**Communication**

**Induction of Rb-associated Protein (RbAp46) by Wilms’ Tumor Suppressor WT1 Mediates Growth Inhibition**

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The Wilms’ tumor suppressor gene, *wt1*, encodes a zinc-finger transcription factor, WT1, that plays an important role in controlling urogenital development. Previously, WT1 has been shown to inhibit cell growth and to repress transcription initiated from the promoters of a number of growth-promoting genes. However, few physiological target genes that are transcriptionally activated by WT1 have been established. Using suppression subtractive hybridization polymerase chain reaction, we isolated a WT1 target gene that is up-regulated about 15-fold in cells expressing WT1. The gene was identified as retinoblastoma suppressor (Rb)-associated protein 46 (RbAp46), a nuclear protein that interacts physically with Rb and is a component of the human mSin3 co-repressor complex. Cells transfected with RbAp46 cDNA formed fewer colonies than the control cells, and RbAp46 suppressed the growth rate (by about 2-fold) of transfected cells. In the developing kidney and gonad, RbAp46 exhibits an expression pattern similar to that of WT1. We conclude that RbAp46 has strong growth inhibition activity and may function as an important mediator of WT1’s function.

The Wilms’ tumor susceptibility gene, *wt1*, at chromosome locus 11p13 encodes a C2-H2-type zinc finger protein (1, 2), WT1. Experimental evidence has demonstrated that WT1 functions as a repressor of transcription (3, 4), and a number of potential WT1 target genes have been proposed, including platelet-derived growth factor A-Chain (4), colony-stimulating factor-1 (5) and insulin-like growth factor-I receptor (6), etc. Recently, we demonstrated that WT1 also functions as a potent transcriptional activator (7, 8). Its repressor and activator activities are contained in different regions of the regulatory domain. Therefore, we identified and cloned RbAp46 as a candidate gene up-regulated by WT. RbAp46 was first identified as a major protein from a HeLa cell lysate that specifically bound to an Rb affinity column along with another protein, RbAp48 (16, 17). We also demonstrated that RbAp46 exhibits growth inhibiting activity. In the developing kidney, both WT1 and RbAp46 are expressed in the differentiating nephrogenic region. RbAp46 is also highly expressed in the developing gonad, another site of WT1 expression. Therefore, RbAp46 may function as a downstream target gene of WT1, and its up-regulation by WT1 may have implications for its role in both normal development of the urogenital system and the tumorigenesis of Wilms’ tumor.

**MATERIALS AND METHODS**

**Cell Culture, Growth Curves, DNA Transfections, and Colony Formation Assays—Osteosarcoma cell lines (Saos-2 and U2OS), human embryonic kidney 293 cells, and African green monkey kidney cells (CV-1) were grown in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. Cells were transfected by the calcium phosphate/DNA precipitation method (18). Drug-resistant colonies were selected by growth in G418 (0.5 mg/ml) for 3 weeks. The G418-resistant colonies were then stained with Crystal Violet (4% in 20% methanol) and counted. To establish the rates of cell growth for transfected cells, 5 × 10⁴ cells were seeded in triplicate onto 35-mm dishes. The cells were trypsinized and counted using a hemocytometer every day for 8 days.

**RNA Extraction and Northern Blot Analysis—Total cellular RNA was isolated by the guanidine-isothiocyanate method (19). Ten μg of total RNA was separated by electrophoresis on a 1.2% formaldehyde-agarose gel and blotted onto a nylon membrane (Hybond-N, Amersham Pharmacia Biotech). The blots were prehybridized for 1 h and hybridized for 2 h in Quick-Hybridization solution (Amersham Pharmacia Biotech) at 65 °C. The probes included a L-kb EcoRI cDNA fragment of RbAp46, an actin DNA probe from CLONTECH, a 200-base pair Smal cDNA fragment of *wt1*, and a 2-kb DNA fragment of RbAp48 purchased from Genome Systems Inc., St. Louis, MO. The blots were washed twice with 2× SSC and 0.1% SDS for 15 min at room temperature and twice with 0.1× SSC and 0.1% SDS for 15 min at 55 °C. Blots were autoradiographed using intensifying screens at ~70 °C for 12 h.

**SSH-PCR—** The mRNAs of control 293 and WT1-6 cells were extracted with the QuickPrep mRNA purification kit (Amersham Pharmacia Biotech). The mRNAs were further purified by passing through a

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1 The abbreviations used are: SSH-PCR, suppression subtractive hybridization-polymerase chain reaction; kb, kilobase pair(s); PAGE, polyacrylamide gel electrophoresis; CMV-IE, cytomegalovirus immediate promoter.
second oligo(dT) spin column (5 Prime→3 Prime Inc., Boulder, CO). Two μg of each mRNA were used to perform SSH-PCR with the PCR-Select cDNA subtraction kit (CLONTECH) according to the method recommended by the manufacturer. The cDNA fragments generated by SSH-PCR were cloned into a TA cloning plasmid, pT-Adv (CLONTECH), and sequenced.

cDNA Library Screening and Construction of Expression Plasmids—An Agtl1 human placenta cDNA library was provided by Dr. Evan Sadler (Washington University, St. Louis, MO) and screened using a cDNA fragment generated by SSH-PCR as a probe, as described previously (16). 0.5 x 10⁶ clones were screened, and four positive clones were obtained after tertiary screening. The longest one (~1.9 kb) was subcloned into pBluescript (KS⁺) and fully sequenced.

The wt1 isoform without any alternative splicing was cloned into the pCB6⁺ expression vector as described previously (7). The RbAp46 expression vector was constructed by cloning a 1.9-kb BamHI-KpnI cDNA fragment of RbAp46 from pBluescript into the BglII and KpnI sites of expression plasmid pcB6⁺. The RbAp48 cDNA in pBluescript (KS⁺) was released from the plasmid by digestion with EcoRI and XhoI and cloned into a pGEM7 plasmid (Promega) digested with the same enzymes to produce plasmid pGEM7-p48. The pGEM7-p48 plasmid was digested with EcoRI and XbaI, and the cDNA fragment of RbAp48 was then cloned into the same site in the expression vector pcB6⁺.

Whole-mount in Situ Hybridization—Kidney, gonad, and mesonephros were dissected from wild type mouse embryos at embryonic day 13.0, fixed in 4% paraformaldehyde, and analyzed by whole-mount in situ hybridization as described previously (20). Hybridization with a 1.9-kb digoxigenin-labeled human RbAp46 riboprobe was performed at 52 °C, and the color reaction was detected using the alkaline phosphatase substrates nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Whole-mount samples were photographed at ×5 magnification with a Zeiss MC-100 camera. Kidneys were sectioned at 50 μm after whole-mount in situ hybridization using a Leica VT 1000 M/E vibrating microtome and photographed at ×400 magnification with a Zeiss Axioshot microscope using bright-field illumination.

Western Blot Analysis—Cells were washed with phosphate-buffered saline and lysed with lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.25 mM EDTA, pH 8.0, 0.1% SDS, 1% Triton X-100, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin and leupeptin). The cell lysates in SDS gel loading buffer were boiled for 5 min and separated on a 10% SDS-PAGE gel. After electrophoresis, the proteins were transferred to a nitrocellulose filter (BA85, Schleicher & Schuell). The filter was probed with antibodies and visualized with an alkaline phosphatase-conjugated secondary antibody.

Anti-WT1 antibody (C-180) was purchased from Santa Cruz Biotechnology Inc. Anti-RbAp46 polyclonal antibody was prepared as a custom service from Alpha Diagnostic International by immunizing rabbits with a synthetic peptide corresponding to the last 15 amino acids of RbAp46, the region that shows the least homology to RbAp48. Both antibodies were used at a concentration of 1:1000.

RESULTS

To seek physiological target genes of WT1, we established a human embryonic kidney (HEK) 293 cell line that expresses an exogenous WT1 isoform without alternative splicing. The 293 cell line was chosen because it is derived from human embryonic kidney and therefore is likely to provide the necessary genetic background for WT1 to function. The 293 cells were transfected by a CMV-IE promoter-driven expression vector containing a full-length cDNA encoding WT1. One clone expressed WT1 at a level comparable with that of endogenous WT1 in promyelocytic leukemia HL-60 cells as determined by Western analysis (Fig. 1a). Reverse transcription PCR amplification and DNA sequencing of the exogenous WT1 mRNA in this clone did not reveal any mutations (data not shown), and this clone was used for further investigation. WT1-6 cells also displayed a 2-fold lower growth rate compared with CMV-293 control cells (293 cells transfected with empty expression vector) (Fig. 1b). These data are consistent with other reports that WT1 can inhibit cell proliferation (6, 11, 13) and indicate that exogenous WT1 is functionally active in the WT1-6 cells.

Since WT1-6 and CMV-293 cells share a similar genetic background, a subtraction analysis of expressed genes between these two cell lines is likely to eliminate most genes expressed in common and identify the genes that are subject to regulation by WT1. We performed SSH-PCR with mRNAs from these two cell lines to identify genes that are up-regulated in WT1-6 cells. After two rounds of subtraction by SSH-PCR, we obtained multiple distinct cDNA fragments. More than eight DNA fragments corresponded to different regions of wt1 cDNA, indicating that the subtraction was successful. Three cDNA fragments did not match any DNA sequences in GeneBank™. Two of the other fragments were known genes; one was human fragile X mental retardation protein (FMR-1) and the other was RbAp48, a protein first identified as one that physically interacts with Rb. The levels of RbAp46 transcripts were up-regulated in WT1-expressing 293 cells (WT1-6). Total RNA isolated from the CMV-293 and WT1-6 cells was evaluated for the levels of RbAp46 and actin (a) and WT1, RbAp48, and actin (b) expression by Northern analysis.

To obtain the full-length cDNA of RbAp46, we screened a human placenta cDNA library with a cDNA fragment generated by SSH-PCR as a probe. Four positive clones were obtained after tertiary screening, and the longest one (~1.9 kb)

![Fig. 1. Establishment of a human embryonic kidney 293 cell line expressing exogenous WT1.](image)

![Fig. 2. The levels of RbAp46 transcripts were up-regulated in WT1-expressing 293 cells (WT1-6).](image)
All cells were transfected by calcium-phosphate/DNA precipitation with CMV-IE promoter-driven constructs containing wild type WT1 and RbAp46 (20 μg) linked to the neomycin-resistant gene and selected with G418 for 3 weeks. G418-resistant colonies were stained and counted. The numbers of colonies/dish shown were derived from a representative experiment (±S.D.).

| Cell line | Mock   | Vector | RbAp46 | WT1   | RbAp48 |
|-----------|--------|--------|--------|-------|--------|
| U2OS      | 0 ± 1  | 456 ± 25 | 136 ± 12 | 191 ± 5 | 274 ± 5 |
| Saos-2    | 0 ± 1  | 87 ± 9  | 18 ± 2  | 29 ± 3 | 45 ± 2 |
| CV-1      | 0 ± 1  | 61 ± 4  | 18 ± 5  | 23 ± 7 | ND     |
| 293       | 0 ± 1  | 467 ± 14 | 194 ± 9 | 170 ± 15 | ND     |

To examine whether RbAp46 can mediate some of the functions previously ascribed to WT1, we performed a colony formation assay in several cell lines, including two osteosarcoma cell lines (U2OS and Saos-2), whose growth is known to be suppressed by WT1 (12), CV-1 cells derived from African green monkey kidney and whose growth is also inhibited by WT1 (11), and 293 cells, which have not been tested before. Cells were transfected with expression vectors containing cDNA for WT1 or RbAp46 (driven by the CMV-IE promoter) and the neomycin-resistant (neo<sup>5</sup>) gene and selected with G418 for 3 weeks.

Table I summarizes the results of several independent experiments. In CV-1, 293, U2OS, and Saos-2 cells, both WT1 and RbAp46 suppressed the emergence of neo<sup>5</sup> colonies to a similar degree. These data suggest that like WT1, RbAp46 has growth-inhibiting activity. The functional similarity between WT1 and RbAp46 also suggests that RbAp46 is a downstream mediator of WT1’s function. Interestingly, RbAp48, the RbAp46 homologue, also suppressed the emergence of drug-resistant colonies (albeit with lower efficiency) in the two osteosarcoma cell lines (Table I), indicating that RbAp48 also has growth inhibiting activity.

To determine whether the reduced growth rate of WT1-6 cells results from the induced expression of RbAp46, we transfected 293 cells with a CMV promoter-driven expression vector of RbAp46 and selected transfected cells with G418. The clonally isolated colonies were examined by Northern analysis using RbAp46 cDNA as a probe. A distinct 2.0-kb transcript was highly expressed in the RbAp46-transfected cells (Fig. 3a). Western blot analysis using polyclonal antibody raised against the final 15 amino acids of RbAp46 confirmed that the RbAp46 (~50 kDa) protein was highly expressed in the cloned cells (Fig. 3b). Two clones expressed RbAp46 at levels comparable with that in the WT1-6 cells, as determined by Western analysis (Fig. 3b). Furthermore, Western analysis indicated that the levels of WT1 expression in these two cell lines were similar to that of control CMV-293 cells (data not shown). This result excludes the possibility that overexpression of RbAp46 up-regulates WT1 expression due to a feedback mechanism. These
two cell clones (p46-1 and p46-2) were then used for further investigation.

The p46-1, p46-2, and control CMV-293 cells were maintained in Dulbecco’s modified Eagle’s medium plus 10% fetal calf serum. The growth rate of each cell line was determined by counting the number of cells daily. The cell lines expressing high levels of exogenous RbAp46 (p46-1 and p46-2) had a 2-fold lower growth rate than CMV-293 cells (Fig. 3c), similar to the patterns observed with WT1-6 cells (Fig. 1b). These data indicate that the high levels of RbAp46 reduce growth of transfected 293 cells and suggest that the reduced growth rate of WT1-6 cells may be a result of the up-regulation of RbAp46.

Since WT1 is required for urogenital development (22), we investigated whether RbAp46 is co-expressed with WT1 in the developing kidney and gonad. We examined developing kidney from a 13-day postcoitus mouse embryo by whole-mount in situ hybridization. Our results demonstrated that RbAp46 is highly expressed in condensed metanephric mesenchyme (Fig. 4, B and C), a site of WT1 expression (23, 24). However, unlike WT-1, RbAp46 is not detected in the metanephric epithelial structures or the prospective podocyte layers in S-shaped bodies (Fig. 4C). Whole-mount in situ hybridization also revealed a high level of RbAp46 expression at day 13 postcoitus in the developing gonad (Fig. 4A), another site of WT1 expression. These data suggest that WT1 may up-regulate the expression of RbAp46 during urogenital development and that RbAp46, as a mediator of WT1’s function, may play an important role in modulating cell growth and differentiation during the development of the urogenital system.

**DISCUSSION**

Using SSH-PCR, we cloned the RbAp46 gene as a candidate target gene of WT1. The transcript levels of RbAp46 were up-regulated about 15-fold in 293 cells that express high levels of exogenous WT1. However, the mechanism by which RbAp46 is up-regulated in the WT1-expressing cells is not clear. WT1 may directly up-regulate the transcription of RbAp46 by binding to the promoter region of the RbAp46 gene or may act indirectly by activating an intermediate transcription factor.

RbAp46 strongly inhibits colony formation and reduces the growth rate of transfected 293 cells to a degree similar to WT1, indicating that RbAp46 may be a downstream mediator of WT1’s function. However, apoptosis of cells was not observed in the RbAp46-transfected cells, suggesting that RbAp46 may only mediate the function of WT1 as a growth inhibitor. The induction of p21 and suppression of epidermal growth factor receptor by WT1 may explain the induction of apoptosis by WT1 (10, 12).

What is the mechanism by which RbAp46 inhibits cell growth? The observation that RbAp46 interacts with Rb both in vivo and in vitro (16, 17) suggests that RbAp46 may inhibit cell growth through an Rb-dependent pathway. However, this possibility is unlikely because the growth of Saos-2 cells is strongly suppressed by the expression of exogenous RbAp46 (Table 1), even though Saos-2 cells express a nonfunctional Rb protein lacking the C-terminal region that interacts with RbAp46 (16). These data suggest that inhibition of cell growth by RbAp46 is not dependent on a functional Rb protein.

Interestingly, RbAp46 has recently been demonstrated to exist with human histone deacetylases HDAC1, HDAC2, and RbAp48 in the mammalian transcriptional repressor mSin3 complex (25), suggesting that RbAp46 may be involved in histone metabolism related to the regulation of transcription. One hypothesis is that RbAp46 and/or RbAp48 may regulate histone metabolism by modulating the activities of HDAC1 and HDAC2, which are required for proper regulation of transcription.

In this way, overexpression of regulatory components of mSin3 such as RbAp46 and/or RbAp48 may lead to titration of factors such as other components of the mSin3 complex and misregulation of subsets of genes, which ultimately alters the rate of cell growth. This hypothesis is supported by the observation that RbAp48 also has growth-inhibiting activity.

The observation that RbAp46 is primarily localized in the developing kidney within similar structures that highly express WT1 further supports a role for RbAp46 as a downstream mediator of WT1 function during urogenital development. It is interesting that RbAp46 is expressed with WT1 in the condensed mesenchyme but not in differentiating epithelial structures. WT1 has been postulated to regulate stem cell survival and early differentiation in the metanephric blastema (22). The pattern of RbAp46 expression in the developing kidney and the growth suppression properties of the protein might suggest that it functions to limit cell proliferation of induced mesenchyme and thus permit cell differentiation to proceed.

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