A Functional Interaction with CBP Contributes to Transcriptional Activation by the Wilms Tumor Suppressor WT1*

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The Wilms tumor gene WT1 encodes a zinc finger transcription factor that is required for normal kidney development. WT1 was identified as a transcriptional repressor, based on its suppression of promoter reporters, but analysis of native transcripts using high density microarrays has uncovered transcriptional activation, rather than repression, of potential target genes. We report here that WT1 binds to the transcriptional coactivator CBP, leading to synergistic activation of a physiologically relevant promoter. The physical interaction between WT1 and CBP is evident in vitro and in vivo, and the two proteins are co-immunoprecipitated from embryonic rat kidney cells. The WT1-CBP association requires the first two zinc fingers of WT1 and the adenovirus 5 E1A-binding domain of CBP. Overexpression of this domain of CBP is sufficient to inhibit WT1-mediated transcriptional activation of a promoter reporter, as is co-transfection of E1A. Retrovirally driven expression of either the CBP fragment or of E1A in human hematopoietic cells suppresses the induction by WT1 of its endogenous target gene, p21Cip1. These observations support a model of WT1 as a transcriptional activator of genes required for cellular differentiation.

The tumor suppressor gene WT1 provides a paradigm for the link between normal organ development and cancer. WT1 was originally identified by its inactivation in a subset of Wilms tumors and in the germline of children with genetic predisposition to this pediatric kidney cancer (1–4). Expression of WT1 is restricted to specific cell types in the fetal kidney, gonads, mesothelium, and hematopoietic lineages (5–8). In the developing kidney, WT1 is present at low levels in blastemal stem cells, and very high levels of expression are observed as these cells differentiate to form glomerular podocytes. The critical role of WT1 in normal renal development is demonstrated by the apoptosis of renal blastemal cells in WT1-null mice, leading to complete failure of kidney differentiation (9).

The DNA-binding domain of WT1 is encoded by four C-terminal Cys-His zinc fingers, which mediate recognition of both GC- and TC-rich sequences (10, 11). The affinity of WT1 for these motifs is greatly reduced by the product of an alternatively spliced transcript, in which the three amino acids KTS are inserted between zinc fingers three and four (10, 12). The WT1(−KTS) isoform modulates transcriptional activity of reporter constructs, and its ectopic expression leads to either cell cycle arrest or apoptosis (13–15). In contrast, the WT1(−KTS) isoform is inactive in such assays, and its discrete subnuclear localization has been linked to a potential role in pre-mRNA splicing (16). The N terminus of WT1 encodes a proline/glutamine-rich domain similar to the transactivation domain of other transcription factors, such as Sp1 (17). Fusion of this transactivation domain to a GAL4 DNA-binding domain results in transcriptional repression of a reporter construct (18), and WT1 itself has been shown to repress GC- and TC-rich promoters in transient transfection assays (for reviews, see Refs. 19 and 20). The observation that the transcriptional activity of promoters from many growth-promoting genes, including early growth response 1 (18), insulin-like growth factor 2 (21), insulin-like growth factor receptor (22), platelet-derived growth factor-A (23, 24), and epidermal growth factor receptor (13), is suppressed by WT1 has led to the model that WT1 functions as a tumor suppressor by transcriptional repression of genes required for cellular proliferation (19). However, variations in experimental conditions, including promoter context (11), presence or absence of p53 (25), and even choice of expression vector (26), appear to modulate the properties of WT1 in transient transfection assays, leading to transcriptional activation as well as repression. Furthermore, analysis of cells with inducible expression of WT1 demonstrated that few endogenous genes with putative WT1-repressible promoters are in fact regulated by WT1 in vivo (13).

As a strategy to identify physiologically regulated WT1 target genes, we recently used high density oligonucleotide arrays representing 6,800 genes and expressed sequence tags to compare the expression profile of cells before and shortly after inducible expression of WT1 (27). Ectopic expression of WT1(−KTS) did not result in reduced expression of any transcripts represented on the microarrays. However, a small number of genes were strongly induced following WT1(−KTS) expression, notably amphiregulin (AR), encoding a secreted growth factor of the epidermal growth factor family capable of stimulating cellular differentiation in organ culture systems, and the cyclin-dependent kinase inhibitor p21(Cip1). A physiologically relevant interaction between WT1 and AR was suggested by their precise co-localization within differentiating glomeruli of the developing kidney, and by the ability of recombinant AR to induce epithelial differentiation in cultured kidney rudiments (27). WT1(−KTS) binds to a high affinity site (WRE) in the AR promoter, adjacent to a CRE site. The synergistic transcription activation mediated through the WRE and CRE sites raised the possibility that WT1 itself might interact with CBP/P300, a coactivator known to enhance CRE-dependent transcriptional activation by CRE-binding factors (28). We show here that WT1...
binds to CBP stoichiometrically both in vitro and in vivo, and that this protein interaction contributes to WT1-dependent transcriptional activation. These observations support the role of WT1 as a transcriptional activator, which may participate in the induction of target genes directly, as well as through potential interactions with other transcriptional regulators.

**MATERIALS AND METHODS**

**Cell Culture and Expression Constructs**—NIH3T3 cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. RSTEM cells with inducible WT1 expression (27) were grown at 32 °C in the same media, supplemented with 1 μg/ml tetracycline. For WT1 induction, cultures at 70% confluence were extensively washed with phosphate-buffered saline and allowed to grow for an additional 30 h in medium without tetracycline before protein extraction. CMV-driven constructs encoding WT1(−KTS) and WT1(+KTS) have been previously described (13), as have WT1-deletion constructs (29) and constructs encoding CBP (30), P300 (31), and E1A (32). Constructs encoding different CBP domains were generated by inserting the appropriate polymerase chain reaction fragments into pCMV-myc vector (Invitrogen). The AR promoter reporter (luciferase) PGL2-B-ΔCRE contains the WT1-responsive site and lacks an adjacent CRE site (27).

**Transient Transfections and Luciferase Reporter Assays**—NIH3T3 cells were transfected using the calcium phosphate DNA precipitation method. For protein extraction following transfection with WT1 deletion constructs, 5 μg of each plasmid was used. For luciferase reporter assays, 0.5 μg of CMV-WT1(−KTS) and/or CBP expression plasmids...
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RESULTS AND DISCUSSION

Enhancement of WT1-mediated Transactivation by CBP—We made use of AR, as a potentially physiological WT1-target gene, to test the effect of CBP expression on WT1-mediated transcriptional activation. The WT1-responsive element (WRE) in the AR promoter, 5′-CCCTGCGGGTGG-3′, is located at position −292 to −283, adjacent to a CRE element at position −274 to −267 (27). To restrict our analysis to WRE-dependent transactivation, we deleted the CRE site from a minimal reporter (pGL2-B-ΔCRE), which was co-transfected into NIH 3T3 cells together with cytomegalovirus (CMV)-driven constructs encoding WT1 (−KTS) and CBP. 2-Fold transcriptional activation of the reporter was observed following transfection of small amounts (0.5 μg) of WT1 (−KTS) plasmid alone. Coexpression of CBP (0.5 μg) resulted in 10-fold activation of the reporter, while CBP alone had no effect (Fig. 1). Transfection of CMV-driven P300, a transcriptional coactivator that is closely related to CBP, also enhanced WT1-dependent transactivation, but to a lesser extent.

In Vivo Physical Association between WT1 and CBP—In the fetal kidney, physiological expression of WT1 is restricted to...
mediates its interaction with WT1. Incubation of these reticulocyte lysates with bacterially synthesized GST-WT1(–KTS) or GST-WT1(+KTS) was followed by GST pull-down and autoradiography. A, a schematic representation of the protein-interaction domains of CBP is shown, including the domains involved in its association with nuclear hormone receptors (NR), CRE-binding factors (CREB), and Jun, E1A, and p53. The E1A-binding domain of CBP (amino acids (aa) 1625–1991) also mediates its interaction with WT1.

Domains Involved in the Interaction between WT1 and CBP—We used five deletion constructs spanning the entire length of the WT1 coding region to map the site required for its interaction with CBP. The HA epitope-tagged deletion constructs were transfected into NIH 3T3 cells along with a plasmid expressing CBP, and whole cell extracts were subjected to immunoprecipitation Western analysis (Fig. 3). Equal expression of the transfected constructs was determined, and anti-CBP immunoprecipitates were analyzed for the presence of WT1 using antibody against the HA epitope. A WT1 product lacking the first two zinc fingers failed to co-immunoprecipitate with CBP, indicating that this portion of the WT1 DNA-binding domain is required for this protein interaction.

To identify the domain of CBP required for its interaction with WT1, we incubated a bacterially synthesized protein containing the four WT1 zinc fingers with in vitro translated domains of CBP. This in vitro approach was used to confirm the direct interaction between these two proteins. CBP encodes a protein of 300 kDa, but four domains mediate its known interactions with transcription factors (34–39). Polymerase chain reaction-generated fragments encoding amino acids 1–436 (CBP1: binding site for nuclear hormone receptors), amino acids 437–786 (CBP2: binding site for CRE-binding factors), amino acids 1625–1991 (CBP3: binding site for E1A), and amino acids 1992–2442 (CBP4: binding site for p53) were translated in vitro, and incubated with GST-WT1 affinity matrix. Only CBP3 bound to WT1 (Fig. 4), indicating that the E1A-binding domain of CBP is also responsible for its interaction with WT1. Consistent with the stoichiometric protein interaction observed in vivo, the in vitro association between WT1 and CBP was quantitative, with ~1/3 of input CBP protein associated with WT1. Of note, the WT1(+KTS) isoform also showed high affinity binding to CBP (Fig. 4). The possibility that this alternative splice product of WT1, which has been previously linked to a role in pre-mRNA processing (16), also
plays a role in transcriptional regulation warrants further investigation.

Disruption of the WT1-CBP Interaction Abrogates WT1 Target Gene Expression—Having defined the domains required for the interaction of WT1 with CBP, we sought to determine the effect of disrupting this protein association on WT1-mediated transcriptional activation. We first tested whether CBP3, the domain that mediates the interaction of CBP with WT1 but lacks functional histone acetyltransferase activity, could function as a dominant negative construct in reporter assays. Co-transfection of NIH 3T3 cells with the WRE-containing reporter (pGL2-B-DCRE) along with CMV-WT1 (2KTS) alone (4 μg) resulted in 8-fold transcriptional activation (Fig. 5, upper panel). Coexpression of increasing amounts of CMV-CBP3 (2–8 μg), fused to an SV40-derived nuclear localization signal, resulted in a dose-dependent reduction in activation. In contrast, co-transfection of CMV-driven constructs encoding other domains of CBP that fail to interact with WT1 had no effect (Fig. 5, upper panel), despite equal expression levels (Fig. 5, lower panel). Transcriptional activation by WT1 may therefore be specifically inhibited by disruption of its interaction with endogenous CBP.

Transcriptional coactivation by both CBP and P300 is also known to be disrupted by their physical association with the adenovirus 5 early gene product E1A (31, 40), and the binding of both WT1 and E1A to the CBP3 domain suggests that these proteins may compete for this binding site. We therefore examined the effect of E1A expression on WRE-dependent transcription. Co-transfection of CMV-driven E1A demonstrated a dose-dependent suppression of CBP-mediated coactivation of the WT1-responsive promoter (Fig. 6). To extend these studies to an endogenous WT1-target gene, we examined the effect of E1A expression on induction by WT1 of the cyclin-dependent kinase inhibitor p21Cip1 WT1 directly activates the p21Cip1 promoter, and induction of the endogenous p21Cip1 gene is linked to WT1-mediated cell cycle arrest (14, 21, 41). More recently, we have found that high titer retroviral infection of U937 human leukemia cells with a mouse stem cell virus promoter-driven WT1 construct, leads to p21Cip1 induction and triggers growth.
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Concluding Remarks—We have described a functional interaction between the tumor suppressor gene product WT1 and the transcriptional coactivator CBP. Recruitment of CBP to a target promoter is thought to enhance transcription by providing a platform that facilitates the assembly of additional transcription factors and components of the basic transcriptional machinery, and by mediating histone acetylation (for review, see Refs. 42 and 43). The observation that WT1 encodes a bona fide transcriptional activator that associates with CBP in vivo was unexpected, since WT1 has long been thought to act as a transcriptional repressor, whose function as a tumor suppressor is linked to the repression of proliferation-inducing genes (19). However, the identification of physiologically induced WT1 target genes (27, 44, 45), and the analysis of alterations in vivo during Wilms tumorigenesis may result in the failure of renal cells to differentiate, leading to the persistence of pluripotent stem cells susceptible to malignant transformation.

The interaction between WT1 and CBP also has implications for understanding functional properties of WT1 that have been reported to date. The consequences of WT1 expression in baby

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2 L. W. Ellisen and D. A. Haber, unpublished data.
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rat kidney cells, a well established primary cell transformation model in which cellular immortalization is achieved by expression of adenovirus E1A, should be interpreted with caution, given the likely disruption of WT1-mediated transcriptional activation by E1A (47, 48). These observations also provide insight into the functional and physical association between WT1 and p53. These two proteins do not interact directly in vitro, but are co-immunoprecipitated from cellular lysates, and expression of wild-type p53 suppresses transcriptional activation by WT1, while expression of WT1 inhibits p53-mediated apoptosis (29, 25). CBP is likely to mediate this indirect interaction, since it binds to both WT1 and p53 through adjacent domains, and WT1 zinc fingers 1–2 are required for its interaction with both CBP and p53. Overexpression of either WT1 or p53 may thus modify the transactivational properties of the other (29, 25).

A similar indirect association may underlie the synergistic effect of WT1 and steroidogenic factor 1, an orphan nuclear receptor implicated in gonadal differentiation. WT1 and steroidogenic factor 1 do not appear to bind to a known DNA sequence, nor do WT1 and steroidogenic factor 1, an orphan nuclear receptor implicated in gonadal differentiation. WT1 and steroidogenic factor 1, an orphan nuclear receptor implicated in gonadal differentiation, may modulate the transactivational properties of the other (29, 25).

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