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WT1 modulates apoptosis by transcriptionally upregulating the *bcl-2* proto-oncogene

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The Wilms' tumor suppressor gene, *WT1*, encodes a zinc finger transcription factor that has been demonstrated to negatively regulate several growth factor and cognate receptor genes. However, inconsistent with its tumor suppressor function, WT1 has also been demonstrated to be required to inhibit programmed cell death *in vitro* and *in vivo*. Moreover, anaplastic Wilms' tumors, which typically express wild-type WT1, display extreme resistance to chemotherapeutic agents that kill tumor cells through the induction of apoptosis. Although p53 mutations in anaplastic Wilms' tumors have been associated with chemoresistance, this event is believed to occur late during tumor progression. Therefore, since dysregulated WT1 expression occurs relatively early in Wilms' tumors, we hypothesized that WT1 was required to transcriptionally upregulate genes that provide a cell survival advantage to tumor cells. Here we demonstrate that sporadic Wilms' tumors coexpress WT1 and the anti-apoptotic Bcl-2 protein. Using rhabdoid cell lines overexpressing WT1, we show that WT1 activates the endogenous *bcl*-2 gene through a transcriptional mechanism. Transient transfections and electromobility shift assays demonstrate that WT1 positively stimulates the *bcl*-2 promoter through a direct interaction. Moreover, WT1 expressing cells displaying upregulated Bcl-2 were found to be resistant to apoptosis induced by staurosporine, vincristine and doxorubicine. These data suggest that in certain cellular contexts, WT1 exhibits oncogenic potential through the transcriptional upregulation of anti-apoptotic genes such as *bcl*-2.

Keywords: apoptosis/ Bcl-2/transcriptional regulation/ Wilms' tumor/WT1

Introduction

The Wilms' tumor-associated gene *WT1* encodes a tumor suppressor gene product that is expressed in the developing kidney and in the adult urogenital system (reviewed in Haber and Housman, 1992; Rauscher, 1993; Reddy and Licht, 1996). Consistent with the role of WT1 as a tumor suppressor, expression of WT1 protein has been shown to suppress cell growth in both Wilms' tumors and non-Wilms' tumors (Haber *et al*., 1993; Luo *et al*., 1995; McMaster *et al*., 1995). Although the *WT1* gene is deleted or mutated in nearly all of patients diagnosed with Denys-Drash syndrome (DDS; a syndrome which includes nephropathy, intersex disorders and a predisposition to develop Wilms' tumors), <10% of all sporadic Wilms' tumors contain abnormalities in the *WT1* gene (reviewed in Coppes *et al*., 1993a; Haber and Housman, 1992). Therefore, despite the ability of WT1 to act as a tumor suppressor, 90% of sporadic Wilms' tumors continue to express this protein either because cells have acquired secondary mutations in other genes which affect WT1 tumor suppressor function, and/or because WT1 provides an important survival function in these tumors.

The WT1 protein is a transcription factor which is composed of two functional domains: a proline-glutamine rich domain at the N-terminus, and a zinc finger domain composed of four Cys_2-His_2 zinc fingers at the C-terminus (Haber and Housman, 1992; Rauscher, 1993; Reddy and Licht, 1996). The *WT1* gene yields four alternatively spliced mRNAs: WT1-A, which contains neither alternative splice; WT1-B, which includes an N-terminal splice which codes for an additional 17 amino acids; WT1-C, which includes a C-terminal splice which results in a three amino acid insertion (KTS); and WT1-D, which contains both alternate exons (Haber and Housman, 1992; Rauscher, 1993; Reddy and Licht, 1996). These different isoforms are expressed at a ratio of 1:2.5:3.8:8.3 in the fetal kidney (Haber *et al*., 1990). WT1-A and WT1-B proteins were originally demonstrated to bind to the same DNA consensus sequence, 5′-GCGGGGGCG-3′, as the structurally related early growth response 1 (Egr-1) protein (Rauscher *et al*., 1990). However, additional DNA sequences have been identified which display a higher level of affinity for the WT1 protein (Wang *et al*., 1993b; Hamilton *et al*., 1995; Nakagama *et al*., 1995). Because the alternative splice II donor site results in the insertion of three amino acids (KTS) between zinc fingers 3 and 4 of WT1-C and WT1-D proteins, these two isoforms recognize related but distinct DNA sequences (Drummond *et al*., 1994; Wang *et al*., 1995). Therefore, not only do differences in DNA-binding sequences suggest that WT1-A and WT1-B may regulate a different subset of genes than KTS-containing WT1 proteins, but WT1 isoforms may also exhibit different biological functions (Englert *et al*., 1995; Larsson *et al*., 1995).

Consistent with the role of WT1 as a tumor suppressor protein, this transcription factor has been demonstrated in transient co-transfections to repress several cellular promoters, most of which include growth factor and cognate receptor genes (Rauscher, 1993; Reddy and Licht *et al*., 1996). However, only a few genes, including the

epidermal growth factor receptor (EGFR), the insulin-like growth factor receptor (IGFR), and the platelet-derived growth factor (PDGF), have been shown to be repressed endogenously (Gashler *et al*., 1992; Englert *et al*., 1995; Werner *et al*., 1995). Besides being a potent repressor of transcription, WT1 is a transcription factor with strong transactivating potential (Reddy *et al*., 1995; Wang *et al*., 1995). Recently, a number of genes have been demonstrated to be positively regulated by WT1-A and WT1-B isoforms, but not by KTS-containing WT1 proteins (Wang *et al*., 1993a; Nichols *et al*., 1995; Cook *et al*., 1996; Kim *et al*., 1998).

The importance of WT1 in normal urogenital development establishes an anti-apoptotic role for WT1. Homozygous deletion of the *WT1* gene in the mouse germline results in embryonic lethality at day 11 of gestation due to a failure of kidney and heart development (Kreidberg *et al*., 1993). In $WT1^{-/-}$ knockout animals, cells of the metanephric blastema, which are required for normal kidney development, fail to proliferate and undergo programmed cell death (Kreidberg *et al*., 1993). These studies suggest that during kidney development, WT1 either functions to repress pro-apoptotic genes or gene products, or that WT1 acts as potent transcription factor to activate WT1-responsive genes required to overcome programmed cell death. Consistent with this first scenario, WT1 has been reported to modulate programmed cell death by interacting directly with p53 and inhibiting p53-mediated apoptosis (Maheswaran *et al*., 1995). Since apoptosis is believed to act as a defense against malignant transformation, it is possible that the anti-apoptotic nature of WT1 may potentiate oncogenesis. In support of this idea, WT1 expression has been reported in a number of cancers, including mesotheliomas, erythro- and myeloid-leukemias (Amin *et al*., 1995; Pritchard-Jones and King-Underwood, 1997). In erythro- and myeloid-leukemia cells, WT1 expression is associated with proliferation and maintenance of an immature cell phenotype (Phelan *et al*., 1994; Sekiya *et al*., 1994). Importantly, it has been demonstrated that the loss of WT1 expression sensitizes myeloid cells to undergo programmed cell death, suggesting that WT1 contributes to oncogenesis by inhibiting apoptosis (Algar *et al*., 1996).

In an attempt to identify WT1-regulated genes that provide protection from programmed cell death, we searched for anti-apoptotic genes that were upregulated in response to WT1 expression. Here, we demonstrate that Bcl-2 expression coincides with WT1 protein levels in sporadic Wilms' tumors. Additionally, we show that WT1 expression upregulates endogenous *bcl*-2 mRNA levels by transcriptionally activating the *bcl*-2 promoter through a high-affinity WT1-binding site. Importantly, we found that cells expressing WT1-B and Bcl-2 are resistant to staurosporine-, vincristine- and doxorubicine-induced apoptosis. Bcl-2 and related proteins have been shown to inhibit apoptosis by regulating the mitochondrial permeability transition, and the subsequent release of apoptosis-inducing factor (AIF) and cytochrome *c* (reviewed in Reed, 1997; Kroemer, 1997; Green and Reed, 1998; Thornberry and Lazebnik, 1998). This cellular response is then responsible for the activation of caspase-9 and the induction of apoptosis (Green and Reed, 1998; Thornberry and Lazebnik, 1998). Our findings have significant implications not only for understanding Wilms' tumor progression, but also for normal kidney development, by identifying *bcl*-2 gene as one of the important proto-oncogenes positively regulated by WT1.

Results

Primary Wilms' tumors express both WT1 and Bcl-2

More than 90% of all sporadic Wilms' tumors express wild-type WT1 (Little *et al*., 1992; Coppes *et al*., 1993b), suggesting that WT1 does not function as a tumor suppressor gene product in human nephroblastomas. As late-stage Wilms' tumors are resistant to apoptotic-inducing chemotherapeutic agents (Beckwith, 1996; Farie *et al*., 1996), and since cells of the metanephric blastema from the $WT1^{-/-}$ knockout animals are susceptible to programmed cell death (Kreidberg *et al*., 1993), we were interested in determining whether WT1 expression was associated with the expression of known anti-apoptotic proteins. Although the *bcl*-2 promoter has been shown to be negatively regulated by the overexpression of WT1 in transient transfection assays (Hewitt *et al*., 1995; Heckman *et al*., 1997), we were interested in whether primary sporadic Wilms' tumors displayed coordinate expression of WT1 and the anti-apoptotic Bcl-2 protein. Like fetal kidney, Wilms' tumors typically express all four WT1 isoforms (Haber *et al*., 1990; see Figure 1A). To address whether Wilms' tumors coexpress both WT1 and Bcl-2, total proteins were isolated from nine randomly selected primary sporadic Wilms' tumors and Western blot analysis was performed. As shown in Figure 1B, eight out of nine Wilms' tumors analyzed displayed the full-length WT1 protein (52–54 kDa), while one tumor expressed a smaller (36 kDa) WT1 immunoreactive band. Interestingly, Wilms' tumors which strongly expressed WT1 immunoreactive bands (52–54 kDa) also displayed high levels of Bcl-2 protein (Figure 1B), while the tumor which displayed the lower WT1 immunoreactive band (36 kDa) failed to express detectable Bcl-2 expression (Figure 1B, lane 3). Only one of the Wilms' tumors analyzed, which expressed high levels of WT1 protein (52–54 kDa), failed to express an abundance of Bcl-2 protein (Figure 1B, lane 9). The differences in Bcl-2 protein levels observed in Wilms' tumor samples were not due to uneven protein loading since re-analysis of the blots demonstrated equal levels of actin protein (Figure 1B). Additionally, Wilms' tumor samples, which were known to contain WT1 mutations, failed to display increases in Bcl-2 expression, supporting the idea that WT1 positively regulates Bcl-2 expression (data not shown). These results indicate that many sporadic Wilms' tumors express both WT1 and Bcl-2 protein and suggest that WT1 does not negatively regulate endogenous *bcl*-2 expression in nephroblastomas, but rather may positively regulate expression of this gene.

Cells stably expressing WT1-B display upregulated bcl-2 transcripts and protein

To determine more directly whether cells expressing WT1 displayed a coordinate increase in Bcl-2 expression, we analyzed derivatives of the rhabdoid tumor cell line G401 (Weissman *et al*., 1987) which stably express either the WT1-B or WT1-C isoforms (McMaster *et al*., 1995).

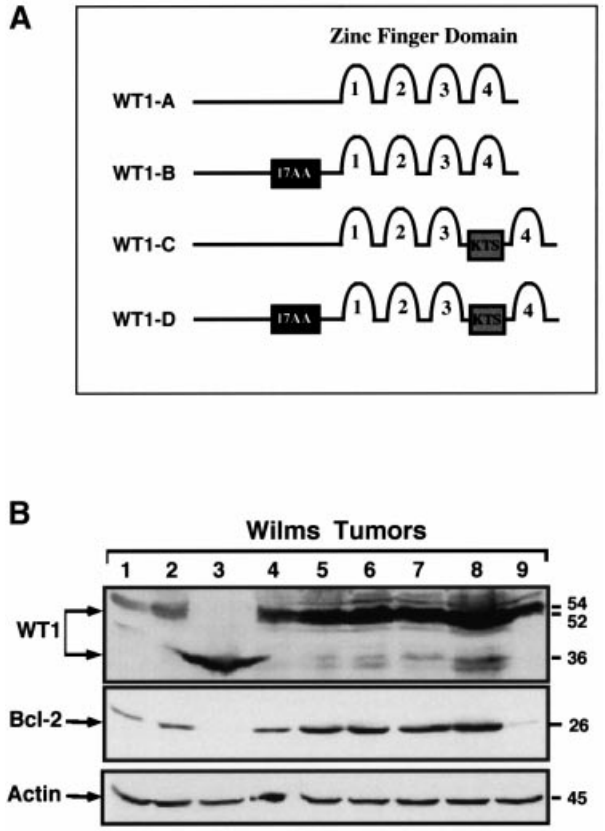


Fig. 1. Coordinate expression of WT1 and Bcl-2 in sporadic Wilms' tumors. (**A**) A diagrammatic representation of the four alternatively spliced WT1 isoforms. The *WT1* transcript contains two alternative splice sites, which results in four distinct transcripts. Alternative splice I, encoded by exon 5, results in a 17 amino acid insertion. Alternative splice II uses an alternative splice donor site located between exons 9 and 10, which results in the insertion of three amino acids [lysine, threonine and serine (KTS)]. Because the KTS insert occurs between zinc fingers 3 and 4, KTS-containing WT1 proteins display altered DNA-binding specificity. (**B**) Bcl-2 immunoblot analysis was performed using total proteins isolated from nine randomly selected sporadic Wilms' tumor specimens. Proteins were quantitated using the Bio-Rad assay and 50 μg per lane were resolved on a 10% polyacrylamide gel. Proteins were transferred and immobilized on a nitrocellulose membrane, and protein expression was analyzed using anti-WT1 (C-19), anti-Bcl-2 (100) or anti-Actin (I-19) primary antibodies, all obtained from Santa Cruz Biotech. Horseradish peroxidase-conjugated anti-mouse, anti-rabbit and anti-goat secondary antibodies (Promega) were detected using ECL (Amersham). Protein molecular weights were determined using prestained rainbow markers (Amersham).

G401 cells fail to express endogenous WT1, and therefore have been used to measure WT1-mediated gene regulation (McMaster *et al*., 1995; Werner *et al*., 1995). Since the alternative splice site II inserts or removes the three amino acid sequence (KTS) between zinc fingers III and IV and changes the DNA-binding specificity of WT1 (Drummond *et al*., 1994; Wang *et al*., 1995), it was important to compare G401 cells which express either WT1-B, a protein isoform which lacks the KTS, or WT1-C, a protein which contains the KTS amino acid insert (Figure 1A). Total proteins were isolated from Lan-5, a neuroblastoma cell line which expresses high levels of endogenous Bcl-2 protein (Hanada *et al*., 1993), and from parental G401, G401-Neo control, or from three representative G401 subclones stably expressing either WT1-B or WT1-C

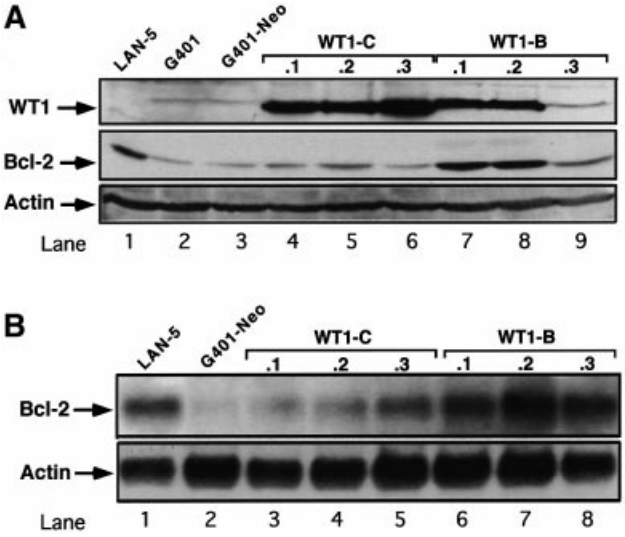


Fig. 2. Rhabdoid cell lines stably expressing WT1-B display elevated endogenous Bcl-2. (**A**) The Bcl-2 anti-apoptotic protein is upregulated in clones expressing WT1-B, but not in clones expressing the WT1-C isoform. Immunoblots of cellular lysates from parental G401, G401-Neo, and from WT1-B and WT1-C clones were analyzed for the expression of Bcl-2 and WT1 proteins. Total proteins (50 μg per lane) were resolved on a 10% polyacrylamide gel, transferred to a nitrocellulose membrane and analyzed with anti-Bcl-2-, WT1- and actin-specific antibodies (Santa Cruz). (**B**) Elevated Bcl-2 protein levels, observed in WT1-B expressing cells, is associated with increased *bcl*-2 transcription. Total RNAs were isolated from Lan-5, G401-Neo control and from WT1-B and WT1-C stable clones, using the TriZol RNA solution (Life Science Inc.). RNAs (10 μg per lane) were resolved on a 0.8% formaldehyde agarose gel, and transferred to a Zeta blot membrane (Bio-Rad, Hercules, CA). Bcl-2 and β actin-specific transcripts were detected by analyzing Northern blots with a ^{32}P-labeled specific random-labeled cDNA probe and blots were subjected to autoradiography.

proteins. As shown in Figure 2A, G401 clones expressing WT1-B or WT1-C displayed immunoreactive bands (52 and 54 kDa) which were not observed in either the parental or G401-Neo control cells. Interestingly, cells stably expressing the WT1-B protein displayed a higher level of Bcl-2 protein expression than G401-Neo cells (Figure 2A, compare lane 2 with lanes 6–8). Moreover, the two cell lines which displayed the highest levels of WT1-B expression (namely, clones WT1-B.1 and WT1-B.2) also showed the highest levels of Bcl-2 expression (Figure 2A). In contrast, all three cell lines expressing the WT1-C isoform failed to displayed significant differences in Bcl-2 protein expression (Figure 2A, compare lane 2 with lanes 3–5). The increased levels of Bcl-2 protein observed in the WT1-B expressing cells were specific to this proto-oncoprotein, since other anti-apoptotic proteins, namely Bcl-x_L, Mcl-1 or A1, failed to show differences in expression (data not shown). Although WT1 has been demonstrated to stabilize p53 and prolong the half-life of this tumor suppressor protein (Maheswaran *et al*., 1993, 1995), immunoblot analysis failed to display differences in p53 protein levels between G401-Neo and WT1-expressing clones (data not shown). These results indicate that G401 cells expressing WT1-B demonstrate an elevation in Bcl-2 protein expression. In contrast, cells expressing WT1-C, an isoform which contains the alternative splice II (KTS) site and which is known to demonstrate limited DNA-binding activity, failed to positively upregulate endogenous Bcl-2 protein expression (Figure 2A).

Since our data indicated a differential regulation between the WT1-B and WT1-C isoforms in G401 cells, it was important to determine whether the increased levels of Bcl-2 protein expression (Figure 2A) were due to an elevation in endogenous *bcl*-2 gene expression. To address this question, total RNAs were isolated from Lan-5, and from G401-Neo cells and G401 cells expressing either WT1-B or WT1-C isoforms. In agreement with our immunoblotting analysis, G401 cells expressing WT1-B displayed an elevation in *bcl*-2 encoding transcripts, while G401-Neo and clones expressing WT1-C displayed lower levels of *bcl*-2 transcripts (Figure 2B). Equal amounts of total RNAs were loaded on to the RNA gels, since no significant differences in the levels of transcripts encoding β-actin were detected between the G401-Neo and the WT1-expressing subclones (Figure 2B). Although the WT1-B.3 subclone expressed higher levels of *bcl*-2 transcripts, for unknown reasons this increase in message did not correlate with Bcl-2 protein expression (Figure 2A and B). Importantly however, these data demonstrate that G401 cells which express WT1-B contain elevated levels of *bcl*-2 mRNA, while cells expressing WT1-C fail to show significant increases in *bcl*-2 expression.

WT1 positively regulates bcl-2 through a transcriptional mechanism

To determine whether the increase in *bcl*-2 mRNA levels observed in stably expressing WT1-B cells was due to the ability of WT1 to upregulate the *bcl*-2 promoter region, transient transfection assays were performed. G401 cells expressing either WT1-B (WT1-B.2), WT-1C (WT1-C.1) or vector control (G401-Neo) were transiently co-transfected with a luciferase reporter plasmid containing the *bcl*-2 promoter (see Figure 4). As shown in Figure 3A, WT1-B.2 cells displayed a significantly higher level of *bcl*-2 promoter activity than did either G401-Neo or WT1-C.1 cells. To determine whether the increase in *bcl*-2 promoter activity observed in WT1-B.2 cells was due to WT1-B-mediated transcription, cells were co-transfected with the DDS allele of WT1-B(394 R→W). This mutation disrupts the third zinc finger of WT1 (reviewed in Reddy and Licht, 1996) and creates an isoform which does not bind DNA and actively interferes with WT1 function (Pelletier *et al*., 1991a). This mutant acts as a dominant-negative inhibitor by heterodimerizing with WT1 and repressing transcriptional activity mediated by wild-type WT1 proteins (Haber *et al*., 1990; Reddy *et al*., 1995). As shown in Figure 3A, WT1-B was responsible for elevated *bcl*-2 promoter activity, since the expression of WT1-B(394 R→W) effectively reduced luciferase activity observed in WT1-B.2 cells. Moreover, expression of the WT1-B(394 R→W) plasmid did not significantly diminish the luciferase activity in either G401-Neo or WT1-C.1 cells, indicating that the dominant-negative protein was not responsible for non-specifically inhibiting basal *bcl*-2 promoter activity in transfected cells (Figure 3A). The difference in *bcl*-2 promoter activity observed in WT1-B.1 cells was not due to differences in transfection efficiencies, since similar levels of β-galactosidase expression were observed following transient co-transfection experiments in G401-Neo, WT1-B.2 and WT1-C.1 cells (Figure 3B). Additionally, WT1-B.2 cells transiently expressing the dominant-negative WT1-B(394 R→W) protein also did not show a decrease in β-galactosidase activity, suggesting that the decrease in *bcl*-2 promoter activity was due to a loss of WT1-B-mediated transcriptional activity and was not due to cytotoxic effects of WT1-B(394 R→W) expression (Figure 3B). These results indicate that WT1-B expression in G401 cells results in increased transcription of the *bcl*-2 promoter.

Since the *bcl*-2 promoter has been previously reported to be repressed by WT1 (Hewitt *et al*., 1995; Heckman *et al*., 1997), it was important to elucidate whether the ability of WT1 to positively upregulate the *bcl*-2 promoter region was a cell type-specific phenomenon. Transient transfection experiments were performed in three different cell lines: CV-1, Saos-2 and HeLa. CV-1 is an immortalized green monkey kidney cell line that does not express endogenous WT1. Expression of WT1-A in these cells has been shown to positively upregulate a WT1-responsive reporter in transient transfection assays (Reddy *et al*., 1995). Saos-2 is an osteosarcoma cell line which fails to express WT1 protein and has been used to demonstrate WT1-mediated transcriptional regulation (Maheswaran *et al*., 1993, 1995, 1998; Englert *et al*., 1995). HeLa is a human cervical epithelial line which has been used to characterize the WT1-induced repression of the *bcl*-2 promoter (Hewitt *et al*., 1995). As shown in Figure 3C, WT1-A positively upregulated the *bcl*-2 promoter in a dose-dependent manner in both Saos-2 and CV-1 cells. However, similar to previous reports (Hewitt *et al*., 1995), expression of WT1-A in HeLa cells resulted in a dose-dependent repression of the basal level of the *bcl*-2 promoter (Figure 3C). These results indicate that WT1 can differentially modulate the *bcl*-2 promoter in a cell type-specific manner, and suggest that the ability of WT1 to regulate the *bcl*-2 promoter is dependent not only on the levels of WT1 expressed, but also on the cell type analyzed.

Because our data suggest that WT1 isoforms which lack the KTS insert could upregulate the *bcl*-2 promoter (Figure 3A and C), we sought to determine whether KTS-containing WT1 isoforms could positively transactivate the *bcl*-2 promoter. To address this, transient transfections were performed in Saos-2 cells. As shown in Figure 3D, WT1-A and WT1-B expression in Saos-2 cells positively transactivated the *bcl*-2 promoter, however, WT1-C and WT1-D, as well as the dominant-negative mutant WT1-B(394 R→W), failed to activate the *bcl*-2 promoter. Consistent with the results observed in Figure 2A and B, transfection experiments indicated that the *bcl*-2 promoter is transcriptionally upregulated by both WT1-A and WT1-B isoforms but not by KTS-containing WT1-C and WT1-D proteins, or the dominant-negative WT1-B(394 R→W) mutant.

WT1 directly regulates the bcl-2 promoter

Experiments shown in Figures 2 and 3 suggest that the ability of WT1-A and WT1-B isoforms to effectively regulate the *bcl*-2 promoter was dependent on the DNA-binding specificity of these proteins, since WT1-C, WT1-D, and WT1-B(394 R→W) failed to positively regulate the *bcl*-2 promoter activity. Therefore, these results suggested that WT1 might regulate the *bcl*-2 promoter through a direct interaction with DNA target elements located within its regulatory region. To address

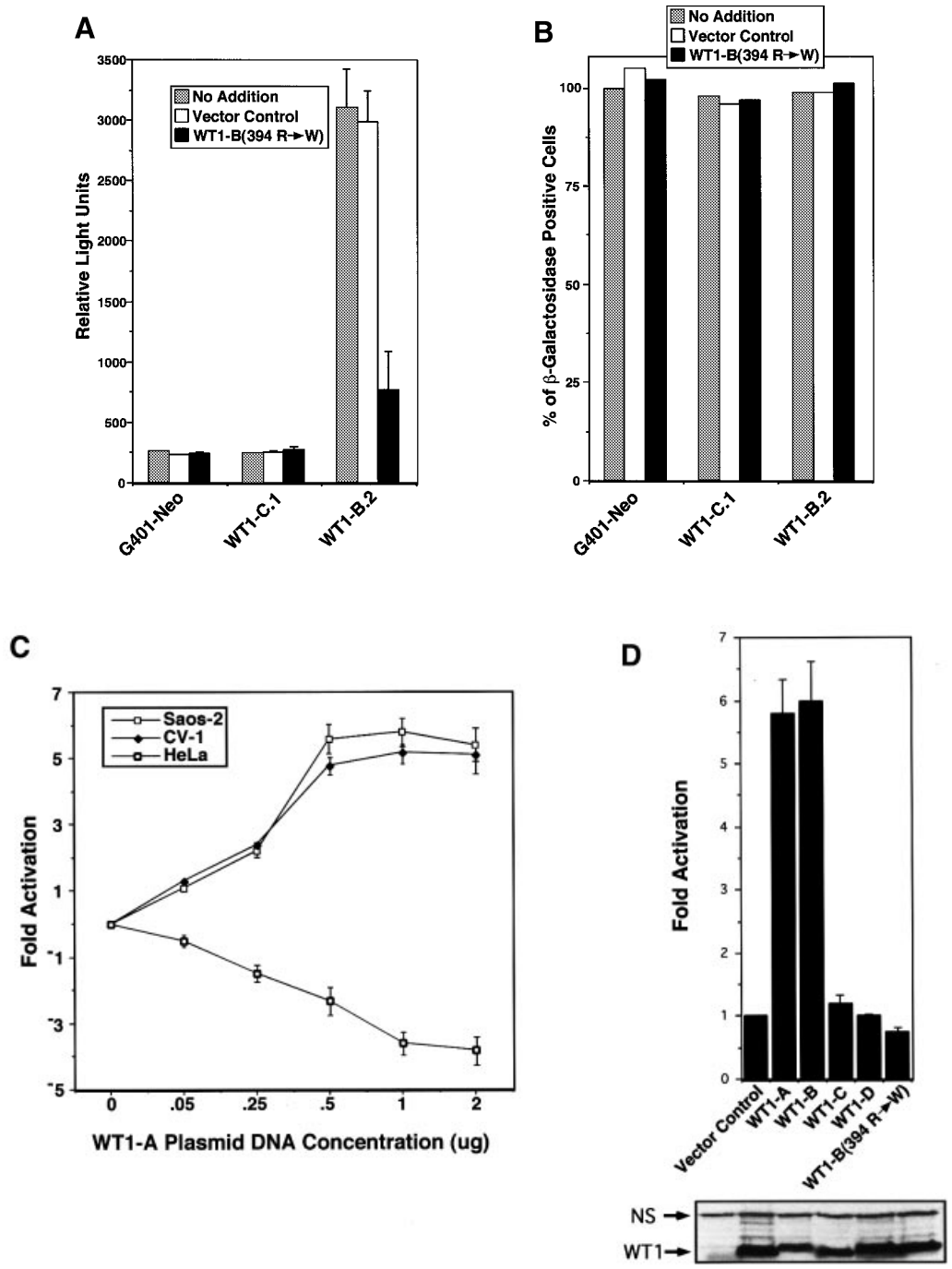


Fig. 3. WT1 positively regulates *bcl*-2 through a transcriptional mechanism. (**A** and **B**) WT1-B expression is responsible for upregulating the *bcl*-2 promoter in WT1-B.2 cells. G401-Neo, WT1-B.2 and WT1-C.1 cells were transiently co-transfected with both the *bcl*-2 XH-LUC reporter and CMV-LacZ (2 μg per 100 mm dish) using lipofectamine reagent (Life Sciences Inc.). In addition, some groups were transfected with plasmids encoding either the dominant-negative mutant WT1-B(394 R→W) or the empty pCMV vector control (1 μg each). Cell extracts were harvested 48 h following the start of transfection. Proteins were quantitated using Bio-Rad reagent and analyzed for both luciferase and β-galactosidase activity, as described in the Material and methods. Data presented in (A) represents the mean ± SD of three independent experiments, while (B) shows data from a single representative experiment. (**C**) WT1 expression upregulates the *bcl*-2 promoter in a dose-dependent and cell type-specific manner. CV-1, HeLa and Saos-2 cells were co-transfected with the *bcl*-2 XH-LUC reporter (2 μg) and either the WT1-A or the empty vector control (pCMV) at varying concentrations (0, 0.05, 0.25, 0.5, 1 and 2 μg). All cell groups were transfected with DNA (4 μg total) and concentrations were normalized using varying amounts of Bluescript $SK^{+/-}$ plasmid (Stratagene). Extracts were harvested 48 h following transfection and analyzed for luciferase activity. Data presented represents the mean ±SD of three independent experiments performed in duplicate. (**D**) Only the KTS minus WT1 isoforms are capable of positively upregulating the *bcl*-2 promoter. Saos-2 cells were transfected with the *bcl*-2 XH-LUC reporter (2 μg) and with plasmids encoding various WT1 isoforms, the mutant WT1-B(394 R→W) or the vector control (1 μg each). Cell extracts were collected 48 h following transfection and luciferase assays were performed. Data represent the average ±SD of three separate experiments. To confirm that expression vectors were encoding the various WT1 transgenes, Western blot analysis was performed on cell extracts. Protein extracts (50 μg/lane), from transiently transfected into Saos-2 cells, were resolved on a 10% polyacrylamide gel, transferred to nitrocellulose and analyzed for WT1 expression using a anti-WT1 antibody (C-19, Santa Cruz). WT1-specific immunoreactive bands, as well as non-specific bands (NS) are indicated with an arrow.

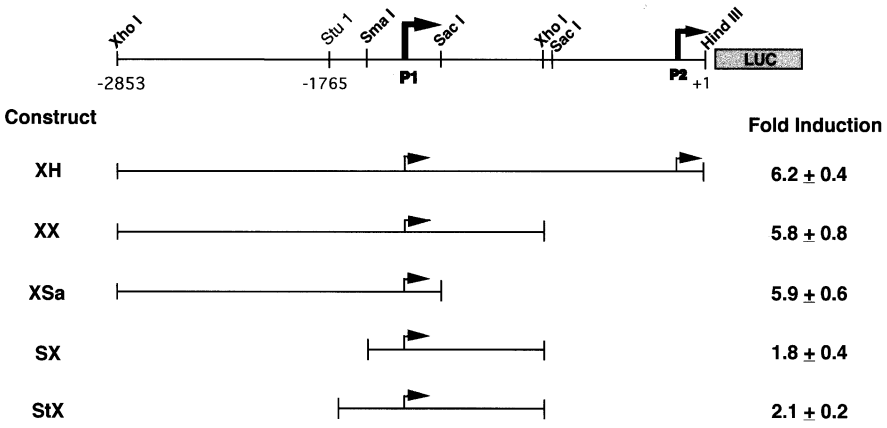


Fig. 4. WT1 positively regulates the *bcl*-2 regulatory region through a domain located upstream of the P_1 promoter. Deletions within the *bcl*-2 regulatory region were generated and cloned in front of the DNA encoding the luciferase reporter gene (LUC), as described in the Materials and methods. Saos-2 cells were co-transfected with various *bcl*-2 LUC constructs (2 μg each) along with either the vector control or with an expression vector encoding the WT1-A isoform (1 μg each). Cell extracts were harvested 48 h following transfection and luciferase activity was analyzed. Fold induction was determined by establishing the increase in *bcl*-2 promoter activity following co-transfection with the WT1-A construct above activity observed following transfection with the empty vector control. Basal promoter expression for each reporter construct was determined by arbitrarily establishing cells co-transfected with *bcl*-2 XH-LUC and the vector control as 100%. Data presented here represent the average of at least four independent experiments and the standard deviations are shown. Reporter constructs are named based on their restriction fragments. Sites are as indicated:X, *Xho*I; H, *Hin*dIII; S, *Sma*I; Sa, *Sac*I and St, *Stu*I.

this, a series of *bcl*-2 promoter constructs were made in which various sequences within the *bcl*-2 regulatory region were deleted (Figure 4). Saos-2 cells were transfected with various deletion constructs containing the 5′ regulatory region of the *bcl*-2 promoter and either the empty vector control, with an expression plasmid encoding WT1-A. The fold induction of the *bcl*-2 promoter activity induced by WT1-A was determined by normalizing the basal promoter construct activity of cells co-transfected with the empty vector control. As shown in Figure 4, *bcl*-2 XH-, XX-, and XSa-LUC reporter constructs were all positively activated by WT1-A. However, both the SX- and StX-LUC reporter constructs displayed a loss of WT1-A-induced *bcl*-2 promoter activity. These results indicated that sites located between –2853 and –1765 were required for WT1 to positively regulate the *bcl*-2 promoter (Figure 4). Importantly, like WT1-A, transactivation of the *bcl*-2 promoter by WT1B was also localized to this same region in Saos-2 cells (data not shown). Although WT1 has been previously demonstrated to directly interact with DNA-binding elements located proximal to the P_1 promoter in the *bcl*-2 regulator region (Hewitt *et al*., 1995; Heckman *et al*., 1997), in our hands, WT1-A positively regulated the *bcl*-2 promoter through a unique region located 337 to 1088 nucleotides upstream of the major transcription start site (Figure 4).

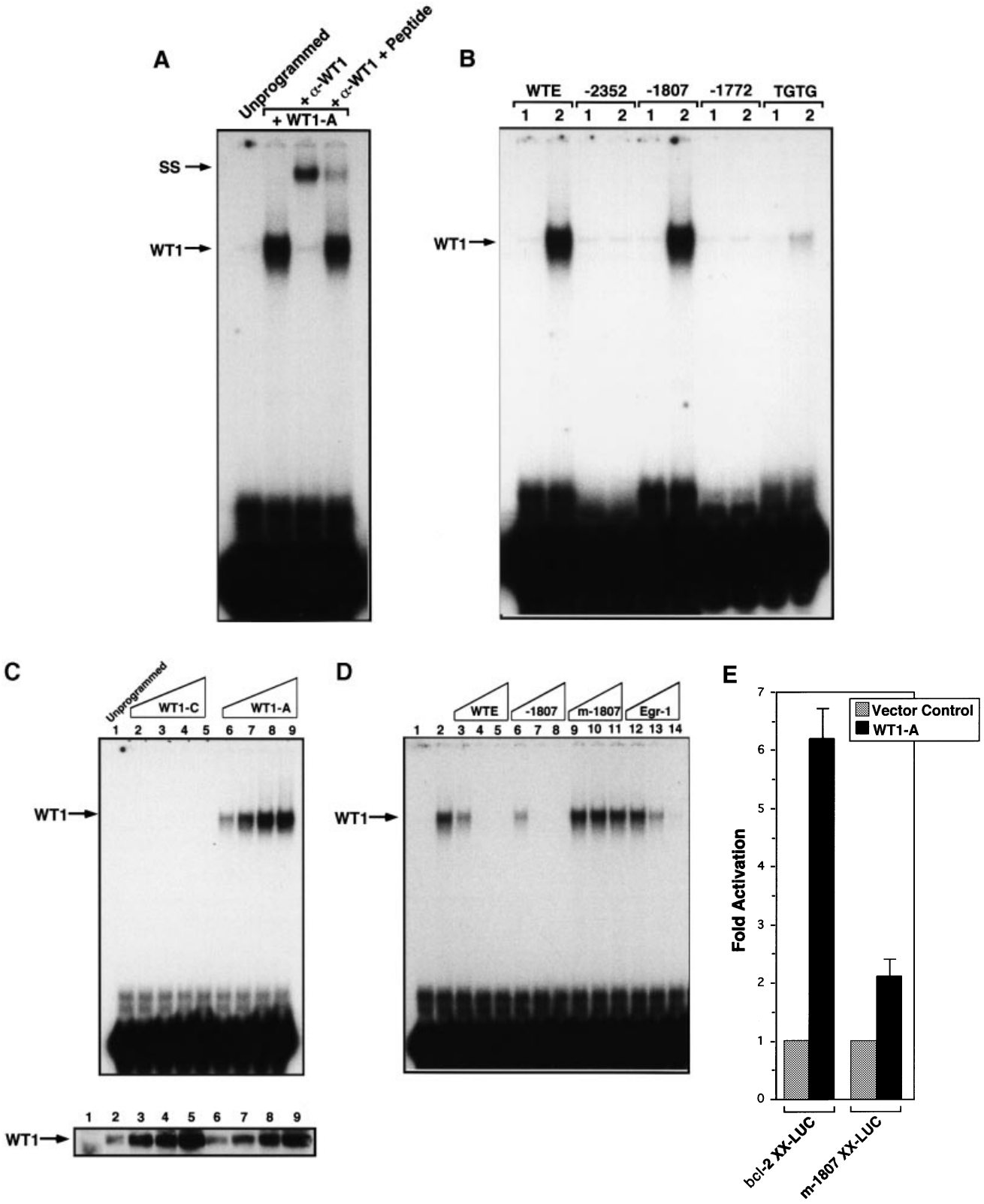
The bcl-2 promoter contains a high-affinity WT1 consensus site

To identify whether WT1 positively regulated the *bcl*-2 promoter through a direct interaction, the –2853 to –1765 base pair (bp) region was analyzed for potential WT1 DNA-binding sites. Because WT1-C and WT1-D isoforms display a different specificity for DNA base recognition than WT1-A and WT1-B, and because we have found that the expression of these two forms of WT1 was incapable of positively upregulating the *bcl*-2 promoter (Figure 3A and D), we focused primarily on DNA consensus sites which preferably bind WT1-A and WT1-B isoforms. Based on proposed WT1 zinc finger-DNA contacts for isoforms lacking KTS, a degenerative WT1 consensus site has been proposed $GCG^{T}/_{G}GGG^{A}/_{T}$ or $_{C}G^{T}/_{G}NN$ (Reddy and Licht, 1996). Using this sequence, three potential sites were identified in the *bcl*-2 promoter region encompassing the –2853 to –1765 bp region which were ⩾80% homologous to the degenerative consensus site. In addition, a TGTGTGTG-rich element, which has previously been demonstrated to bind WT1-A and WT1-B isoforms preferentially (Bickmore *et al*., 1992), was also identified within the –2018 to –2061 bp upstream of the *bcl*-2 translation initiation site.

To determine whether WT1 directly interacts with sites located within the *bcl*-2 promoter region, electromobility shift assays (EMSAs) were performed. As expected, *in vitro* translated WT1-A effectively bound the high affinity WT1 positive control element (WTE, 5′-GCG-TGGGAGT-3′; Nakagama *et al*., 1995), as determined by supershifting the DNA–protein complex with a WT1-specific antibody (Figure 5A). Moreover, the WT1 antibody was specific for the *in vitro* translated protein and was effectively inhibited with WT1-specific peptide (Figure 5A). As shown in Figure 5B, of the four potential WT1 DNA-binding sites, only the –1807 site (5′-GCG-TGGGTGT-3′), which displayed 100% identity with the degenerative consensus site, effectively bound WT1-A protein. Interestingly, the TGTGTGTG-rich element only weakly bound *in vitro* translated WT1-A protein (Figure 5B). To elucidate whether the –1807 site is preferentially recognized by WT1-isoforms which lack the KTS insert between zinc fingers 3 and 4, this element was analyzed for WT1-C and WT1-A DNA binding. Consistent with previous reports using the WTE site (Nakagama *et al*., 1995), the –1807 element was not recognized by the KTS-containing WT1-C isoform, while the WT1-A protein demonstrated a dose-dependent binding, as determined by EMSAs (Figure 5C).

To determine whether the –1807 site was a high affinity-binding site for WT1A, EMSAs were performed using

excess cold-competitor double-stranded oligonucleotide. As shown in Figure 5D, the –1807 site displayed similar affinity for the WT1 protein as the WTE site. However, a single nucleotide substitution (5′-GCGTAGGTGT-3′), which is predicted to alter the binding of the third zinc finger of WT1, completely abolished the ability of this double-stranded oligonucleotide to compete for WT1-A binding (Figure 5D). Although the Egr-1 consensus element (5′-GCGGGGGCG-3′) was also able to compete for WT1 DNA-binding to the –1807 site, the Egr-1 recognition sequence was less effective than either the WTE or the –1807 site (Figure 5D). Thus, these results indicate that the *bcl*-2 promoter region contains a high-affinity WT1 site, –1807, which strongly binds the WT1-A protein.



To elucidate whether the –1807 site is required for WT1-mediated regulation of the *bcl*-2 promoter, site-directed mutagenesis analysis was performed. As shown in Figure 5E, site-directed mutagenesis of the –1807 site resulted in a reduction in WT1-A-mediated *bcl*-2 promoter activity, compared with the wild-type *bcl*-2 XX-LUC reporter. Since WT1 has been previously reported to interact with the *bcl*-2 regulatory region through an additional element located near the P_1 promoter (Hewitt *et al*., 1995; Heckman *et al*., 1997), this site may account for the remaining *bcl*-2 promoter activity observed in the m-1807 XX-LUC reporter following WT1-A expression (Figure 5D). In our hands, this previously published site (5′-GCGCGGGAGG-3′) only weakly bound *in vitro* translated WT1-A protein in EMSAs and this oligonucleotide sequence was less efficient at competing for WT1-A binding than either the WTE or the –1807 site (data not shown). Collectively, our results indicate that WT1 protein is capable of directly interacting with the *bcl*-2 promoter region through the –1807 element. Transfection experiments confirm that this site significantly contributes to the ability of WT1 to positively transactivate the *bcl*-2 promoter region.

WT1 rescues cells from agents that kill by the induction of apoptosis

Although WT1 has been implicated in tumor suppression by virtue of its ability to inhibit expression of cell survival factors required for cell proliferation and for suppression of apoptosis (Rauscher, 1993; Reddy and Licht, 1996), we have found that WT1-A and WT1-B isoforms are capable of upregulating the *bcl*-2 proto-oncogene. Based on our studies, we were interested in determining whether the expression of WT1 protein would alter cellular sensitivity to apoptotic stimuli. To address this question, WT1-B.2, WT1-C.1 and G401-Neo cells were subjected to cytotoxic concentrations of staurosporine, doxorubicine and vincristine, and cell viabilities were analyzed. These agents were selected because they are known to kill cells by the induction of apoptosis, and because Bcl-2 has been shown to confer cellular resistance to programmed cell death induced by these compounds (reviewed in Reed, 1995; Decaudin *et al*., 1998). Moreover, doxorubicine and vincristine are used to treat post-operative Wilms' tumors (D'Angio *et al*., 1989).

As shown in Figure 6A, G401-Neo and WT1-C.1 cells displayed a loss of cell viability following the addition of staurosporine, doxorubicine or vincristine. In contrast, WT1-B.2 cells were resistant to cell death induced by these genotoxic agents (Figure 6A). To elucidate whether the loss of cell viability was due to the induction of apoptosis, cells were treated with staurosporine and cell morphologies were analyzed by phase-contrast microscopy. As shown in Figure 6B, WT1-C.1 and G401-Neo cells displayed hallmark characteristics of apoptosis, including membrane blebbing, nuclear condensation, and retraction of cellular processes, following the addition of staurosporine. However, unlike these cell lines, no visible signs of apoptosis were observed in WT1-B.2 cells following the addition of staurosporine for 8 h (Figure 6B). To ensure that staurosporine was initiating cell killing through an apoptotic mechanism, cells were analyzed for the appearance of deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL). Although both G401-Neo and WT1-C.1 cell populations displayed numerous TUNEL-positive cells following staurosporine treatment, WT1-B.1 cells were resistant to staurosporine-induced apoptosis (Figure 6B). To determine the degree of resistance to staurosporine-induced apoptosis exhibited by WT1-B.2 cells, cells were treated with increasing concentrations of staurosporine and genomic DNA was isolated and analyzed for the presence of DNA fragmentation. Both G401-Neo and WT1-C.1 cells displayed DNA fragmentation patterns characteristic of apoptosis at very low concentrations of staurosporine (25–50 nM). In contrast, the WT1-B.2 cells were extremely resistant to staurosporine and failed to display fragmented DNA patterns even up to doses as high as 100 nM (Figures 6B). Cellular resistance to staurosporine-induced apoptosis was not due

Fig. 5. WT1-A interacts with the *bcl*-2 promoter through a high-affinity site. (**A**) *In vitro* translated WT1-A displays specific binding to the high-affinity WT1 site. EMSAs were performed by incubating a ^{32}P-labeled double-stranded DNA probe containing the high-affinity WT1 element (WTE) with either *in vitro* translated WT1-A protein, or unprogrammed reticulocyte lysate (unprogrammed), and DNA–protein complexes were resolved on a 5% non-denaturing polyacrylamide gel. In order to demonstrate specificity of the gel shift complex, DNA–protein complexes were incubated with either an anti-WT1-specific antibody (C-19), either alone or in combination with a WT1-specific immune peptide inhibitor, and antibody supershift experiments were performed. 'SS' indicates the position of the WT1 + antibody supershift complex. (**B**) WT1-A directly binds to a DNA element located within the *bcl*-2 promoter region. EMSAs were performed by incubating either unprogrammed reticulocyte lysate (lane 1) or WT1-A (lane 2) with ^{32}P-labeled double-stranded oligonucleotides corresponding to either the positive control WTE (Nakagama *et al*., 1995) or to four potential WT1 DNA-binding sites located within the *bcl*-2 promoter (–2352, –1807, –1772 and TGTG). DNA–protein complexes were resolved on a non-denaturing polyacrylamide gel. (**C**) WT1-A, but not WT1-C, displays dose-dependent DNA-binding to the –1807 element from the *bcl*-2 promoter. Unprogrammed reticulocyte lysate (6 μl, lane 1) and increasing concentrations (1, 2, 4 and 6 μl) of *in vitro* translated WT1-C (lanes 2–5) and WT1-A (lanes 6–9) were incubated with a ^{32}P-labeled double-stranded oligonucleotide corresponding to the –1807 site. DNA–protein complexes were resolved on a polyacrylamide gel and WT1 gel mobility shift complexes are shown. WT1 immunoblot analysis (lower panel) of unprogrammed reticulocyte lysate (lane 1) and increasing concentrations (1, 2, 4 and 6 μl per reaction) of *in vitro*-translated WT1-C (lanes 2–5) and WT1-A (lanes 6–9), where immunoreactive bands were detected using a WT1-specific primary antibody (C-19). *In vitro*-translated WT1-C and WT1-A protein both migrate at ~52 kDa following SDS–PAGE. (**D**) The –1807 site located within the *bcl*-2 promoter region binds WT1-A with high-affinity. Competition assays were performed by pre-incubating *in vitro* translated WT1-A protein with either no competitor (lane 2) or increasing concentrations (50-, 100- and 200-fold excess) of unlabeled, double-stranded DNA-binding elements, corresponding to WTE (lanes 3–5), –1807 (lanes 6–8), mutant –1807 (m-1807, lanes 9–11), or the EGR-1 site (lanes 12–14) before the addition of 40 000 c.p.m. of ^{32}P-labeled –1807 DNA probe. EMSA were performed as described above. (**E**) The –1807 element contributes to WT1-A-mediated *bcl*-2 promoter activity. Saos-2 cells were transiently co-transfected with the wild-type *bcl*-2 XX-LUC, site-directed mutant m-1807 XX-LUC (2 μg), and with either the empty vector control (pCMV) or with the WT1-A construct (1 μg). Cell extracts were harvested 48 h following transfection, and equal amounts of protein extracts were analyzed for luciferase activity. The results presented represent the mean ±SD of three independent experiments.

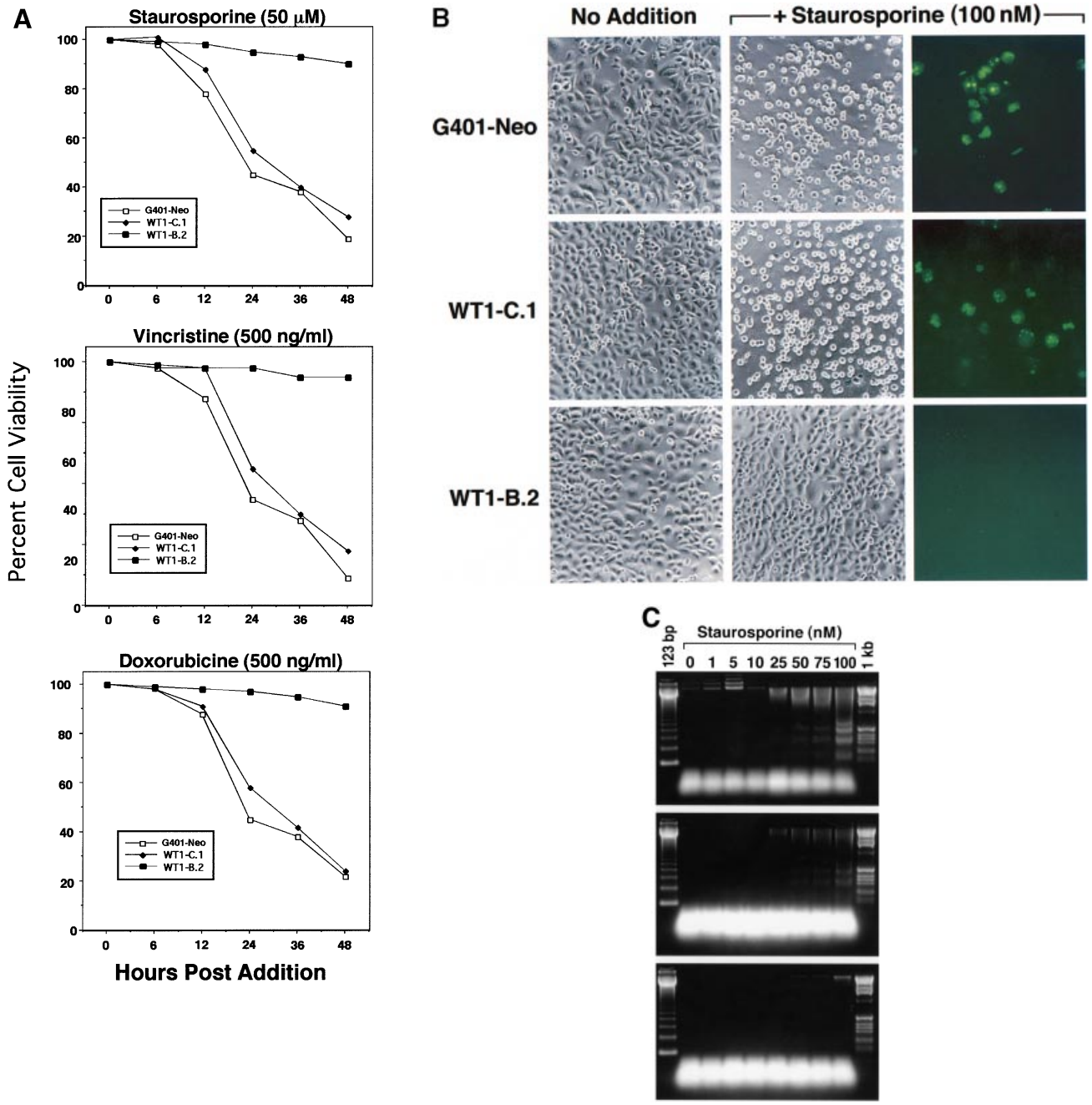


Fig. 6. WT1-B expression confers resistance to programmed cell death induced by genotoxic agents. (**A**) WT1-B expressing cells are resistant to genotoxic agents. G401-Neo, WT1-B.2, and WT1-C.1 cells were subjected to staurosporine (50 nM), vincristine (500 ng/ml) or doxorubicine (500 ng/ml). The percent cell viability was determined over the time course indicated by performing Trypan blue dye exclusion analysis. Data represented is one of three experiments all of which produced similar results. (**B**) Phase contrast micrographs (×20) and TUNEL staining of cells following staurosporine addition. G401-Neo, WT1-C.1, and WT1-B.2 cells were either left untreated or subjected to staurosporine (100 nM) for 8 h. After staurosporine addition cells where photographed and then fixed in 4% paraformaldehyde, and TUNEL staining was performed using the ApopTag reagent (Oncor). Non-adherent cells demonstrating signs of apoptosis appear as refractile by phase contrast and nuclear fragmentation is confirmed by the presence of TUNEL-positive cells. (**C**) WT1-B expressing cells display extreme resistance to DNA fragmentation induced by staurosporine. G401-Neo (top panel), WT1-C.1 (middle panel) and WT1-B.2 (bottom panel) cells were treated with increasing concentrations of staurosporine (1–100 nM). Eight hours post-staurosporine addition, cells were collected and DNAs were isolated and analyzed for the presence of nucleosomal length fragments on a 2% agarose gel. DNA markers (123 bp and 1 kb) are shown in the first and last lanes of each gel. The fast migrating EtBr-stained band located at the bottom of each lane is digested total RNA and demonstrates equal loading of nucleic acids.

to clonal variation, since two additional G401 clones expressing WT1-B also displayed cellular resistance to staurosporine (data not shown). Thus, expression of WT1-B in G401 cells confers significant resistance to cell killing induced by pro-apoptotic agents, such as staurosporine, doxorubicine or vincristine.

WT1-B-mediated resistance to apoptotic stimuli is associated with Bcl-2 expression

To further examine whether WT1-B expression correlated with both increased Bcl-2 expression and with increased resistance to apoptosis, subclones of WT1-B.2 cells were isolated which no longer expressed the WT1-B protein.

As shown in Figure 7A, these subclones failed to display elevated Bcl-2 protein levels compared with the parental WT1-B.2 cell line. Moreover, these subclones were sensitive to staurosporine-induced apoptosis, as detected by analyzing cell culture supernatants for the presence of histone-associated DNA fragments (Figure 7B). Therefore, these results support our findings that WT1-B is associated with elevated Bcl-2 protein expression and suggest that WT1-induced expression of the *bcl*-2 proto-oncogene may be responsible for the observed resistance to apoptosis-inducing agents.

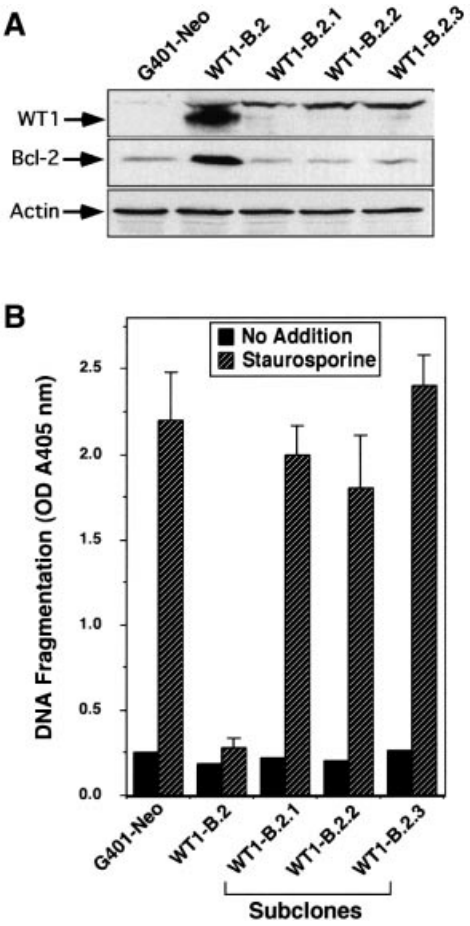


Fig. 7. WT1-B expression is associated with elevated Bcl-2 protein levels and heightened resistance to staurosporine-induced apoptosis. (**A**) Spontaneous loss of WT1-B expression correlates with a loss of Bcl-2 expression. Total protein extracts were isolated from G401-Neo, WT1B.2, and from three WT1-B.2 subclones (namely WT1-B.2.1, .2.2 and .2.3). Proteins (50 μg) were resolved on a 10% polyacrylamide gel, transferred to nitrocellulose, and analyzed for WT1, Bcl-2 and actin expression by Western blot analysis. (**B**) WT1-B subclones, which no longer express WT1, demonstrate a downregulation of Bcl-2 expression and are sensitive to staurosporine-induced apoptosis. G401-Neo, WT1-B.2 and WT1-B.2.1, WT1-B.2.2, and WT1-B.2.3 were either treated with staurosporine (100 nM) or left untreated. Eight hours after staurosporine addition, cellular supernatants were isolated and analyzed for the presence of histone-associated DNA fragments using the Cell Death Detection Elisa Plus (Boehringer Mannheim, Germany). Data represents the average ±SD of three experiments.

Discussion

WT1 exhibits both tumor suppressor and oncogenic properties

Although it has been demonstrated by numerous laboratories that WT1 overexpression functions to block growth of transformed cells *in vitro* and *in vivo* (Reddy and Licht, 1996), it is unclear whether endogenous WT1 functions as a tumor suppressor or as an oncogene in nephroblastomas. The high incidence of wild-type WT1 expression in sporadic Wilms' tumors strongly supports the idea that WT1 does not display growth inhibitory function, but instead may provide a cell survival advantage to tumors that express this transcription factor. This hypothesis is supported by the fact that wild-type WT1 is expressed not only in sporadic Wilms' tumors, but also in mesotheliomas and myeloid- and erythro-leukemias (Amin *et al*., 1995; Pritchard-Jones and King-Underwood, 1997). Consistent with this idea, WT1 has been demonstrated to be required to overcome apoptosis and potentiate proliferation in myeloid leukemia cell lines, suggesting that WT1 provides a cell survival function in tumor cells by overcoming pro-apoptotic signals (Algar *et al*., 1996). It is now well accepted that tumorigenesis is influenced by cellular responses to pro- and anti-apoptotic signals (Dragovich *et al*., 1998). This concept was first established when it was found that t(14;18) translocations in follicular small cleaved-cell non-Hodgkin's lymphomas led to the dysregulation of the *bcl*-2 gene. B-cells containing the t(14;18) translocation display a selective advantage relative to their normal counter parts, and clonally expand due to reduced cell death (reviewed in Yang and Korsmyer, 1996). This mechanism for clonal expansion is based on selective cell survival advantage where Bcl-2 contributes to neoplastic cell expansion by delaying or preventing apoptosis. Although resistance to pro-apoptotic stimuli is critical for tumor development, increased cellular resistance also plays an important role for cancer therapy (Fisher, 1994) (see Discussion).

Transcriptional regulation by WT1

WT1 is a transcription factor whose activity is modulated through protein–protein interactions involving both co-activators (Madden *et al*., 1993; Wang *et al*., 1995) and co-repressors (Jonstone *et al*., 1996). Therefore, the ability of WT1 to regulate transcriptional activation or repression may depend on the expression of secondary molecules that modulate WT1 activity. This notion is supported by observations by us and others that the levels of WT1 expression as well as the cellular background in which it is expressed determine whether WT1 functions as a transcriptional activator or a repressor of gene expression (Reddy and Licht, 1996). Consistent with these reports, we find that in some cell types, WT1 can repress transcription in transient transcription assays (Figure 3C).

Mounting evidence suggests that dysregulated WT1 expression in Wilms' tumors is associated with a loss of its transcriptional repressive properties. In contrast, WT1 expression in Wilms' tumors may act to positively upregulate gene targets that were once repressed by WT1 during kidney development. For example, the *Pax*-2 gene encodes a transcription factor that is required for normal kidney development (Ryan *et al*., 1995). It has been suggested that WT1 may repress transcription of the *Pax*-2 gene, because WT1 has been shown to repress this promoter in transient transfection assays, and because during kidney development an inverse correlation between the pattern

of *Pax*-2 and WT1 expression has been observed (Ryan *et al*., 1995). Despite this evidence, the lack of *Pax*-2 expression in WT1$^{-/-}$ knockout mice suggests that WT1 may positively upregulate *Pax*-2 rather than repress this gene target (Kreidberg *et al*., 1993). Consistent with our findings for *bcl*-2, *Pax*-2 gene expression is persistent in sporadic Wilms' tumors, suggesting that *Pax*-2 may be a transcriptional target of dysregulated WT1 expression in these nephroblastomas. In fact, many of the genes which have been reported to be repressed by WT1 in promoter transfection assays, including *IGF*-1R, *IGF*II, *Pax*-2 and *WT1* itself, are overexpressed in Wilms' tumors (Reeve *et al*., 1985; Scott *et al*., 1985; Fraizer *et al*., 1987; Werner *et al*., 1993; Reddy and Licht, 1996).

WT1 positively activates the bcl-2 promoter

Although WT1 has been shown to negatively regulate the promoter regions of many different genes (Haber and Housman, 1992; Rauscher, 1993; Reddy and Licht, 1996), very few genes have been demonstrated to be upregulated by this transcription factor (Wang *et al*., 1993b; Nichols *et al*., 1995; Cook *et al*., 1996; Kim *et al*., 1998). Other reports have shown that WT1 binds to and represses through GC-rich elements located near the P_1 promoter region of *bcl*-2 in HeLa cells (Hewitt *et al*., 1995; Heckman *et al*., 1997). Additional negative regulatory elements have also been identified in the *bcl*-2 5′-untranslated region which are probably independent of WT1 expression (Young and Korsmeyer, 1993). However, using a longer region of the *bcl*-2 promoter, we demonstrated here that a novel element located in the upstream regulatory region is critical for WT1-mediated transcription in Saos-2 cells. The WT1 regulatory element described in this study consists of a high-affinity WT1-binding site located 374 bps upstream of the major transcription initiation site of the *bcl*-2 promoter. To our knowledge, the –1807 site is the first naturally occurring high-affinity site located within a promoter region. In agreement with previous reports, this site displays a greater affinity for WT1 than the Egr-1 consensus site (Rauscher *et al*., 1990; Nakagama *et al*., 1995). Consistent with our data demonstrating that WT1-A and WT1-B transcriptionally upregulate the *bcl*-2 promoter, we find that *in vitro* translated WT1-A, but not WT1-C, effectively recognized the high-affinity WT1 site. The TGTGTG-rich region within the *bcl*-2 promoter only weakly bound *in vitro* translated WT1-A protein and did not appear to be important for WT1-mediated activity of the *bcl*-2 promoter in transient transfection reporter gene assays.

Both WT1 and Bcl-2 are required for kidney development

In normal mouse kidney development, WT1 is expressed for only a short period of time, from embryonic day (E) 9 to E16 (Pritchard-Jones *et al*., 1990; Buckler *et al*., 1991; Pelletier *et al*., 1991b; Armstrong *et al*., 1992). Interestingly, in agreement with the *bcl*-2 proto-oncogene being transcriptionally regulated by WT1, Bcl-2 protein is expressed maximally from E12.5 to E16.5 (Novack and Korsmeyer, 1994). Therefore, WT1 expression in the developing mouse kidney precedes Bcl-2 expression temporally, and correlates well with progenitor-type cells, which characteristically display resistance to apoptotic cues present in these developing tissue structures. Moreover, Bcl-2 expression, like WT1, is critical for kidney development. Compared with wild-type litter mates, Bcl-2$^{-/-}$ mice have smaller kidneys which contain fewer nephrons and display a greater susceptibility to apoptosis within blastemas at E11.5 and E12 (Novack and Korsemyer, 1994). Thus, like WT1$^{-/-}$ knockout mice, results for Bcl-2$^{-/-}$ animals support the idea that Bcl-2 expression is important in maintaining cell survival during inductive interactions between epithelium and mesenchyme in the embryonic kidney. Although homozygous loss of Bcl-2 does not result in embryonic lethality, the Bcl-2$^{-/-}$ animals develop polycystic kidney disease with marked dilation of proximal and distal tubules and collecting ducts which results in renal failure (LeBrun *et al*., 1993; Veis *et al*., 1993; Nakayama *et al*., 1994; Novack and Korsemyer, 1994; Sorenson *et al*., 1995). Interestingly, patients suffering from Wilms' tumor, which display loss of WT1 due to chromosomal deletion or mutation, characteristically present with dysplastic kidneys and renal failure (Kissane and Dehner, 1992; Fernbach and Feinstein, 1995; Homsy *et al*., 1997).

WT1 expression modulates programmed cell death

Despite the fact that the pro-apoptotic function of WT1 has been associated with the transcriptional repression of growth factors and growth factor receptor genes (Englert *et al*., 1995), most sporadic Wilms' tumors persistently express high levels of WT1 and, in turn, late-stage tumors can display extreme resistance to programmed cell death (Beckwith, 1996; Faria *et al*., 1996). Although WT1 has been shown to modulate programmed cell death by inhibiting p53-dependent apoptosis (Maheswaran *et al*., 1995), expression of WT1-B did not alter the levels of p53 expression in our cell lines. These results would suggest that in our system WT1-induced resistance to apoptosis was unlikely to be due to the ability of WT1 to inhibit p53 tumor suppressor function. Instead, our studies suggest that WT1 can inhibit staurosporine-, vincristine- or doxorubicine-induced programmed cell death by transcriptionally upregulating the *bcl*-2 proto-oncogene. The ability of WT1 to suppress apoptosis in response to vincristine and doxorubicine *in vitro* may be clinically significant, since combinations of these two agents are the chemotherapeutic drugs of choice when treating post-operative Wilms' tumors (D'Angio *et al*., 1989). The survival rate for patients suffering from Wilms' tumors, like most cancers, is dependent on early detection and is based on the stage of tumor progression (Beckwith, 1996). Although mean survival for patients diagnosed with stages I and II is extremely good, late stage tumors that exhibit hematogenous metastases to the abdomen and lung have markedly reduced survival rates (D'Angio *et al*., 1989).

Stage IV tumors which display diffuse anaplasia, a histological marker of adverse prognosis for Wilms' tumors, have increased resistance to therapy (Beckwith, 1996; Faria, 1996). Importantly, this histological subtype commonly displays mutations within the *p53* allele, and this loss of functional p53 tumor suppressor protein has been proposed to contribute to chemoresistance displayed by anaplastic Wilms' tumors (Bardeesy *et al*., 1995). This raises an important point with regard to our studies. Although our data indicates that WT1-B expression correl-

ates well with increased Bcl-2 expression and subsequent resistance to apoptosis, it is important to point out that in our system we are already starting out with a fully transformed and tumorigenic cell line. Thus, our results may not reflect the temporal series of mutational events that occur *in vivo* during the course of Wilms' tumor development. Therefore, it will be important to determine the pattern of WT1 and Bcl-2 expression in Wilms' tumors and to determine whether their expression correlates with chemotherapy-induced response rates. For example, it is possible that WT1-mediated Bcl-2 upregulation is an early event and might play a secondary role to p53 mutational events which develop later during tumor progression. Thus, combined WT1-regulated increases in Bcl-2 and the loss of p53 function may culminate in chemoresistance observed in anaplastic Wilms' tumors. In conclusion, our studies suggest that WT1 may potentiate oncogenesis and tumor progression by transcriptionally upregulating the *bcl*-2 proto-oncogene, and that this mechanism may contribute to the heightened resistance to chemotherapy-induced apoptosis in diffuse anaplasia Wilms' tumors.

Materials and methods

Cell culture

The rhabdoid (G401), osteosarcoma (Saos-2), human cervical carcinoma (HeLa), human neuroblastoma (Lan-5), and African green monkey kidney (CV-1) cell lines were all grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. The stable G401 clones used in this manuscript, G401, G401-Neo, WT1-B.1, WT1-B.2, WT1-B.3, WT1-C.1, WT1-C.2 and WT1-C.3, have been described previously as G4016TGc6, WTF 116.7A, 116.13A, 116.14B, 116.15B, 116.26A, 116.26B and 116.58B, respectively (McMasters *et al*., 1995). G401 stable clones were maintained as described (McMasters *et al*., 1995). Cell viabilities were determined by seeding 3×10^5 cells per 35 mm plate 18 h before the addition of staurosporine (50 nM), vincristine (500 ng/ml) or doxorubicin (500 ng/ml), adherent and non-adherent cells were collected over the time course indicated, and cells were stained using Trypan blue reagent (Sigma, St Louis, MO).

Western and Northern blot analysis

Western blot analysis and protein concentrations were performed as described previously (Mayo *et al*., 1997). Nine randomly selected snap-frozen sporadic Wilms' tumors were desegregated in liquid nitrogen using a pestle and mortar and cells were lysed using RIPA buffer. Total proteins (50 μg/lane) were resolved on a 10% SDS–polyacrylamide gel, and transferred to nitrocellulose membranes. WT1, Bcl-2 and actin proteins were analyzed using a 1:1000 dilution of rabbit polyclonal WT(C-19), mouse monoclonal Bcl-2(100) or goat polyclonal actin(I-19) obtained from Santa Cruz Biotechnology, Santa Cruz, CA. HRP-labeled anti-rabbit, anti-mouse (Promega, Madison, WI) and anti-goat (Upstate Biotechnology, Lake Placid, NY) secondary antibodies were used at 1:8000 dilution, and were detected using the enhanced chemiluminescence (ECL) system (Amersham Life Science Inc., Gaithersburg, MD). Total RNAs were isolated from subconfluent Lan-5, G401-Neo, and G401 subclones expressing either WT1-B or WT1-C using TRIzol (Life Technologies Inc., Arlington Heights, IL). RNAs (10 μg) were resolved on a 0.8% formaldehyde agarose gel and transferred to Protran (Schleicher & Schuell, Dassel, Germany). Transcripts encoding *bcl*-2 were detected using a ^{32}P-labeled random primed probe derived from either the human *bcl*-2 or from β-actin cDNAs, and hybridizations were carried out in Quickhyb (Stratagene, La Jolla, CA). Membranes were washed to remove non-specific radiolabeled probe and blots were subjected to autoradiography.

Construction of reporter plasmids

The 2.8 kb sequence encompassing the *bcl*-2 promoter region (Miyashita *et al*., 1994), was subcloned into the multiple cloning site of the pGL2-Basic plasmid (Stratagene), containing the firefly luciferase gene. This was accomplished by performing a partial *Xho*I–*Hin*dIII digest and subcloning the 2855 bp fragment into compatible sites within the pGL2-basic reporter, creating the *bcl*-2 XH-LUC construct. The XX-LUC construct was made by subcloning the 2100 bp *Xho*I fragment into pGL2-basic and selecting for subclones in the 5′→3′ orientation. The XSa construct was created by digesting the XX-LUC construct with *Sac*I, blunt ending the site using Klenow enzyme, digesting with *Xho*I, and directionally subcloning the *Xho*I–*Sac*I/blunt 1563 bp fragment into pGL2-Basic containing a blunted *Hin*dIII site and a sticky *Xho*I overhang. The SX-LUC reporter was generated by digesting the XX-LUC construct with *Sma*I, gel purifying the reporter fragment and re-ligating, thus preserving the *Sma*I–*Xho*I 877 bp sequence. The StX-LUC reporter was made by digesting the XX-LUC reporter with *Stu*I and *Nhe*I, gel purifying the reporter fragment, blunt ending the *Nhe*I restriction site using the Klenow enzyme, and re-ligating the plasmid. The *bcl*-2 promoter sequences containing a site-directed mutation within the –1807 WT1 high affinity site (m-1807 XX-LUC) was created by PCR amplificating a 73 bp *Stu*I fragment using a 5′ primer (5′-GCCTGAGCAGAAGGCCCCGCGC**ACACCT*ACGC**-3′) and a 3′ primer (5′-AGTGGGTGGCGCGGGCGGCACAGGCCTCCCCGCGG-GCCCCGC**GCGTA*GGTGT**-3′). The 5′ primer spanned across the –1807 element and was engineered with a mutation which destroyed the WT1 DNA-binding motif and converting it to a mutant –1807 site (5′-GCGTAGGTGT-3′). The PCR amplified *Stu*I fragment containing the site-directed m-1807 site was subcloned in to *Stu*I-digested and gel-purified XX-LUC reporter to create the m-1087 XX-LUC reporter. All reporter plasmids were confirmed by performing restriction digests and nucleotide sequence analysis.

Luciferase and β-galactosidase assays

Subconfluent cells were transfected using the lipofectamine procedure (Life Technologies Inc.). Unless otherwise stated, cells were transfected with 2 μg of luciferase reporter and with 0.2 μg of pCMV-Lac Z (pcDNA 3.1 His/Lac Z, Invitrogen) alone, or with 1 μg of either WT1 expression plasmid, or the vector control (pCMV). Cells were lysed in 0.25 M Tris (pH 7.6) and 100 μg of protein was analyzed for luciferase activity as described (Ausubel *et al*., 1989). Additionally, cell extracts were standardized with respect to β-galactosidase activity, which served as both a transfection efficiency and cell survival control. β-galactosidase expression levels were determined by analyzing either soluble β-galactosidase activity in cell lysates using the β-galactosidase Enzyme Assay System (Promega) or by performing X-gal staining on 0.5% gluteraldehyde fixed cells as described previously (Mayo *et al*., 1997). All experiments were performed in duplicate and repeated at least three times.

Electrophoretic mobility shift assays

WT1-A and WT1-C proteins used in EMSAs were translated from pSP6-based vectors using TNT rabbit reticulocyte lysate system (Promega). The pGEM-WT1A and pGEM-WT1C plasmids have been described previously (Madden *et al*., 1991; Kim *et al*., 1998). The unprogrammed reticulocyte lysate control is the reaction product generated after the addition of the pGEM-7Z empty vector. For EMSAs, WT1-A and WT1-C were translated as cold proteins, and protein concentrations were approximated by performing dual reticulocyte lysate reactions in the presence of [^{35}S]methionine, resolving proteins on an SDS–polyacrylamide gel, and comparing expression levels to a known [^{35}S]methionine-labeled standard. Synthetic DNA oligomers used for DNA-binding analysis are as follows: the high-affinity WTE element (Nakagama *et al*., 1995) 5′-GCGTGGGAGT-3′; and elements present in the *bcl*-2 promoter including: –2352 (5′-GCGTGCGTGT-3′), –1807 (5′-GCGTGG-GTGT-3′) and –1772 (5′-GCCGCGGGCG-3′). Oligonucleotides were engineered so that the complementary strands leaves a 4 bp *Sal*I overhang. The TGTGTG-rich element was generated by hybridizing a 23 bp element (5′-GACACACACACACACACACACACACACT-3′) to a 45 bp element (5′-TGTGAGTGTGTGTGGAGTGTGTGTGTG-TGTGTGTGTGTGTGTGTC-3′). To generate radiolabeled probes for EMSAs, annealed oligonucleotides were filled-in using [α-^{32}P]dCTP and Klenow. Binding reactions were prepared by combining *in vitro* translated WT1 proteins with 5 mM HEPES pH 7.9, 10% glycerol, 25 mM KCl, 0.05 mM EDTA, 1 μg of poly[d(I-C)] (Sigma) and 40 000 c.p.m. of radiolabeled probe (~0.05 ng) for 15 min at room temperature. For antibody supershift assays and competition experiments, reaction mixtures containing *in vitro* translated WT1 protein were pre-incubated (10 min at room temperature) with either 1 μg of WT1-specific antiserum (WT-19, Santa Cruz), 1 μg of WT1 antiserum plus 1 μg of peptide inhibitor, or with non-radioactive double stranded oligonucleotides (50-, 100- or 200-fold molar excess) before the addition of the radiolabeled gel shift probe. DNA–protein complexes were resolved on a 5%

non-denaturing (30:1) acrylamide to bis-acrylamide gel in 1× TGE at a 20 mA.

TUNEL analysis and DNA fragmentation

G401-Neo, WT1-B.2 and WT1-C.1 cells were plated onto 2×2 cm coverslips at 1×10^5 cells per 6-well plate. Eighteen hours later, cells were treated with 100 nM staurosporine. Ten hours post-staurosporine addition, cells were fixed with 4% paraformaldehyde for 20 min, washed twice with PBS and TUNEL analysis was performed according to the manufacturer's recommendations (Oncor, Gaithersberg, MD). Incorporation of dUTP was detected using a fluorescein-conjugated anti-digoxigenin antibody and visualized by fluorescent microscopy. Apoptosis-induced genomic DNA fragments were analyzed as described previously (Sandstrom and Buttke, 1993). G401-Neo, WT1-B.2 and WT1-C.1 cells were seeded at 1×10^7 cells per 100 mm dish and 18 h later cells were treated with increasing concentrations of staurosporine (0, 1, 5, 10, 25, 50, 75, and 100 nM). Eight hours post-staurosporine addition cells were harvested, and DNAs were prepared and resolved on a 2.0% agarose gel containing ethidium bromide.

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