase reporter (p21-Luc) system was used. Results showed that p53 alone had a modest stimulatory effect on p21 promoter activity (Fig. 5) compared with the p53-nonexpressing control (data not shown). However, such an effect was significantly inhibited when cells were co-transfected with TIP30, showing that TIP30 can inactivate the p21-promoting ability of p53.

ACKNOWLEDGMENTS

This study was supported by a grant from the National R&D Program for Cancer Control, Ministry for Health and Welfare, Republic of Korea (0920240), and by a Korean-Hungarian Joint Laboratory Program grant (UGM0021011) to K.H. Han from the Korea Research Council of Fundamental Science and Technology (KRCF).

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