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The Interaction of KCTD1 With Transcription Factor AP-2α Inhibits Its Transactivation

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ABSTRACT

AP-2 is a transcription factor implicated in mammalian development, cell proliferation, apoptosis, and carcinogenesis. To identify potential AP-2α-interacting partners, a yeast two-hybrid screen was performed in human brain cDNA library. One of the identified clones encodes potassium channel tetramerization domain-containing 1 (KCTD1). We demonstrated the novel KCTD1–AP-2α interaction in vitro by GST pull-down assays and in vivo by co-immunoprecipitation assays and mapped the interaction domains to the N-termini of both proteins. In addition, we observed that the two proteins were completely co-localized in the nuclei of mammalian cells. Transient transfection assays using four promoters containing AP-2-binding sites confirmed that KCTD1 significantly repressed AP-2α-mediated transactivation through the BTB domain, whereas KCTD1 siRNA strongly relieved KCTD1-mediated repression of AP-2α transcriptional activity, and other BTB domain proteins such as PDIP1, KCTD10, and TNFAIP1 did not markedly inhibit the transcriptional activity of AP-2α, suggesting that KCTD1 specifically acts as a negative regulator of AP-2α. Finally, we found that KCTD1 interacted with three major members of the AP-2 family and inhibited their transcriptional activities. Taken together, our results indicate the novel function of KCTD1 as the transcriptional repressor for AP-2 family, especially for AP-2α. J. Cell. Biochem. 106: 285–295, 2009.

KEY WORDS: AP-2α; INTERACTION; KCTD1; BTB DOMAIN; TRANSCRIPTIONAL REPRESSION

The AP-2 transcription factor family consists of five different genes known as AP-2α, AP-2β, AP-2γ, AP-2δ, and AP-2ε [Eckert et al., 2005]. They share a conserved structure, possessing an N-terminal proline/glutamine-rich activation domain, central DNA-binding region, and a C-terminal helix-span-helix dimerization motif [Williams and Tjian, 1991a, b]. AP-2 factors could bind to a consensus DNA sequence, 5-GCCN$_3$GGC-3 [McPherson and Weigel, 1999] and many genes with the AP-2-binding sites have been identified, such as human metallothionein IIa [Imagawa et al., 1987] and simian virus 40 (SV40) [Mitchell et al., 1987].

To date, the functions of the AP-2 family members have been extensively studied. AP-2 genes play important roles in the embryogenesis and development, mice knockouting AP-2α, AP-2β, or AP-2γ exhibit lethal phenotypic defects in neural tube, face, eye, heart, skin, urogenital tissues, or extraembryonic trophoblasts and usually die at the birth [Zhang et al., 1996; Moser et al., 1997; Auman et al., 2002; Werling and Schorle, 2002; Eckert et al., 2005]. AP-2 proteins have been shown to be implicated in many different types of cancer, such as mammary carcinoma [Pellikainen et al., 2002; Douglas et al., 2004], melanoma [Huang et al., 1998; Tellez

Abbreviations used: AP-2, activator protein-2; BTB, broad-complex, tramtrack, bric-a-brac; ER, estrogen receptor; GST, glutathione-*S*-transferase; KCTD1, potassium channel tetramerization domain containing 1; Luc, luciferase; P21 (WAF1/Cip1), cyclin-dependent kinase inhibitor 1A; PDIP1, polymerase delta-interacting protein 1; siRNA, small interfering RNA.

Xiaofeng Ding and Chang Luo contributed equally to this work.

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et al., 2003], and glioma [Heimberger et al., 2005]. Moreover, loss of AP-2α proteins has been reported in several cancers, and such loss may predict for poor survival and the enhancement of tumorigenicity [Douglas et al., 2004]. Accordingly, AP-2 family members regulate an increasing number of cancer-associated genes including IGF IR [Turner et al., 1998], MCAM/MUC18, c-KIT [Huang et al., 1998; Bar-Eli, 2001], VEGF [Ruiz et al., 2004], and transforming growth factor-α [Wang et al., 1997]. In addition, AP-2 participates in cellular growth and differentiation [Auman et al., 2002] and directly activates the promoter of several related genes such as p21WAF/CIP [Zeng et al., 1997], erbB-2 [Bosher et al., 1996], and ERα [McPherson et al., 1997].

More importantly, AP-2 regulates so many different genes in that AP-2 is capable of forming the heterodimers through the protein–protein interactions to affect the transcription of various AP-2 downstream targets by modulating AP-2 transcriptional activity. So far, many AP-2-interacting proteins have been identified. For example, Yin Yang 1 interacted with AP-2α and enhanced the ERBB2 gene expression in mammary cancer cells [Begon et al., 2005]. And our previous results showed that GAS41 associated with AP-2β and stimulated AP-2β-mediated transactivation, at least in part, by enhancing the DNA-binding activity of AP-2β [Ding et al., 2006]. The Wwox tumor suppressor protein interacted with AP-2γ and triggered redistribution of nuclear AP-2γ to the cytoplasm, hence suppressing its transactivation function [Aqeilan et al., 2004].

To identify new proteins that interact with transcription factor AP-2α, we performed a two-hybrid screen using AP-2α as bait and identified a novel BTB domain-containing protein KCTD1 as a potential factor. The interaction between KCTD1 and AP-2α was confirmed in vitro by GST pull-down assays and in vivo by co-immunoprecipitation and co-localization analyses. Moreover, KCTD1 strongly inhibited the transactivation of AP-2α through the BTB domain. Furthermore, KCTD1 inhibited the transcriptional activities of other AP-2 family members. These results supported a direct interaction between KCTD1 and AP-2α and further suggested the transcriptional repression of AP-2α by KCTD1.

MATERIALS AND METHODS

VECTOR CONSTRUCTION

The bait plasmid pDBLeu-AP-2α was generated by inserting the entire coding sequence of human AP-2α in frame with the GAL4 DNA-binding domain of pDBLeu (Invitrogen), and the same full-length sequences of AP-2α were cloned into pCMV-Myc (Clontech) and pGEX-4T-1 (Amersham). The truncations of AP-2α (1–165 a.a. and 165–438 a.a.) were introduced into pGEX-4T-1. Human full-length KCTD1 (1–257 a.a.), KCTD1N (1–130 a.a.), and KCTD1C (125–257 a.a.) were inserted into pQE-N3 (Qiagen) and pCMV-HA (Clontech) as described recently [Ding et al., 2008]. Expression vectors for HA-PDIP1, HA-KCTD10, and Myc-TNFAIP1 were constructed by the insertion of full-length PDIP1, KCTD10, and TNFAIP1 to pCMV-HA or pCMV-Myc. The pCMV-Myc-AP-2β fusion plasmid was described previously [Ding et al., 2006], and the AP-2γ coding region was introduced into the pCMV-Myc vector. The mutants AP-2α D52A, P61R, and L107A/L108A were obtained by PCR amplification using site-directed mutagenesis with two complementary oligos containing the desired change.

Reporter plasmid A2-Luc containing three copies of AP-2-binding sites in the promoter region of human metallothionein IIa gene has been prepared previously [Williams and Tjian, 1991a; Ding et al., 2006]. The erbB-2-Luc reporter plasmid was constructed by inserting human c-erbB-2 promoter sequence between −300 and +40 including one AP-2-binding site to pTAL-Luc [Bosher et al., 1996]. Reporter plasmid P21-Luc was generated by inserting human P21 promoter sequence (from −121 to +16) containing two AP-2-binding sites to pTAL-Luc [Zeng et al., 1997]. Reporter plasmid ER-Luc (−3500/+230) was obtained by inserting human ER promoter including two AP-2-binding sites to pTAL-Luc [McPherson and Weigel, 1999]. All constructs have been sequenced for verification. Four siRNA sequences targeting KCTD1 (AF542549) were from the positions (siRNA1, 153–171; siRNA2, 474–492; siRNA3, 680–698; siRNA4, 756–774) relative to the start codon and synthesized by Shanghai GenePharma Co. Ltd (China).

YEAST TWO-HYBRID SCREEN

Yeast two-hybrid screening was performed as described previously [Ding et al., 2006] using pDBLeu-AP-2α as bait and pPC86-human brain cDNA library (Invitrogen) as prey. Yeast strain MaV203 was sequentially transformed with pDBLeu-AP-2α and human brain cDNA library. Approximately 2×10^6 yeast transformants were screened and false-positive clones were excluded by retransforming the prey DNA to MaV203. Positive clones were verified by using X-galactosidase filter assays and then sequenced in both directions.

GST PULL-DOWN ASSAYS AND WESTERN BLOTTING

In vitro interactions between AP-2α and KCTD1 were examined by GST pull-down assays as described previously [Ding et al., 2006]. Briefly, GST and His fusion proteins were expressed and purified, respectively (Amersham). Five micrograms of GST fusion proteins was incubated with equal amount of His recombinant proteins in binding buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 0.05% NP-40, and 5 mM DTT) at 4°C for 2 h before glutathione-sepharose 4B beads were added. After a further 2 h, proteins bound to the beads were extensively washed, resuspended in SDS–gel loading buffer and boiled. The proteins were resolved by 10–15% SDS–polyacrylamide gels and detected by Western blotting with mouse monoclonal antibodies against GST (Santa Cruz Biotech) or His-tag (Clontech).

CELL CULTURE AND TRANSIENT TRANSFECTIONS

HeLa, HBL100, and HEK293FT cells were cultured in DMEM medium supplemented with 10% fetal calf serum, glutamine, penicillin, and streptomycin. Cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C. Cells were transfected with plasmid DNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For siRNA transfection, the same method was used. Lipofectamine 2000-siRNA or -DNA complexes were prepared at room temperature for 20 min. Cells were incubated with complexes in growth medium without serum and antibiotics for 4–6 h and then transferred to complete medium, transfected cells were harvested after a further 30 h.

CO-IMMUNOPRECIPITATION ASSAYS

HEK293FT cells in 10 cm dishes were grown to 80% confluence, and co-transfected with 10 μg of Myc-AP-2α and 10 μg of HA-KCTD1, HA-KCTD1N, or HA-KCTD1C or only transfected with 10 μg of Myc-KCTD1. After 36 h, the cells were harvested and lysed in RIPA buffer [50 mM Tris–HCl (pH 7.2), 150 mM NaCl, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS and protease inhibitors]. The lysates were precipitated using rabbit polyclonal antibodies against Myc-tag, HA-tag (Santa Cruz Biotech), or KCTD1 we developed [Ding et al., 2008] and protein A/G plus agarose (Santa Cruz Biotech); the immunoprecipitates were separated by 15% SDS–polyacrylamide gels and analyzed with mouse monoclonal antibodies against HA-tag, Myc-tag, or AP-2α (Santa Cruz Biotech).

IMMUNOFLUORESCENCE ANALYSIS

HeLa cells were cultured on glass coverslips in a 12-well plate, grown to 70% confluence and transfected with the indicated plasmids. After 24 h, cells were treated as described previously [Ding et al., 2006]. The primary antibodies used were mouse monoclonal anti-Myc antibodies while the secondary antibodies were Alexa 594 goat anti-mouse antibodies. For the analysis of endogenous protein localization, HeLa and HBL100 cells were grown on glass coverslips. Endogenous AP-2α proteins were recognized by mouse monoclonal anti-AP-2α antibodies and Alexa 594 goat anti-mouse antibodies (Molecular Probes), while endogenous KCTD1 proteins were recognized by rabbit polyclonal anti-KCTD1 antibodies and Alexa 488 goat anti-rabbit antibodies (Molecular Probes). The nucleus was stained with Hoechst 33258 (Sigma). The fluorescent signals were analyzed using a fluorescence microscope (Zeiss Axioskop 2).

LUCIFERASE REPORTER ASSAYS

HEK293FT cells were cultured in 12-well plates and transfections were carried out with reporter plasmids A2-Luc, erbB-2-Luc, P21-Luc, or ER-Luc and the indicated plasmids using Lipofectamine 2000. The pCMV-LacZ vector was co-transfected in all experiments as an internal control, and β-galactosidase activity was used to normalize for different transfection efficiencies. The total amount of transfected DNA was kept constant by adding pCMV-Myc empty plasmids. Thirty-six hours after transfection, cells were lysed and the luminescence was measured using the luciferase assay system (Promega, Madison, WI) in a TD-20/20 Luminometer (Turner Design). Each transfection was performed in triplicate wells and replicated with similar results in at least three independent experiments.

RESULTS

KCTD1 INTERACTS WITH TRANSCRIPTION FACTOR AP-2α

To identify proteins interacting with transcription factor AP-2α, we carried out yeast two-hybrid screening. The full-length AP-2α was cloned as a fusion pDBLeu-AP-2α with the GAL4 DNA-binding domain, and yeast strain MaV203 was sequentially transfected with pDBLeu-AP-2α and a human brain cDNA library cloned as a fusion pPC86-cDNA with the GAL4 activation domain. After screening with the pDBLeu-AP-2α clone, we identified multiple AP-2-interacting proteins, including the previous reported ubiquitin-conjugating enzyme E2I (Ube2i) [Eloranta and Hurst, 2002] and some novel partners. One of them isolated is a full-length cDNA clone encoding potassium channel tetramerization domain containing 1 (KCTD1) (Fig. 1). Our recent reports show that the KCTD1 protein contains a highly conserved BTB domain in its N-terminus, and KCTD1 is a nuclear protein that functions as a transcriptional repressor and mediates homomeric protein–protein interactions [Ding et al., 2008].

To further confirm the interaction between KCTD1 and AP-2α in yeast, the pDBLeu-AP-2α plasmid was reintroduced into MaV203 yeast strain along with the pPC86-KCTD1 plasmid. The interaction was confirmed by the X-Gal assay. As shown in Figure 1, blue colonies (image in gray scale) were observed only in colonies containing pDBLeu-AP-2α and pPC86-KCTD1, positive control C including pPC97-CYH2^s and pPC86-dE2F and positive control D expressing pPC97-Fos and pPC86-Jun, but not in the controls containing pDBLeu-AP-2α and pPC86, pDBLeu and pPC86-KCTD1, or negative control A expressing pPC97 and pPC86. As reported, KCTD1 includes one conserved domain known to be important for protein–protein interaction, namely BTB domain [Ding et al., 2008].

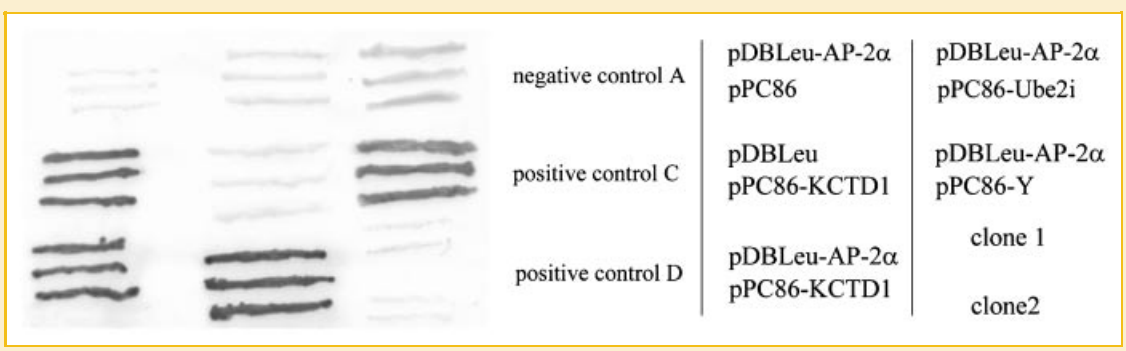

Fig. 1. The identification and confirmation of AP-2α-interacting partners in yeast two-hybrid screen. The colonies were grown on SC-Leu-Trp-His+30 mM AT plates after transformation with pDBLeu-AP-2α and pPC86-human brain cDNA library and then duplicated onto a nylon membrane for the X-Gal assay. The sequences of positive clones were further analyzed and retransformation assays were performed. Other controls were also set. Y indicated another novel AP-2α-interacting factor, but no interactors were preyed in clones 1 and 2.

The KCTD1 protein was truncated into two peptides, and the truncated KCTD1N protein contained the complete BTB domain (Fig. 2A).

Next, we examined the physical interactions between KCTD1 and AP-2α in vitro by GST pull-down assays. Full-length and truncated AP-2α (Fig. 2A) were bacterially expressed as GST fusion proteins and purified (Fig. 2B), while full-length and truncated KCTD1 were bacterially expressed as His fusion proteins and purified (Fig. 2C, D). As shown in Figure 2C, KCTD1 bound to the full-length GST-AP-2α fusion protein but not to GST alone, demonstrating that KCTD1 and AP-2α interact directly. Furthermore, KCTD1 bound specifically to GST-AP-2αN fusions containing only the N-terminal AP-2α activation domain (1–165 a.a.). In contrast, GST-AP-2αC fusions containing the C-terminal AP-2α DNA-binding domain and dimerization domain (165–438 a.a.) did not bind to KCTD1. Thus, the activation domain is sufficient to mediate the binding of AP-2α to KCTD1.

We also investigated whether BTB domain of KCTD1 is necessary for their interactions. From Figure 2D, we found that KCTD1N bound to GST-AP-2αN, but not to GST, while KCTD1C did not bind to either GST-AP-2αN or GST. These results indicated that the BTB domain of KCTD1 is critical for its interaction with AP-2α and the interaction domains between the two proteins are mapped to the N-termini of both AP-2α and KCTD1.

Since KCTD1 and AP-2α interact directly in vitro, we tested whether overexpressed KCTD1 and AP-2α in HEK293FT cells could interact in vivo. HEK293FT cells were transiently transfected with expression plasmids pCMV-Myc-AP-2α and pCMV-HA-KCTD1 or pCMV-HA-truncated KCTD1. The lysates were immunoprecipitated with rabbit polyclonal antibodies against Myc-tag, HA-tag, or control IgG, and the immunoprecipitates were detected with mouse monoclonal antibodies against HA-tag and Myc-tag, respectively. The expression of four proteins was firstly demonstrated in Western blotting (Fig. 3A, upper panel). KCTD1 and KCTD1N were detected in immune complexes of Myc-AP-2α, but not KCTD1C. Rabbit preimmune IgG did not precipitate any band (Fig. 3A, lower panel). Likewise, AP-2α was detected in immunoprecipitates of HA-KCTD1 or HA-KCTD1N but not in those of HA-KCTD1C, whereas mouse preimmune IgG did not recognize target bands (Fig. 3B). Because KCTD1 interacts with AP-2α in the case of protein overexpression, we determined whether KCTD1 associates with endogenous AP-2α. Immunoblots of AP-2α and immunoprecipitates of KCTD1 were detected with mouse monoclonal antibodies against AP-2α, endogenous AP-2α could be precipitated by Myc-KCTD1 but not by control rabbit IgG (Fig. 3C). Therefore, these data suggested that KCTD1 and AP-2α interact in vivo.

KCTD1 CO-LOCALIZES WITH AP-2α

Because the strong interaction between KCTD1 and AP-2α was found in GST pull-down and co-immunoprecipitation assays, we investigated whether the two proteins are present in the same

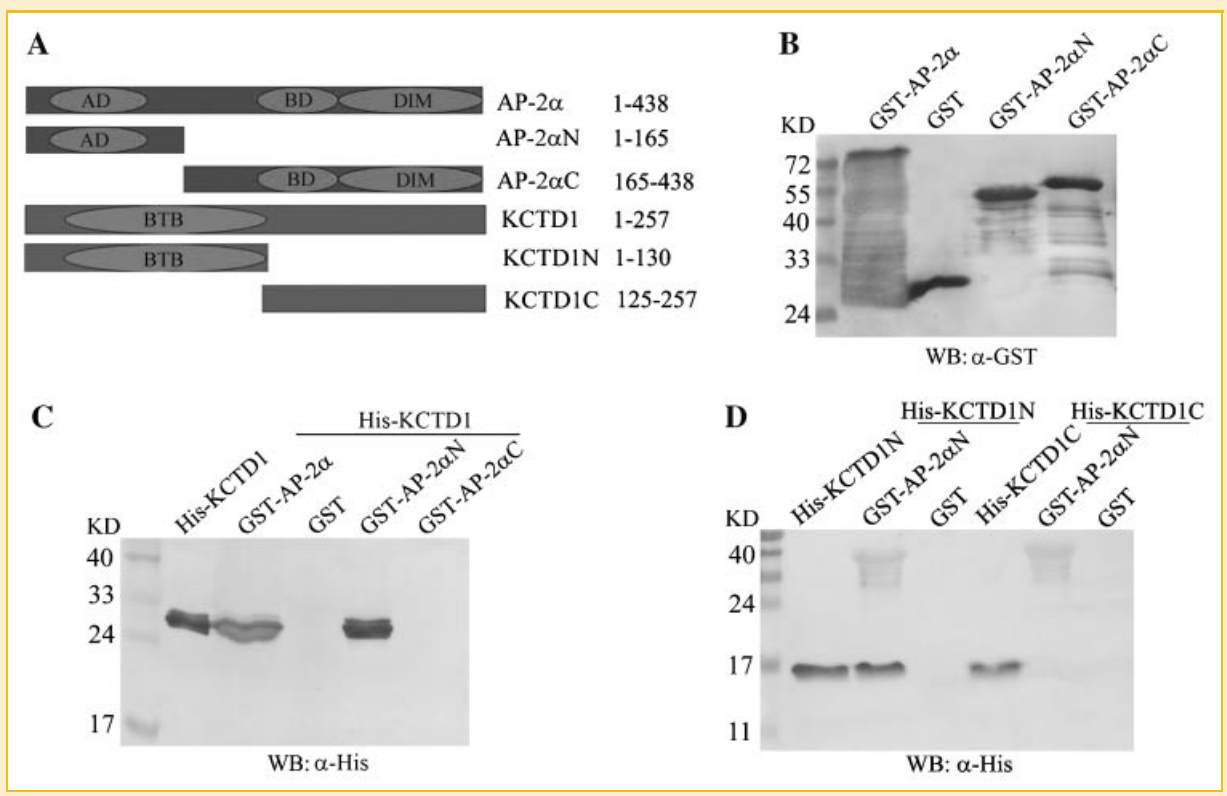

Fig. 2. Interaction of KCTD1 and AP-2α in vitro. A: Schematic representation of the domain structure of AP-2α and constructs of AP-2α used in the study (upper panel). AD, activation domain. BD, DNA-binding domain. DIM, dimerization domain. Schematic representation of the domain structure of KCTD1 and constructs of KCTD1 used in the study (lower panel). B: Expressed and purified GST-AP-2α fusion proteins run on a 10% SDS–polyacrylamide gel. C: The GST fusion proteins above were used in GST pull-down assays with His-KCTD1. D: The same pull-down assays were performed to analyze the interaction between AP-2αN and KCTD1N or KCTD1C.

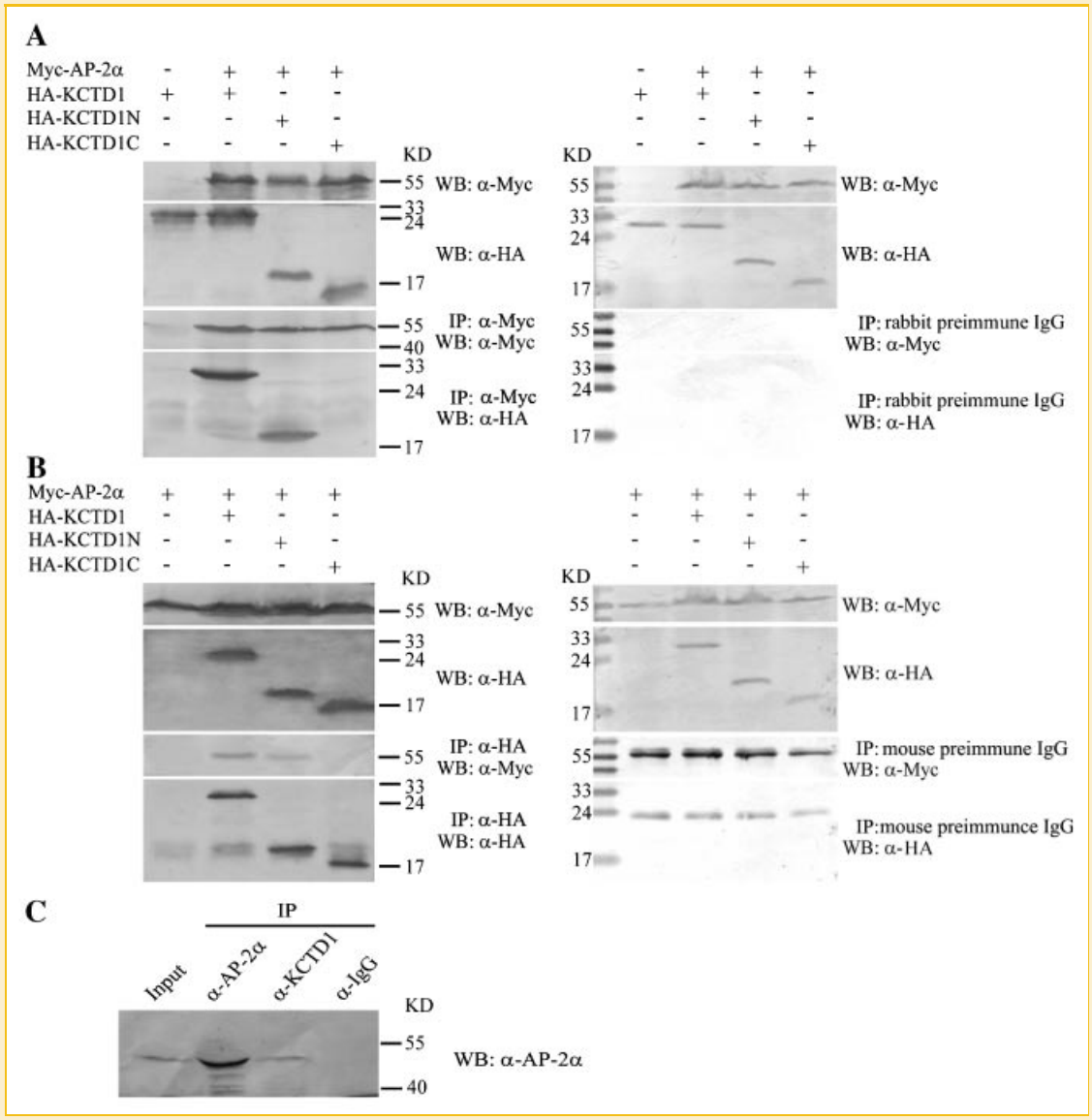

Fig. 3. Interaction of KCTD1 and AP-2α in vivo. A: HEK293FT cells were transfected with the indicated expression vectors. Cell lysates were immunoblotted with mouse monoclonal antibodies against Myc-tag and HA-tag to verify protein expression (top panel), and were immunoprecipitated with rabbit polyclonal anti-Myc antibodies followed by immunoblotting with mouse monoclonal antibodies against Myc-tag and HA-tag (bottom panel). Rabbit preimmune IgG was used as negative control. B: The similar experiments were performed as indicated in subpart A, lysates were prepared and checked for protein expression (top panel), immunoprecipitates with rabbit polyclonal anti-HA antibodies were immunoblotted with mouse monoclonal antibodies against Myc-tag and HA-tag (bottom panel). Mouse preimmune IgG was used as negative control, and IgG heavy chain band (53 kD) and light chain band (22.5 kD) were detected. C: HEK293FT cells were only transfected with pCMV-Myc-KCTD1. Immunoblot analysis with mouse monoclonal anti-AP-2α antibodies of Myc-KCTD1 precipitated proteins from the total extract of HEK293FT cells. The protein extract was used as positive control. Immunoprecipitates with rabbit polyclonal anti-AP-2α antibodies (Santa Cruz Biotech) further demonstrated AP-2α expression and immunoprecipitates with rabbit preimmune IgG were used as negative control.

cellular structure in vivo. We first analyzed the localization of overexpressed GFP-AP-2α and Myc-KCTD1 truncations, we found that both Myc-KCTD1 and Myc-KCTD1N were co-localized with GFP-AP-2α in the nuclei, whereas KCTD1C did not interact with AP-2α, they could not localize together in most cells and KCTD1C was mainly localized in the cytoplasm (Fig. 4A). Neither KCTD1 nor KCTD1N affected the localization of AP-2α.Likewise, HeLa cells were cultured in glass coverslips and then stained with specific antibodies to AP-2α and KCTD1. As shown in Fig. 4B, endogenous KCTD1 proteins significantly localized in the nuclei and completely merged with endogenous AP-2α proteins by the anti-KCTD1 and anti-AP-2α antibody detection. Similar results were obtained in human breast cell line HBL100. These data indicated that KCTD1 co-localizes with AP-2α and further demonstrated both proteins exist in the same complex in vivo.

KCTD1 SIGNIFICANTLY INHIBITS THE TRANSACTIVATION OF AP-2α

The mammary cancer cell lines MDA MB 453 and T47D expressed high levels of AP-2α proteins; AP-2α protein levels were low in MCF7 cells and no AP-2α proteins were detected in HBL100 and

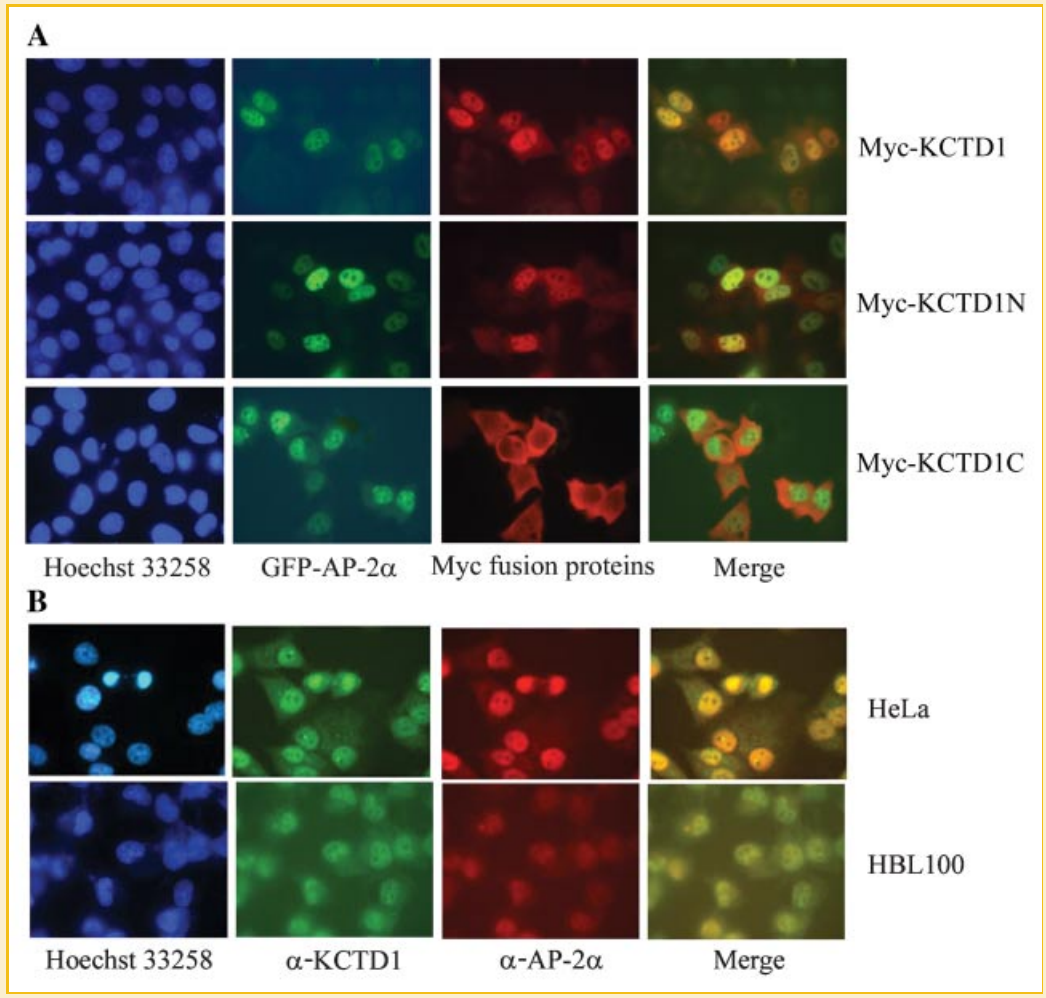

Fig. 4. Localization analysis of KCTD1 and AP-2α proteins. A: Localization of overexpressed KCTD1 truncations and GFP-AP-2α in HeLa cells by immunofluorescence analysis. KCTD1 truncations were detected with mouse monoclonal anti-Myc antibodies and Alexa 594 goat anti-mouse secondary antibodies. B: Localization of endogenous KCTD1 and AP-2α in mammalian cells. Endogenous KCTD1 was detected with rabbit polyclonal anti-KCTD1 antibodies and Alexa 488 goat anti-rabbit secondary antibodies, while endogenous AP-2α was detected with mouse monoclonal anti-AP-2α antibodies and Alexa 594 goat anti-mouse secondary antibodies. Nuclei were stained with Hoechst 33258. Yellow signal indicated the overlap of the two proteins. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

MDA MB 231 cells [Bosher et al., 1996; Begon et al., 2005], whereas KCTD1 was expressed in all these cell lines detected by RT-PCR, whether these cells were normal or malignant [Ding et al., 2008]. Moreover, we demonstrated the interaction between KCTD1 and AP-2α, a functional significance could exist in their interaction. We decided to examine whether the interaction between KCTD1 with AP-2α alters the transcriptional activity of AP-2α, we transfected the A2-Luc, erbB-2-Luc, P21-Luc, ER-Luc reporter plasmids or expression plasmids in HEK293FT cells as indicated in Fig. 5A. AP-2α activated the A2, erbB-2, P21, and ER promoters to a different level (two to threefold) as previously reported [Bosher et al., 1996; Zeng et al., 1997; McPherson and Weigel, 1999]; KCTD1 alone gave a slight decrease of the promoters in HEK293FT cells due to endogenous AP-2α expression (Fig. 3C). Co-transfection of KCTD1 and AP-2α significantly reduced the luciferase activity (3- to 13-fold) when compared to the transfection of AP-2α alone. To exclude cell-specific effects, the repression was tested in another two cell lines. Similar results were got in NIH3T3 and HBL100 cells using A2-Luc reporter gene, KCTD1 strongly inhibited the transactivation function of AP-2α (data not shown). These results indicated that KCTD1 interacts with AP-2α and dramatically inhibits AP-2α-mediated transactivation.

Most of the BTB domains have been found to repress transcriptional regulation [Soubry et al., 2005; Qi et al., 2006]. Our recent studies show that KCTD1 inhibits the transcriptional activity via its BTB domain like other BTB family proteins [Deltour et al., 1999; Li et al., 1999], though KCTD1 transcriptional repression is in a TSA-insensitive manner [Ding et al., 2008]. We next investigated whether KCTD1 inhibits the transcription activity of AP-2α over the promoters through the BTB domain. As expected, KCTD1N and AP-2α repressed the transactivation of the four promoters, similar to full-length KCTD1 and AP-2α, whereas KCTD1C lacking the BTB domain and AP-2α did not show significant changes in promoter activity compared with AP-2α alone (Fig. 5B). Thus, our data demonstrated that the BTB domain of KCTD1contributes to transcriptional inhibition of AP-2α.!

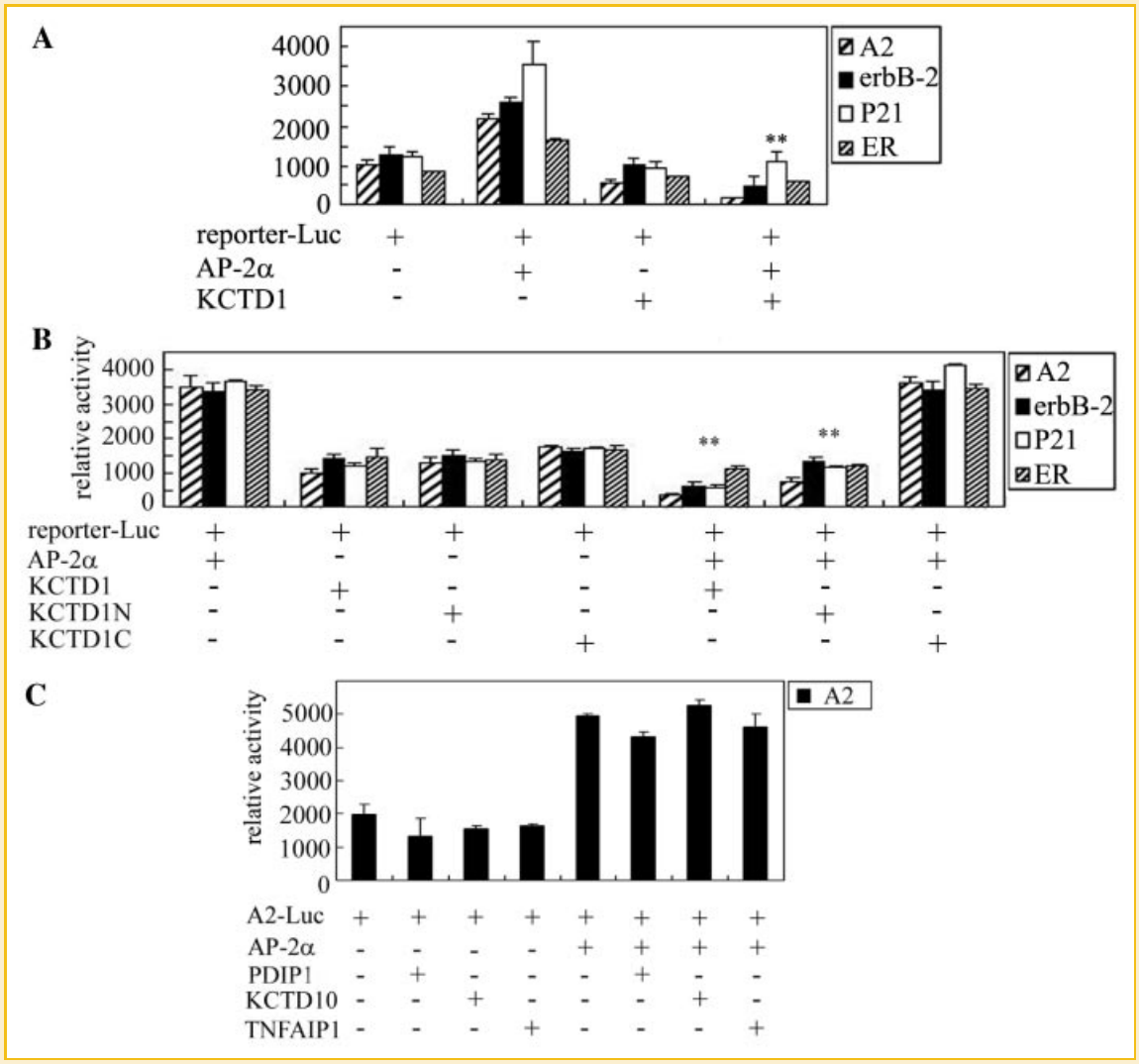

Fig. 5. The inhibitory effects of KCTD1 on the transactivation of AP-2α in HEK293FT cells. A: HEK293FT cells were transfected with four promoter plasmids or with AP-2α or with KCTD1 or with both AP-2α and KCTD1. B: The same transfections were performed as subpart A with four promoter plasmids or with full-length KCTD1 or with truncated KCTD1. C: HEK293FT cells were transfected with A2-Luc reporter plasmid or with AP-2α or with PDIP1 or with both AP-2α and PDIP1. Relative luciferase activities represent mean ± SD from at least three independent experiments after normalization to β-galactosidase activities. $^{**}P < 0.01$ compared with controls.

As many BTB proteins described so far functioned as the transcriptional repressors [Lee et al., 2002; Soubry et al., 2005], we asked the question of whether other members of the BTB protein family inhibit the transcriptional activity of AP-2α. We next analyzed the influence of the PDIP1 family (PDIP1, KCTD10, TNFAIP1) containing the conserved BTB domain [Zhou et al., 2005a, b] on AP-2α transcription activity. From Figure 5C, no statistically significant reduction in the transactivation potential of AP-2α was observed for PDIP1, KCTD10, or TNFAIP1, even if these proteins displayed high expression levels. Therefore, KCTD1 inhibits the transcriptional activity of AP-2α but neither do other BTB family proteins.

KCTD1 SPECIFICALLY INHIBITS THE TRANSACTIVATION OF AP-2α

The above results indicated that KCTD1 significantly inhibits AP-2α-mediated transactivation. To gain the further supporting evidence, we attempted to knock down KCTD1 gene expression using siRNA and asked whether siRNA-mediated KCTD1 knockdown alleviated the inhibition effects. Four siRNA sequences targeting different regions of KCTD1 mRNA were made and their abilities to knock down KCTD1 expression in transfected HEK293FT cells were confirmed by Western blotting and luciferase assays. As shown in Figure 6A, B, these data consistently revealed that the four sequences could knock down KCTD1 gene in different levels, especially siRNA3. The siRNA3 strongly relieved KCTD1-mediated repression of AP-2α transcriptional activity by 4.8-fold. Therefore, the siRNA3 was used to more efficiently knock down KCTD1. We found that the siRNA greatly alleviated the transcription inhibition of KCTD1 on all four promoters compared with negative control siRNA in the case of KCTD1 overexpression (Fig. 6C), whereas KCTD1 siRNA slightly alleviated the repression of endogenous KCTD1 protein on AP-2 downstream promoters, partly due to the low KCTD1 background (Fig. 6D). These findings indicated that KCTD1 specifically inhibits the transactivation of AP-2α.

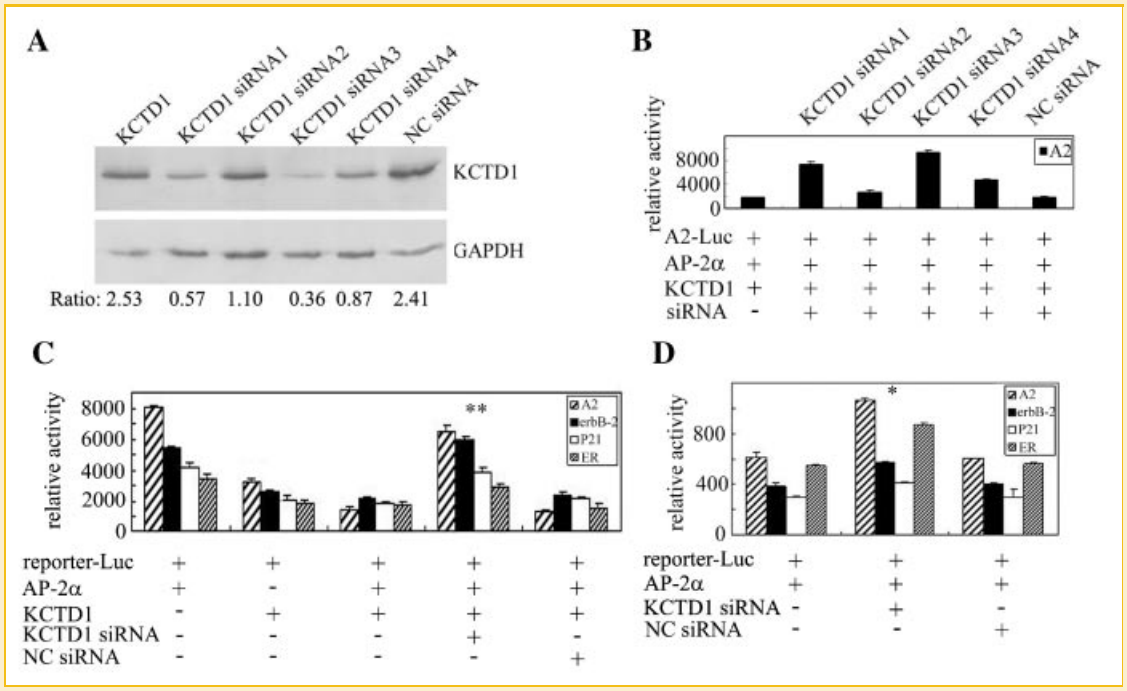

Fig. 6. The effects of KCTD1 siRNAs on the transactivation of AP-2α in HEK293FT cells. A: HEK293FT cells were transfected with pCMV-Myc-KCTD1 and KCTD1 siRNAs, the KCTD1 knockdown efficiency was detected by Western blotting. GAPDH was used as a loading control. Densitometric analysis of KCTD1/GAPDH ratio was performed with Gel-Pro Analyzer 4.0 software. B: HEK293FT cells were transfected with A2-Luc reporter, AP-2α, KCTD1, and KCTD1 siRNAs, the KCTD1 knockdown efficiency was detected by luciferase assays. C: KCTD1 siRNA3 was used in the transfections with four promoter plasmids or with the plasmids indicated above when KCTD1 was overexpressed. D: KCTD1 siRNA was used in the transfections with four promoter plasmids or with the plasmids indicated above under the condition of endogenous KCTD1 background. Relative luciferase activities represent mean ± SD from at least three independent experiments after normalization to β-galactosidase activities. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with controls.

KCTD1 INHIBITS THE TRANSCRIPTIONAL ACTIVITY OF THE AP-2 FAMILY MEMBERS

KCTD1 interacts with transcription factor AP-2α and inhibits its transactivation. And the region of AP-2α interacting with KCTD1 was mapped to the N-terminal activation domain. Although there are less homology in the N-terminal activation domains than the C-terminal DNA-binding and dimerization domains of AP-2 family members, the majority of the aromatic and proline residues are conserved between them, such as a PY motif FPPPY [Yagi et al., 1999]. Therefore, we next examined the possible effects of KCTD1 on the transcriptional activities of other AP-2 family members. As shown in Figure 7A, in the same manner, KCTD1 inhibited the transactivation of AP-2γ, but less strongly than AP-2α. Although the overexpression of AP-2β in HEK293FT cells induced the AP-2β transcriptional self-interference as in PA-1 cells [Kannan and Tainsky, 1999], KCTD1 still repressed the transcriptional activity of AP-2β by 50% compared with AP-2β only. By co-immunoprecipitation assays, we found that KCTD1 not only interacted with AP-2α, but also interacted with AP-2β and AP-2γ (Fig. 7B). We then analyzed the critical residues in the activation domain of AP-2 interacting with KCTD1. Three mutants of AP-2α were constructed to analyze the effects of KCTD1, and these amino acids are highly conserved in AP-2 family proteins [Wankhade et al., 2000]. KCTD1 still inhibited the transcriptional activities of both AP-2α D52A and AP-2α L107A/L108A to the same extent as wild-type AP-2α, but the transcriptional inhibition of AP-2α P61R was, at least in part, decreased by KCTD1 (Fig. 7C), indicating that the PY motif might be important in the interaction between KCTD1 and the AP-2 family members. These results confirmed that KCTD1 interacts with the N-terminal activation domain of AP-2 family and inhibits their transcriptional activities.

The three transcription factors can form homodimers, and the AP-2β and AP-2γ proteins can bind DNA in a form of a heterodimer with AP-2α. And AP-2β and AP-2γ can also bind to DNA as a heterodimeric complex [Bosher et al., 1996]. We therefore tested whether KCTD1 influences the transcriptional activity of AP-2 heterodimers. KCTD1 could inhibit the transcriptional activity of three heterodimeric complexes comparable to the activity of AP-2 in the absence of KCTD1 (Fig. 7D). Although AP-2α and AP-2γ activated four promoters more strongly than AP-2α and AP-2β or AP-2β and AP-2γ, KCTD1 inhibited the transcriptional activity of AP-2 heterodimers in different levels. These data strengthened the conclusion that KCTD1 has the inhibitory effects on AP-2 family members.

DISCUSSION

The present study addressed the BTB protein, KCTD1, interacts with transcription factor AP-2α, and inhibits AP-2α-mediated transcriptional activation. The interactions between the two proteins were confirmed by GST pull-down assays and co-immunoprecipitation

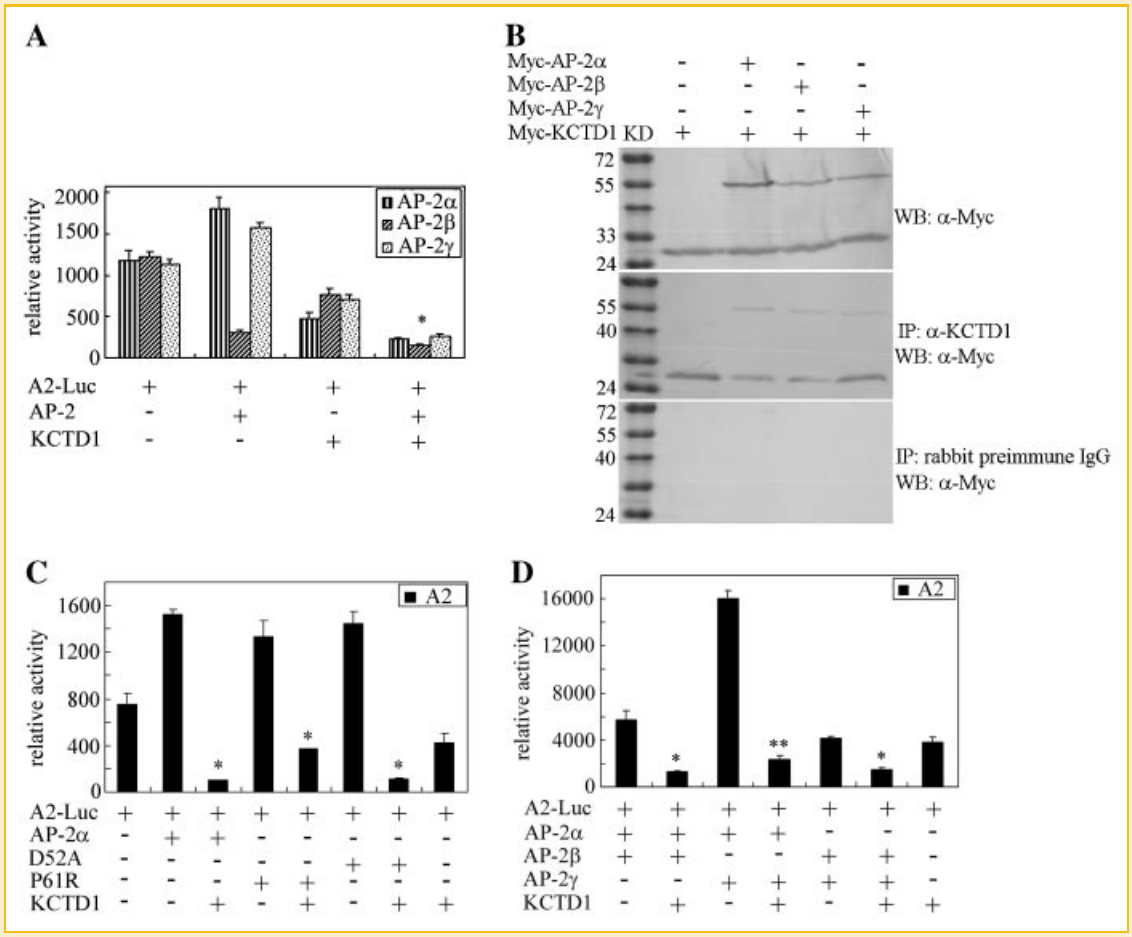

Fig. 7. The inhibitory effects of KCTD1 on the transcriptional activity of AP-2 family members in HEK293FT cells. A: HEK293FT cells were transfected with A2 reporter plasmid or with AP-2α, AP-2β or AP-2γ or with KCTD1 or with both AP-2 and KCTD1. B: HEK293FT cells were transfected with pCMV-Myc-KCTD1 and AP-2 family members. Cell lysates were immunoblotted with mouse monoclonal anti-Myc antibodies to detect protein expression (top panel) and were immunoprecipitated with rabbit polyclonal anti-KCTD1 antibodies followed by immunoblotting with mouse monoclonal anti-Myc antibodies (middle panel). Rabbit preimmune IgG was used as negative control (bottom panel). C: HEK293FT cells were transfected with A2 reporter plasmid or with wild-type AP-2α, or mutants. D: The same transfections were performed with A2 reporter plasmid or AP-2α and AP-2β or AP-2α and AP-2γ or AP-2β and AP-2γ or both AP-2 and KCTD1. Relative luciferase activities represent mean ± SD from at least three independent experiments after normalization to β-galactosidase activities. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with controls.

assays. And the immunofluorescence analysis results show that KCTD1 and AP-2α were localized in the same subcellular structures. Only the BTB domain of KCTD1 interacted with AP-2α, while the non-BTB region did not. The BTB domain is required for its interaction with AP-2α, this domain is a highly conserved motif of 100-amino acid mostly identified at the N-terminus of proteins, the domain has been demonstrated to mediate the protein–protein interactions and target proteins to regulate gene transcription [Li et al., 1999]. This type of BTB domain-mediated interaction has been reported to be implicated in transcriptional repression [Dhordain et al., 1997]. The mutations of critical amino acid residues in the BTB domain resulted in the loss of PLZF BTB-dependent repression [Melnick et al., 2002]. Our studies indicated that KCTD1 significantly inhibits AP-2α-mediated transactivation.

When KCTD1 siRNA was introduced to examine its effects on the transcriptional regulation, we found a marked down-regulation of KCTD1 protein by small interference RNA, and the KCTD1-mediated transcriptional repression was significantly alleviated. Therefore, KCTD1 plays an important role in regulating AP-2α transcriptional activity. Moreover, KCTD1 is required to inhibit the transactivation function of AP-2α because AP-2α transcriptional activity was reversed if KCTD1 was silenced. Further supports show that KCTD1 and AP-2α proteins exist together, physically interact and functionally co-operate in transcriptional regulation. However, other BTB domain proteins such as PDIP1, KCTD10, and TNFAIP1 did not influence the transcriptional activities of AP-2α. The interaction domain of AP-2α with KCTD1 is located in the N-terminal moiety, which only includes the activation domain. Although the domain is less conserved between all members of the AP-2 family, the transcriptional activities of other AP-2 family members are repressed by KCTD1, further investigation indicated that KCTD1 could interact with AP-2 family members (AP-2α, AP-2β, and AP-2γ).

Unlike other BTB family proteins, KCTD1 does not contain any other domains, indicating that KCTD1 is a unique BTB protein that may contribute differentially than other BTB family members to cellular functions. BTB domain proteins predominantly serve as transcriptional repressors, some BTB proteins recruit a co-repressor

complex containing N-CoR, mSin3A, SMRT, and HDAC with an HDAC activity [Deltour et al., 1999]. Our recent study showed that KCTD1 transcriptional repression is unaffected by the HDAC inhibitors, TSA, and sodium butyrate. The HDAC complex is not involved in KCTD1 repression [Ding et al., 2008]. Moreover, we found KCTD1 did not affect the DNA-binding activity of AP-2α (data not shown) possibly because the KCTD1-binding domain in AP-2α is located to the activation domain, not to the DNA-binding domain of AP-2α protein. Furthermore, KCTD1 overexpression in HBL100 cells did not enhance protein degradation of AP-2α detected by Western blotting and immunofluorescence analysis (data not shown), which is consistent with the previous report that the C-terminal 90 amino acids of AP-2α are responsible for protein stability [Li et al., 2006]. Therefore, KCTD1 does not affect the DNA-binding activity and protein stability of AP-2α. As we have known, the transcription of eukaryotic genes is regulated by the combined action of multiple transcription factors, general transcription factors, cofactors, and mediators that regulate the activities of transcription factors. Protein–protein interactions play important roles in the transcriptional activity among these factors. Indeed, the BTB domain in KCTD1 has long been recognized as an interface for protein–protein interactions. Therefore, one possible explanation is that KCTD1 represses AP-2α-mediated transactivation by the recruitment of co-repressors devoid of HDAC activity like other BTB transcription factors [Deltour et al., 1999], and competitive binding to AP-2α and excluding transactivators [Qin et al., 2005]. Another potential mechanism is that KCTD1 could negatively interfere with components of the basal transcriptional machinery, as shown for SMRT/N-CoR with TFIIB and TAFII32 [Muscat et al., 1998]. Further experiments are needed to testify these speculations.

The BTB domain proteins have been demonstrated to participate in other cellular functions including cellular proliferation, apoptosis, ion channel assembly, protein degradation, and human cancer [Nakayama et al., 2006]. The biological consequences of the interaction between KCTD1 and AP-2α were estimated by examining the transcriptional activities of AP-2α-dependent promoters. KCTD1 significantly inhibits the transactivation of AP-2α-mediated downstream genes, such as erbB-2. The erbB-2 gene is overexpressed mostly in breast carcinomas, which is a marker of a poor prognosis [Klapper et al., 2000]. And erbB-2 gene amplification and increased transcriptional activation by AP-2 are responsible for erbB-2 overexpression. Many studies showed a positive association between nuclear AP-2α and erbB-2 overexpression in clinical breast cancer series, and inactivation of AP-2 proteins in erbB-2-overexpressing breast cancer cells reduced the erbB-2 promoter activity by 50%, suggesting their function in erbB-2 expression [Pellikainen and Kosma, 2007]. We found that KCTD1 repressed the transcriptional activity of AP-2α on the erbB-2 promoter. Thus, KCTD1 may function in breast cancer development due to the inhibition of erbB-2 expression. Moreover, AP-2 proteins are involved in the estrogenic regulation in breast tissues, and transactivate human ERα promoter. There is evidence that nuclear AP-2α was positively associated with ER expression in early-stage breast cancer [Turner et al., 1998; Pellikainen et al., 2002]. KCTD1 inhibition on AP-2α-mediated ER expression may further suggest that KCTD1 plays important roles in controlling the expression of specific genes in breast cancer, this may provide a new sight into the treatment of human breast cancer.

Even though the exact mechanism through which KCTD1 helps inhibit the transactivation of AP-2α and their physiological function, is yet unclear, our results demonstrate for the first time, that a BTB protein, KCTD1, can associate with the transcription factor AP-2α and function as its transcriptional repressor. Although KCTD1 transcriptional repression is common to other AP-2 family members as well, AP-2 transcriptional regulation is specific to KCTD1, not to other BTB family proteins (PDIP1, KCTD10, and TNFAIP1) tested, suggesting KCTD1 protein may be important in the regulation of the family behavior.

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