Transcriptional regulatory mechanisms in IGF-I receptor gene expression

by

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Abstract

The insulin-like growth factor-I receptor (IGF-IR) mediates the majority of the biological actions of IGF-I and IGF-II, which include cellular proliferation, differentiation, and tumorigenesis. Since cell-surface receptor number is consistently correlated with receptor gene expression, transcriptional control of IGF-IR gene expression is a critical part of overall IGF action. Previously, the WT1 and Sp1 transcription factors have been shown to modulate IGF-IR gene expression. WT1 function in particular is complex due to the existence of four naturally occurring splice variants resulting from two alternative splice sites (exon 5 and a three amino acid KTS insertion between zinc fingers 3 and 4 that results from the use of an alternative splice donor site at the 3' end of exon 9) and RNA editing in exon 6. Additionally, there are a number of clinically relevant mutations. The existence of overlapping and adjacent recognition sites for WT1 and Sp1 in the IGF-IR promoter sequence implies that these factors may functionally and physically interact.

This thesis addresses three specific aspects of WT1 function in the context of IGF-IR gene regulation. 1, The differential activity of WT1 splicing and RNA-edited variants and mutations with respect to IGF-IR promoter activity in transient transfection assays. 2, The self-association of wild-type and mutant WT1 amino-termini in the yeast two-hybrid system. 3, The domain requirements for Sp1 activation of the IGF-IR gene promoter and functional and physical interactions of Sp1 and WT1.

Transient cotransfection of WT1 splice variant expression vectors and IGF-IR promoter-luciferase reporter constructs in CHO cells demonstrated that WT1 repression of the IGF-IR promoter is strongly influenced by the KTS insertion. WT1 proteins lacking the KTS insertion, regardless of the presence or absence of the exon 5 sequence, repressed stronger than WT1 proteins containing the KTS insertion. The amino- and carboxy-terminal domains of WT1 also exhibited partial repression activity, consistent with a requirement for both DNA binding and protein-protein association for maximum repression by WT1. In addition, the RNA-edited form of WT1, as well as versions lacking the major proline-rich domain or carrying a WAGR syndrome-associated point mutation in exon 3, were unimpaired in their transcriptional repression activity.

The self-association capability of different version of the WT1 proteins was addressed using the yeast two-hybrid system. Although wild-type dimers demonstrated self-association, homodimers of the RNA-edited form of WT1 as well the proline-rich domain deleted and the WAGR-associated mutations exhibited severely disrupted oligomerization capability as assessed with two reporter genes, β-galactosidase and histidine synthase.

The potential functional interaction of WT1 and Sp1 in IGF-IR promoter regulation was addressed using reporter gene assays and transient cotransfection of expression plasmids encoding these factors. Transient transfection of Sp1 deletion constructs in Sp1-deficient Drosophila Schneider cells localized Sp1 domains required for IGF-IR activation. The carboxy-terminal D domain and the amino-terminal glutamine and serine/threonine-rich domains are critical for Sp1 activation of the IGF-IR promoter. In addition, a region between the second glutamine-rich domain and the zinc-finger domain, the C domain, apparently functions as a transcriptional inhibitory domain. Cotransfection of WT1 and Sp1 resulted in WT1 attenuation of Sp1 activation of the IGF-IR promoter. The possible physical interaction of WT1 and Sp1 was examined using yeast two-hybrid assays and coimmunoprecipitation. The yeast two-hybrid assays failed to detect any direct association between WT1 and Sp1. However, coimmunoprecipitation of Sp1 with WT1 was observed, with only the unphosphorylated form of Sp1 being immunoprecipitated with WT1.

In summary, these data indicate that 1, the differential activity of WT1 splice variants in IGF-IR promoter repression is due primarily to the KTS insertion between zinc fingers 3 and 4, which disrupts DNA binding. 2, RNA editing, deletion of the major proline-rich region, or introduction of a WAGR syndrome-associated point mutation in exon 3 impaired self-association, but did not affect transcriptional repression, suggesting that WT1 self-association is not required for WT1 transcriptional regulatory activity. 3, WT1 and Sp1 functionally and potentially physically interact. Thus, a major mechanism through which WT1 effects transcriptional repression may involve attenuation of Sp1 activation.

Introduction

1. IGF systems

The insulin-like growth factor(s) were first discovered as molecules that mediated the stimulation of cartilage growth by growth hormone (GH) in an *in vitro* assay system and originally termed sulfation factor (Salmon and Daughaday, 1957). The biological significance of sulfation factor was expanded to stimulation of DNA synthesis (Daughaday and Reeder, 1966), proteoglycan synthesis (Hall and Uthne, 1971), and protein synthesis (Salmon and DuVall, 1970). Due to this broad range of biological functions, the sulfation factor was renamed somatomedin C (Daughaday et al., 1972). During the period in which characterization of the biological activities of the sulfation factor was done, a study was begun to identify factors in serum that could stimulate insulin-like effects (Froesch et al., 1966). This factor, termed nonsuppressible insulin-like activity (NSILA), was thought to be distinct from insulin since it exhibited effects that were not inhibited by an anti-insulin antibody. Although initial purification attempts of both sulfation factor and NSILA were not successful, the highly purified factors stimulated glucose incorporation into fat and sulfate incorporation into cartilage (Froesch et al., 1976). These findings suggested that these molecules were identical or very similar to each other. In 1976, the amino acid sequence of NSILA was determined and it was shown that the protein had 48% similarity to pro-insulin. From this evidence, one of the NSILAs was renamed insulin-like growth factor-I (IGF-I) (Rinderknecht and Humbel, 1978a). Subsequently, another bioreactive NSILA molecule was identified and termed insulin-like growth factor-II (IGF-II) (Rinderknecht and Humbel, 1978b). Later, somatomedin C was purified from human plasma and its amino acid sequence confirmed that somatomedin C was, in fact, identical to IGF-I (Svoboda et al., 1980).

Biological studies based upon tissue extracts moved to utilizing cultured cells in the late 1970s. The tissue culture system and highly purified ligands made ligand-receptor binding studies possible . Since insulin binding to the insulin receptor had been characterized, the possibility of IGF-I binding to insulin receptor were assessed (Hintz et al., 1972). Although IGF-I could bind to the insulin receptor, it was shown that iodinated IGF-I ligand binds to a receptor that is distinct from the insulin receptor (Marshall et al., 1974). Also, a novel receptor appeared to bind to IGF-II with higher affinity than IGF-I and not to insulin at all (Rechler et al., 1980). This evidence suggested an independent receptor for each IGF. Later, cross-linking studies demonstrated differences of the molecular sizes of the IGF-I, -II, and insulin receptors, further supporting the existence of three different receptors for these factors. (Massague and Czech, 1982). Subsequently

cloning of the insulin (Ebina et al., 1985) , the IGF-I (Ullrich et al., 1986), and IGF-II receptors (Morgan et al., 1987) were demonstrated.

During the last decade, IGF systems have been one of the major areas in growth factor research. The biological actions of the IGFs have been expanded to cell cycle regulation, cell proliferation and differentiation, and cell death. The advent of molecular techniques allowed the cloning not only of IGF ligands and their receptors, but more recently of the IGF-binding proteins (IGFBPs). Six IGFBPs have been cloned and sequenced. The physiological roles of IGFBPs are broad; to act as transporters for IGFs in plasma, to prolong the half lives of IGFs, to localize IGFs in a tissue- and cell-specific manner, to change IGF binding affinity for their receptors and modulate IGF actions, and to function independently (Jones and Clemmons, 1995).

(1). Structural features of IGF molecules and receptors

(a). IGF-I and -II

IGF-I is a single-chain polypeptide consisting of 70 amino acids and has a significant homology to proinsulin (Rinderknecht and Humbel, 1978a). IGF-II is also a singular chain polypeptide that contains 67 amino acid residues (Rinderknecht and Humbel, 1978b). Primary sequences of the IGFs shows 70 % identity to one another and 50 % homology to the A and B domains of human insulin. This structural similarity is conserved in various vertebrate species.

(b). IGF-I receptor (IGF-IR)

The IGF-IR is a heterotetrameric complex composed of two extracellular α subunits and two membrane-spanning β subunits. Those subunits are covalently bound through disulfide bonds between two α subunits and the α and β subunits. The IGF-IR is synthesized as a single preproprotein containing 1367 amino acids. After removal of the first 30 amino acids as a signal peptide and posttranscriptional modification through glycosylation, the IGF-IR is cleaved at amino acids 707-710 (consisting of Arg-Lys-Arg-Arg basic amino acids) to produce α (706 amino acids) and β (627 amino acids) subunits (Ullrich et al., 1986). The α subunit contains a cysteine-rich ligand binding domain and binds IGF-I with a dissociation constant (K_d) of 0.2-1nM (Steele-Perkins et al., 1988). IGF-II and insulin also bind to the IGF-IR, but with several-fold and more than 100-fold lower affinities, respectively (Germain-Lee et al., 1992 and Steele-Perkins et al., 1988). The β subunit anchors the receptor in the cell membrane and has a tyrosine kinase domain in its cytoplasmic region, which shares 84 % identity with insulin receptor kinase domain (Czech, 1989). Ligand binding induces conformation changes in the receptor and

autophosphorylation, resulting in activation of the intrinsic tyrosine kinase and trans-autophosphorylation of several tyrosine residues in the β subunit. Through those phosphorylated tyrosines, the receptor recruits various substrates and adaptor/docking molecules and triggers downstream signal transduction cascades leading to physiological effects.

(C). IGF-II receptor (IGF-IIR)

The IGF-IIR is identical to the mannose-6-phosphate receptor (Kornfeld, 1992). Structurally, the IGF-IIR is considerably distinct from IGF-IR and the insulin receptor. the IGF-IIR is a single polypeptide chain (2451 amino acids) and divided into three regions; a large extracellular domain (more than 90% of the size), a transmembrane domain, and a small cytoplasmic domain (Morgan et al., 1987). The IGF-IIR contains 15 contiguous segments that maintain 8 cysteine residues at corresponding positions in the extracellular region, in which there are two mannose-6-phosphate binding sites (Repeat motifs 1-3 and 7-9) and an IGF-II binding site (Repeat motif 11) (MacDonald et al., 1988). Unlike other growth factor receptors, the IGF-IIR lacks an obvious catalytic activity in its cytoplasmic domain. However, the intracellular domain is thought to be involved in endocytosis (Lobel et al., 1989) and lysozomal enzyme sorting into subcellular compartments (Johnson and Kornfeld, 1992). The predominant function of the IGF-IIR in IGF action is to act as a clearance receptor for extracellular IGF-II (Wang et al., 1994 and Lau et al., 1994).

(2). Signaling pathways of IGF systems

IGF molecules mainly utilize the IGF-IR as a signal transducer to elicit their biological actions (Werner, 1991). Upon ligand binding, the IGF-IR is autophosphorylated on tyrosine residues 1131, 1135, and 1136 in its tyrosine kinase domain and the intrinsic tyrosine kinase is activated (Gronborg et al., 1993 and Kato et al., 1994). Several tyrosine residues are subsequently phosphorylated within the juxtamembrane and the carboxy-terminal domains and play a critical role in IGF-IR activity. Tyrosine 950 provides a core binding site for insulin receptor substrate-1 (IRS-1) (Miura et al., 1995 and Yamasaki et al., 1992) and ras GTPase-activating protein (GAP) (Seely et al., 1995). Tyrosine 1316 in the carboxy-terminal end is involved in the binding of p85, the PI-3 kinase regulatory subunit, and the SH-PTP2 tyrosine phosphatase (Blakesley et al., 1996 and Seely et al., 1995).

IRS-1 is a major substrate protein for IGF-IR. IRS-1 is recruited to the receptor upon ligand stimulation and tyrosine phosphorylated at multiple sites. Those tyrosine residues are in YMXM motifs or a related context, which are targets for src homology-2

(SH2) domain-containing proteins. The phosphorylated tyrosine residues then provide target sites for SH2 proteins, including the PI-3 kinase regulatory subunit (p85), the SH-PTP2 protein tyrosine phosphatase (Sugimoto et al., 1994), and adaptor proteins Nck and Grb2. The IRS-1 adaptor molecule, then, mediates divergent IGF signals by recruiting and activating downstream molecules.

Another SH2-containing docking protein, Shc, is also phosphorylated by the IGF-IR (Pronk et al., 1993). Shc is a substrate for other receptor tyrosine kinases, including the epidermal growth factor receptor (EGFR) and the platelet-derived growth factor receptor (PDGFR) (Pelicci et al., 1992 and Yokote et al., 1994). From the sequence motif, YMXM, surrounding the phosphotyrosine residues, the SH2 domain of Shc could potentially bind to IGF-IR. However, no binding site for Shc has been identified (Sasaoka et al., 1994).

In IGF-IR signaling, multiple phosphorylated tyrosines have distinct functions, leading to differential downstream signaling. This signaling involves, at least, the activation of the MAP kinase cascade through Grb2, the PI3-kinase cascade and AKT/PKB, and effects downstream of the SH-PTP2 phosphatase.

(3). Physiological functions

(a). Cell cycle progression

The important roles of IGFs and other growth factors in cell cycle progression come from studies using mouse fibroblasts. Quiescent cells in G0 are induced into the G1 phase by so-called competence factors, which include PDGF and basic fibroblast growth factor (bFGF or FGF-2). However, either IGF-I or EGF is required for subsequent progression into S phase and through the cell cycle (Baserga and Rubin, 1993 and Pardee, 1989). Introduction of the proto-oncogene c-myb, SV40 T-antigen, and EGF overexpression bypasses the requirement for IGF-I, presumably by the induction of endogenous IGF expression (Pietrzkowski et al., 1992, Porcu et al., 1992, and Travali et al., 1991). The major role of PDGF as a competence factor is thought to induce IGF-I expression, and the establishment of an autocrine loop involving IGF-I and IGF-IR obviates the competence factor requirement in BALB/c-3T3 cells (Baserga and Rubin, 1993 and Pietrzkowski et al., 1992). Recent evidence shows that in IGF-IR-overexpressing BALB/c-3T3 cells, another competence factor, bFGF, causes tyrosine phosphorylation of the IGF-IR β subunit chain by an unknown mechanism, and abrogates the IGF-I requirement (Yoshinouchi et al., 1993). Also, IGF-IR overexpression reduces competence factor requirement in other cell lines, including hematopoietic cells (McCubrey et al., 1991) and NIH 3T3 cells (Kaleko et al., 1990).

(b). Cell growth

The role of the IGF system in cell cycle progression correlates with its function in somatic cell growth. It has been reported that IGF-I and -II potentiate cellular proliferation in many tissues and cells (Stewart and Rotwein, 1996). Studies from pituitary-deficient rats treated with IGF-I and transgenic mice expressing human IGF-I peptide demonstrated enhanced body growth and proportional size increases of various organs. IGF also promotes these organs' growth rates. Effects of IGF systems in fetal and embryonic growth come from studies of transgenic mice with homozygously disrupted IGF-I and -II genes. Those knock-out mice lacking combinations of IGF-I, -II, and their receptors show dwarfism and postnatal death, in most of the cases, from respiratory failure and muscle hypoplasia (Baker et al., 1993 and Liu et al., 1993a).

(c). Development and differentiation

The role of IGF action in differentiation has been demonstrated in many tissues, including skeletal muscle, skin, bone, and the nervous system (Stewart and Rotwein, 1996). Transgenic mice lacking IGF-I, -II, or IGF-IR exhibit muscle dysgenesis, skin hypotrophy, delay of bone ossification, and reduced number of non-neuronal cells and precursors of oligodendrocytes. Overexpression of IGF-I in mice results in myofiber hypertrophy and restoration of impaired long bone growth in GH-deficient mice (Schoenle et al., 1982). IGF-I also influences viability and differentiation of neuronal cells. In addition, overexpression of IGF-II causes skin and hair hypertrophy (Ward et al., 1994).

(d). Cell death

IGF action in apoptosis establishes IGF-I as a anti-apoptotic agent. The capability of IGFs to inhibit cell death has been well studied in hematopoietic cells. In human erythroid cells, IGF-I is able to inhibit DNA fragmentation and apoptosis caused by serum deprivation (Muta and Krantz, 1993). Also, IGF-I prevents apoptosis in the IL-3-dependent BAF-3 murine lymphocyte line. (Rodriguez et al., 1992). In addition, the c-Myc-induced apoptosis seen in serum-deprived fibroblasts is prevented by IGF-I (Harrington et al., 1994). This evidence supports a function for IGF in the prevention of cell death.

(4). Implications of IGF-IR in tumorigenesis

The IGF-IR is implicated in cellular transformation and tumorigenesis. In the last decade, many growth factor receptors have been identified as cellular homologues of oncogenes, including the v-erb/EGF receptor, the trk/NGF receptor, and the met/HGF receptor. The notion that aberrant expression of growth factor receptors could be a factor in cellular transformation is now well-accepted. In fact, in the case of IGF-IR, a high level of expression is observed in many tumor cell types, including lung, breast, pancreas, kidney, thyroid, and other cancers (Macaulay, 1992 and Werner and LeRoith, 1996). Therefore, much effort has focused on elucidating the role of the IGF-IR in cellular proliferation.

Many studies addressing the relationship between IGF-IR and tumorigenesis have been reported. Overexpression of IGF-IR showed that ligand-dependent transformation in NIH 3T3 and Rat-1 fibroblasts as shown by colony formation in soft agar and formation of tumors in nude mice (Kaleko et al., 1990). The transforming capability of the IGF-IR is blocked by truncated IGF-IRs that lack a tyrosine kinase domain (Hernandez-Sanchez et al., 1995 and Li et al., 1994). Fibroblasts lacking IGF-IR and cells expressing mutant IGF-IRs that contain amino acid substitutions in the cytoplasmic tyrosine residues lose their ability to form tumors in nude mice (Sell et al., 1994, Kato et al., 1994, and Blakesley et al., 1996). Also, αIR-3, a monoclonal antibody against the α subunit of IGF-IR, has been shown to inhibit the proliferation of various tumor cell types; colorectal (Lahm et al., 1994), osteosarcoma (Raile et al., 1994), breast cancer (Peyrat and Bonneterre, 1992), melanoma (Furlanetto et al., 1993), and hematopoietic cells (McCubrey et al., 1991). This evidence indicates that intact IGF-IR function is required for cellular transformation.

The relationship between reduced IGF-IR number and tumorigenicity has been demonstrated by various antisense strategies. Stable expression of an IGF-IR antisense RNA inhibited cell growth in human T98G glioblastoma cells (Ambrose et al., 1994) and colony formation in soft agar in human FO-1 melanoma cells (Resnicoff et al., 1994b). Moreover, although both the FO-1 cells and rat C6 glioblastoma cells develop tumors in nude mice, treatment of these cells with IGF-IR antisense oligonucleotides inhibited tumor development (Resnicoff et al., 1993a and 1993b).

Another strong line of evidence to support IGF-IR tumorigenicity comes from studies of a fibroblast cell line derived from the knockout mouse embryo that lacks IGF-IR alleles (Sell et al., 1993 and 1994). The cell line, named R^-, was shown to be arrested at entering S-phase in serum-free medium with growth factor supplement, in which wild-type littermate W cells grow. Although the R^- cells grow in a medium supplemented with 10% serum, their growth rate is much slower than that of W cells. The striking evidence is that an introduction of SV40 T antigen or the Ha-ras oncogene transforms wild-type littermate

W cells, but not R^- cells. Reintroduction of the IGF-IR into R^- cells restores SV40 T antigen transformation capability (Sell et al., 1993 and 1994). The critical role of IGF-IR in promotion of the SV40 T antigen-mediated cell proliferation and transformation has also been demonstrated in related studies (Porcu et al., 1992 and Valentinis et al., 1994). In addition, it was shown that IGF-IR is also an essential agent for EGFR-mediated transformation. EGF administration to EGFR-overexpressing R^- cells was unable to cause transformation, however, reintroduction of a functional IGF-IR results in EGF-induced accelerated cell cycle progression and growth (Coppola et al., 1994). Furthermore, IGF-IR mediates PDGF induction of proliferating cell nuclear antigen (PCNA) mRNA expression. PCNA is a cofactor of DNA polymerase δ and is required for DNA synthesis. It was shown that IGF-IR was required for the maturation of PCNA mRNA. However, PCNA pre-mRNA levels are increased by PDGF without IGF-IR, indicating the IGF-IR implications in mRNA processing. This could be one possible mechanism through IGF-IR promotes cell cycle progression (Miura et al., 1994).

In summary, the above studies demonstrate that an aberrant expression of IGF-IR is clearly implicated in cellular transformation and tumor development. Therefore, regulation of IGF-IR expression at the transcriptional level is crucial for the control of normal and aberrant cell proliferation.

2. Wilms' tumor

Wilms' tumor is an embryonal malignancy of the kidney with an incidence of 1 in 10,000, making it one of the most common solid tumors in childhood (Matsunaga, 1981). Wilms' tumor is thought to originate from metanephric rests and residual islands of immature kidney, and exhibits a triphasic morphology consisting of undifferentiated blastemal (mesenchymal), stromal, and epithelial elements.

Most cases of Wilms' tumor are unilateral and sporadic with an onset age of 44 months, and not are associated with any other symptoms of disease. However, in a small number of cases (5-7%), the tumor is bilateral and of early onset (33 months), and appears to have a familial pattern (Hastie, 1994). In addition, in rare cases, Wilms' tumor is found as a part of three syndromes, which all predispose to Wilms' tumor; WAGR (Wilms' tumor, aniridia, genitourinary abnormalities, and mental retardation) (Riccardi et al., 1978), Denys-Drash (severe nephropathy and pseudohermaphroditism) (Drash et al., 1970), and Beckwith-Wiedemann (postnatal overgrowth, macroglossia, and increased incidence of hepatoblastoma and rhabdomyosarcoma) (Beckwith, 1969 and Wiedemann, 1964) syndromes.

A genetic component of Wilms' tumor etiology was predicted from the fact that an increased incidence of early onset correlated with bilateral tumors, congenital anomalies, or familial history, whereas late onset correlated with unilateral and sporadic tumors. This fact lead to the hypothesis that a rate-limiting event could be crucial for Wilms' tumor development. The hypothesis was supported from two types of studies. Cytogenetic studies from Wilms' tumor/aniridia patient specimens showed deletions at the 11p region. Although the deletions detected were different from patient to patient, 11p13 markers were commonly absent in all specimens (Riccardi et al., 1978 and 1980). Loss of heterozygosity (LOH) studies demonstrated that 50% of the patients lost 11p markers as a result of chromosome loss/deletion or somatic recombination (Fearon et al., 1984, Koufos et al., 1984, Orkin et al., 1984, and Reeve et al., 1984). However, a loss of markers is not unique to 11p13, and in some cases it was confined to 11p15 markers. This evidence indicated that loss of alleles at 11p13 and/or 11p15 leads to tumorigenesis, implying the presence of tumor suppressor gene(s) at these locations.

3. Wilms' tumor suppressor gene-1 (WT1)

Analyses of WAGR patients, in which cytogenetically detectable large deletions at the 11p13 locus occur, made it possible to identify a putative Wilms' tumor suppressor gene, called WT1 (Call et al., 1990). The WT1 gene encodes a transcriptional factor containing glutamine/proline-rich putative transcriptional regulatory motifs in the amino-terminus and four C_2H_2-type zinc-finger DNA-binding domains in the carboxy-terminus. Four naturally occurring variants of the WT1 proteins are produced as a result of two alternative splice sites in the WT1 mRNA. One involves exon 5, which encodes 17 amino acids in the amino-terminal transcriptional regulatory domain. The second results from the use of an alternative splice junction at the 3' end of exon 9; this results in the insertion of three amino acids (KTS) between zinc fingers 3 and 4. It has been predicted that the insertion of these amino acids would disrupt its DNA-binding capability, since the crystal structure of the related Egr-1 gene product shows that a certain spacer length between each zinc finger is critical for sequence recognition (Pavletich and Pabo, 1991). In addition, WT1 is modified at the posttranscriptional level. RNA editing in exon 6 occurs after transcription of WT1 gene, converting nucleotide U839 to C, which changes leucine 280 to proline (Sharma et al., 1994). Expression of WT1 is tissue- and developmental stage-specific in gonads (Pritchard-Johns et al., 1990), heart, spleen (Call et al., 1990), and nervous system (Sharma et al., 1992), suggesting a role in development and differentiation.

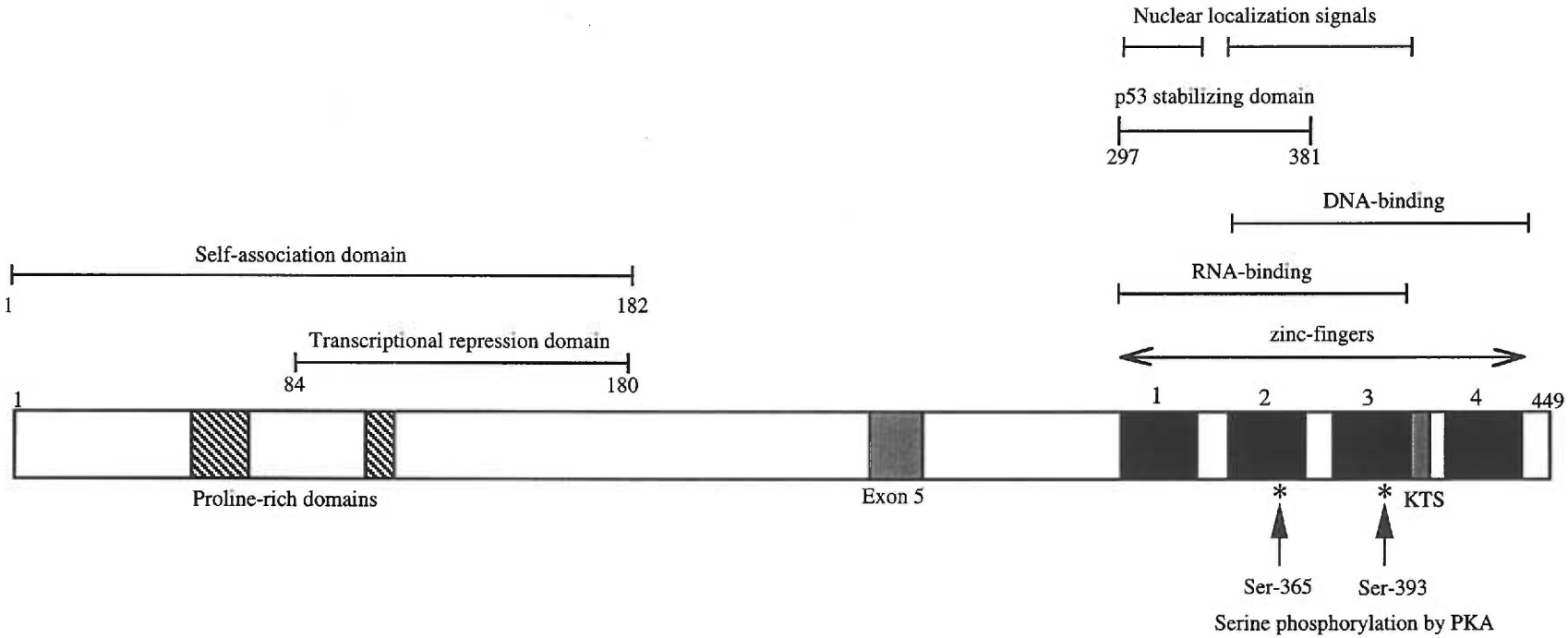

Figure 1. Structural basis of WT1.

WT1 structural domains are shown. In the amino-terminal domain, two proline-rich clusters are found. The first 182 amino acids are involved in self-association. Amino acids 84 to 180 are a transcriptional repression domain in the context of PDGF A-chain gene regulation. At the carboxy-terminus, WT1 contains four zinc-finger DNA-binding motifs that exhibit significant sequence homology with the early growth response (EGR) gene family. Zinc fingers 2, 3, and 4 are involved in DNA binding. More recently, zinc fingers 1, 2, and 3 have been identified as an RNA-binding domain. Although the domain(s) responsible for p53 association has not been determined yet, amino acids 297 to 381 are critical for stabilizing p53 protein. Zinc finger 1 and zinc finger 2 and 3 segments comprise the nuclear localization signal. Ser-365 and Ser-393 are phosphorylated by cyclic AMP-dependent protein kinase (PKA); this modification reduces DNA binding in vitro.

As a DNA-binding transcriptional factor, WT1 recognizes GC-rich sequences; 5'-GCGGGGGCG-3' (Rauscher et al., 1990), 5'-GCGTGGGCG-3' (Hamilton et al., 1995), and (5' TCC 3')$_n$ (Wang et al., 1993). Many growth-related genes that have GC-rich sequences in their promoter regions are negatively regulated by WT1, including those encoding the PDGF A-chain (Wang et al., 1992), IGF-II (Drummond et al., 1992), TGF-β1 (Dey et al., 1994), CSF-1 (Harrington et al., 1993), Egr-1 (Madden et al., 1991), RAR-α (Goodyer et al., 1995), Pax-2 (Ryan et al., 1995), and WT1 itself (Rupprecht et al., 1994). Another molecular aspect of WT1 includes its potential phosphorylation by PKA and PKC (Ye et al., 1996 and Sakamoto et al., 1997), which may alter the repression activity of WT1 (summarized in Figure 1).

Most of the cases of sporadic Wilms' tumor result from nonsense mutations upstream of the zinc-finger domain or large deletions of the gene. Also, a handful of point/deletion mutations in the WT1 gene have been reported in related syndromes. Denys-Drash patients often have missense mutations in the DNA-binding zinc-finger domain (Coppes et al., 1993). WAGR syndrome is due to large chromosome deletions at the 11p13 region, including the aniridia gene, Pax-6. This evidence strongly indicates that a loss of function of WT1 is a part of the etiology of Wilms' tumor.

In transcriptional regulation by WT1, both protein-DNA and protein-protein interactions play a critical role. p53 is one of the proteins thought to associate with WT1 (Maheswaran et al., 1993). p53 is a nuclear protein involved in DNA repair, apoptosis, and transcriptional activation (Ko and Prives, 1996). The association of p53 and WT1 was demonstrated by immunoprecipitation (Maheswaran et al., 1993). The presence of p53 is required for the transcriptional repression activity of WT1. Another study demonstrated that the WT1-p53 physical association stabilizes p53 protein and inhibits p53-mediated apoptosis (Maheswaran et al., 1995). In addition, WT1 self-associates through its animo-terminal domain (Englert et al., 1995, Moffett et al., 1995, and Reddy et al., 1995). However, the role of this self-oligomerization is not clear.

It has been reported that WT1 splice variants have distinct functions. The presence or the absence of the KTS insertion affects DNA-binding specificity and affinity (Reddy and Licht, 1996). A WT1 (+ exon 5) construct repressed the WT1 promoter more than the -exon 5 form of WT1 (Rupprecht et al., 1994). In addition, a WT1 protein containing the +KTS insertion was shown to colocalize with splicing factors as shown by immunostaining, confocal microscopy, and immunoprecipitation techniques, suggesting that WT1 might be involved in mRNA splicing (Larsson et al., 1995). The observation that the +KTS form of WT1 binds preferentially to RNA supports this notion (Caricasole et al.,

1996). In addition, the RNA edited form of WT1 was shown to suppress the Egr-1 promoter less effectively than the non-edited form of WT1 (Sharma et al., 1994).

In 1993, Werner et al. showed that IGF-IR mRNA expression is reciprocally correlated with WT1 mRNA in Wilms' tumor specimens, and the IGF-IR gene was identified as a target of WT1 (Werner et al., 1993). The IGF-IR promoter is TATA and CAAT box-less, but has GC-rich and $(TCC)_n$ elements that are recognized by zinc-finger family of transcription factors. WT1 binds to multiple sites in both the 5'-flanking and 5'-UTR regions of the promoter and negatively regulates its expression (Werner et al., 1994 and Figure 2) although the mechanism of this repression is not fully understood. Moreover, WT1 functions as a cell proliferation suppressor. Reintroduction of WT1 into the WT1-negative rhabdoid tumor-derived G401 cell line represses endogenous IGF-IR gene expression, leading to inhibition of cell growth (Werner et al., 1995).

In the context of IGF-IR gene regulation by WT1, questions still remain. It has been speculated that splice variants of WT1 would have differential effects in the regulation of the IGF-IR gene. It is also likely that syndrome-associated mutant forms of WT1 may have altered functions in IGF-IR gene regulation.

4. Kidney organogenesis

(1). General aspects

Fetal kidney development has been proposed to depend on a series of sequential and reciprocal inductive interactions between an epithelium and mesenchyme. These interactions promote the mesenchyme-to-epithelium transition and continue the epithelium to growth and branch into the ureter and epithelial tubules (Vainio and Muller, 1997). The ureteric bud, which is precursor of the collecting system, induces metanephric mesenchyme condensation. The condensed mesenchyme forms the renal vesicle, in which the cells undergo a mesenchyme-to-epithelial transition. The renal vesicle matures through the comma-shaped and S-shaped bodies, and fuses with the ureteric bud. Associating with glomerulus-forming capillaries, the fused renal vesicle elongates to the proximal tubule and culminates in the genesis of the mature nephron.

(2). IGF-IR and WT1 function in renal development

WT1 is critically involved in renal development. WT1 is expressed in the condensing mesenchyme, renal vesicle, comma/S-shaped bodies, and glomerular epithelium (Pritchard-Jones et al., 1990), indicating its possible role in the mesenchyme-epithelial transition that initiates renal differentiation. This notion is also supported from the

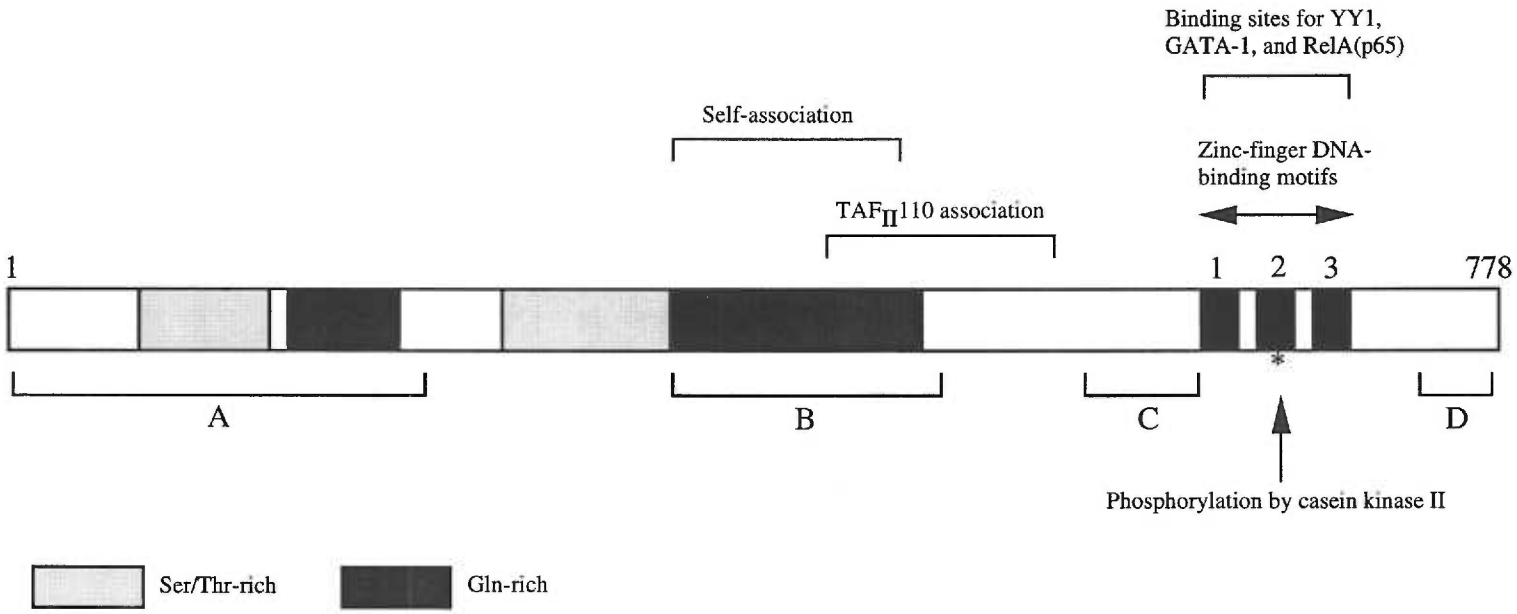

Figure 2. Structural basis of Sp1.

Schematic diagram of Sp1 is shown. Sp1 is a zinc finger type transcriptional activator. Sp1 contains two serine/threonine- and glutamine-rich domains in the amino-terminus shown in light grey and dark gray, respectively. In carboxy terminal region, three zinc fingers are located. Regions A, B, and D are defined as synergistic activation domains. Domain C contains highly charged amino acids. Second glutamine-rich domain is involved in self-association. A region between second glutamine-rich and C domains interacts with a TBP association factor (TAF $_{II}$110). Zinc-finger domains are involved in DNA-binding and an association with transcription factors, such as YY1, GATA1, and the RelA (p65) NF κB subunit. Sp1 is serine/threonine phosphorylated by DNA-dependent protein kinase, cAMP-dependent protein kinase (PKA), and casein kinase II. Casein kinase II phosphorylates Thr579 in zinc finger 3, shown as a asterisk. In addition, Sp1 is highly O-glycosylated.

study of WT1 null mice (Kreidberg et al., 1993). $WT1^{-/-}$ mice showed complete agenesis of the kidneys and lack of ureteric bud formation and apoptosis of metanephric blastema, which occurs in normal renal development. In organ culture, the WT1-deficient mesenchyme fails to respond to heterologous inducer tissues, suggesting that WT1 might regulate the expression signals that induce ureter growth. Therefore, it was proposed that WT1 is an essential factor for the signaling that results in differentiation of the mesenchyme and growth and branching of the ureteric bud.

A role of the IGF system in renal growth and development is supported by the facts that IGF systems are expressed in fetal kidney (Chin and Bondy, 1992) and that the IGF molecules produced in the metanephros are required for growth and development *in vitro* (Rogers et al., 1991). In particular, IGF-IR action in renal development was demonstrated by antisense oligonucleotide strategy (Liu et al., 1993b and Wada et al., 1993). An inhibition of IGF-IR gene expression by antisense oligonucleotides resulted in lack of renal differentiation. Taken together, the requirement for WT1 and IGF-IR in renal growth and development suggested a possibility of functional interaction between WT1 and IGF-IR.

5. Sp1

(1). General aspects

Sp1 is a ubiquitous nuclear protein in most cells, originally identified and characterized as a factor that binds to 21-bp GC-rich repetitive sequences found in the SV40 virus early promoter (Dynan and Tjian, 1985, Kadonaga and Tjian, 1986, and Kadonaga et al., 1987). Sp1 is a zinc-finger type transcriptional activator, composed of amino-terminal glutamine- and serine/threonine-rich domains that are required for transcriptional activation (Courey et al., 1989) and three C_2H_2-type zinc fingers that recognize consensus DNA sequences 5'-GGGGCGGGG-3' (Letovsky and Dynan, 1989) and $(TCC)_n$ (Beitner-Johnson et al., 1995). Although Sp1 is believed to be expressed ubiquitously, some differential expression patterns are observed in certain developmental contexts. Sp1 expression is high in hematopoietic cells (T cells), epithelial cells of thymus, and spermatids, and developmentally regulated in the stomach (Saffer et al., 1991).

The molecular aspects of Sp1 have been studied in great detail. Sp1 is highly O-glycosylated (Jackson and Tjian, 1988) and phosphorylated by a DNA-dependent kinase (Jackson et al., 1990), casein kinase II (Armstrong et al., 1997), and cAMP-dependent protein kinase (Rohlff et al., 1997). Sp1 can self-oligomerize through its amino-terminal domain, and this self-association is enhanced by its DNA binding (Courey et al., 1989 and Pascal and Tjian, 1991). The amino terminus of Sp1 mediates transcriptional activation of

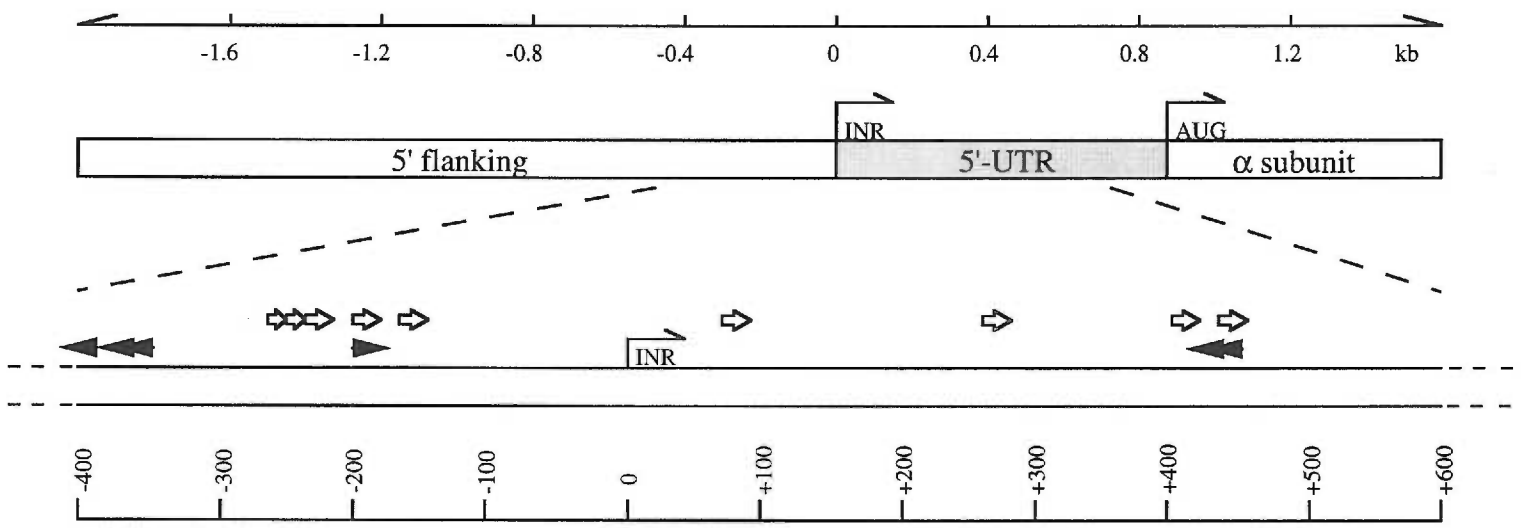

Figure 3. Structure of IGF-IR promoter sequence.

The rat IGF-IR promoter sequence was originally cloned by Werner et al. (1990). A -476 to +640 construct was used in this study. In this region, several binding sites for WT1 and Sp1 were found by DNase I foot-printing: WT1 sites; 6 in 5' flanking and 4 in the 5'-UTR regions (shown as empty arrows). All sites described here contain classical GC-rich sequences, except the most 3' site, which corresponds to a (TCC)n motif that is a second class of WT1 recognition site as shown in the PDGF A-chain promoter. Sp1; 4 in the 5' flanking and 2 in the 5'-UTR region (black arrow heads). Five sites comprise conventional GC-boxes. One site in 5'-UTR is the (TCC)n element that is also a site for WT1 binding. The -199 and +432 regions contain overlapping WT1 and Sp1 sites. INR indicates transcription initiation site.

many genes and an interaction with other transcription factors, such as $TAF_{II}110$ (Gill et al., 1994). The zinc-finger region of Sp1 mediates binding to not only DNA, but also other proteins, including transcription factors YY1 (Lee et al., 1993), GATA-1 (Merika and Orkin, 1995), and the p65 subunit of NFκB (Perkins et al., 1994) (summarized in Figure 2). A critical function of Sp1 in embryonic development was demonstrated by an analysis of Sp1 null mice generated by targeted gene disruption (Marin et al., 1997). The observations of the death of Sp1-deficient mice at day11 of gestation and the cell death of Sp1-/- cells injected into normal host embryos after the commitment of cellular differentiation suggest that Sp1 is essential for early embryonic development but dispensable for cell differentiation.

Recently, Sp1 was identified as an essential factor in the regulation of the IGF-IR gene (Beitner-Johnson et al., 1995). Sp1 is the only factor known to positively regulate IGF-IR expression. Sp1 binds to multiple GC box sequences in the 5' flanking region and one $(TCC)_n$ motif in the 5'-UTR region of the IGF-IR promoter and activates its expression. Those binding sites for Sp1 partially overlap or are adjacent to the ones for WT1 found in the promoter (Figure 3), implying possible functional/physical interactions between WT1 and Sp1.

(2). Functional interactions with another zinc-finger transcription factor, Egr-1

In recent studies, it has been shown that the Egr-1 transcription factor, whose zinc-finger domains are homologous to that of WT1, functionally interacts with Sp1 in cytokine and growth factor gene regulation. Egr-1 and Sp1 coordinate maximum gene expression of the Tissue Factor (Cui et al., 1996) and interleukin-2 genes (Skerka et al., 1995), whose promoters have adjacent and partially overlapping recognition sites for these factors. Egr-1 responds to phorbol 12-myristate 13-acetate (PMA) stimulation and induces PDGF A-chain gene expression by displacing Sp1 at overlapping binding sites in the 5'-flanking promoter region (Khachigian et al., 1995). These findings strongly suggest that the Egr-1/WT1 family of factors may functionally interact with Sp1 in IGF-IR gene regulation.

6. Rationale and aims of study

The transcriptional regulation of the IGF-IR gene by WT1 makes physiological sense in renal development and its aberrant regulation is one of the etiologies that may cause Wilms' tumor and possibly other tumors. WT1 negatively regulates IGF-IR gene expression, which might require other factors and protein-protein interactions. In addition,

recent findings of Egr1-Sp1 interactions support the possibility of functional interactions between WT1 and Sp1.

The hypothesis proposed in this thesis is that naturally occurring variants and clinically related mutants of WT1 have differential activities in IGF-IR gene repression and altered ability to self-associate that might, in part, explain the differential repression of WT1. In addition, the two zinc-finger transcription factors, WT1 and Sp1, differentially regulate and coordinate IGF-IR gene expression through their physical interaction and competition for binding to overlapping recognition sites.

In this thesis, three specific aspects of the hypothesis have addressed.

[Section I]
Examine the differential activity of naturally occurring variants and clinically-related mutants of WT1 in the context of IGF-IR gene regulation employing luciferase reporter gene assays and transient transfections.

[Section II]
Assess the self-association capabilities of WT1 variants in the yeast two-hybrid system.

[Section III]
Study functional/physical interactions of WT1 and Sp1 in IGF-IR gene regulation.
(a). Assess the effects of WT1 and Sp1 in combination on IGF-IR promoter activity using luciferase reporter gene assays and transient cotransfections.
(b). Address the possibility of WT1 and Sp1 physical interactions by means of coimmunoprecipitation and the yeast two-hybrid system.

The results of the proposed studies delineate the complexity of transcriptional regulation by WT1 variants and elucidate the importance of WT1 and Sp1 in IGF-IR gene regulation, whose aberrant expression can lead to tumorigenesis and developmental dysgenesis.

Materials and Methods

Plasmids

Mammalian expression plasmids

The WT1 gene has two alternative splice sites (exon 5 and Lys-Thr-Ser (KTS) insertion at the 3' end of exon 9) generating four naturally occurring variants. Those are designated as (+/+), (+/-), (-/+), and (-/-) depending upon the presence or absence of the exon 5 and KTS insertions, respectively. Human WT1 (+/+) and (-/-) cDNAs cloned into pCB6+ were inserted into pcDNA3 mammalian expression plasmids containing the cytomegalovirus (CMV) promoter (Invitrogen) using EcoR I and Xba I restriction sites. The (+/-) and (-/+) variants were constructed as follows: WT1 amino- and carboxy-teminal fragments were excised with EcoR I/Afl III and Afl III/Xba I, respectively, from parental pcDNA3/WT1 (+/+) and (-/-) plasmids. Those amino- (the presence or absence of exon 5) and carboxy-terminal domains (the presence or absence of KTS insertion) were cloned into a pcDNA3 vector linearized with EcoR I and Xba I in a tripartite ligation.

The amino- and carboxy-terminal domain constructs were created as follows: WT1 (+/+) and (-/-) full-length cDNA fragments excised from parental pCB6+/WT1 (+/+) and (-/-) using Cla I and Hinc II were inserted into pGEM7Zf(+) (Stratagene) linearized with Cla I and Sma I, which is designated as pGEM7Zf(+)/WT1 (+/+) and (-/-). The FLAG peptide (MDYLDDDD) was inserted into the pcDNA3/WT1 (+/+) at the 5' end to generate a pcDNA3/FLAG plasmid, which is recognized by M-series antibodies (KODAK).

```
   EcoRI      M  D  Y  L  D  D  D  D      ClaI
5'-AATTCCACCATGGACTACAAGGACGACGATGACAAGAT  -3'
3'-    GGTGGTACCTGATGTTCCTGCTGCTACTGTTCTAGC-5'
```

This sequence also encodes an ATG initiation codon with a Kozak consensus sequence (CCACC) at the 5' end to facilitate translation initiation. Since this oligonucleotide contains a GATC sequence at the 3' end, the N^6 position of the adenine is methylated by the Dam methylase produced in most E. coli strains, producing a Cla I site resistant to digestion by this restriction enzyme. For subsequent procedures, the pcDNA3/FLAGWT1 (+/+) was introduced into the DMI dam- E. coli strain (Life Technologies). From this pcDNA3/FLAGWT1 (+/+) (dam-) plasmid, two sets of backbone vectors for amino- and carboxy-terminal domain constructs were generated as follows: 1, pcDNA3/FLAGWT1 (+/+) (dam-) was linearized with Xba I and filled-in with Klenow fragment, then cut with Cla I (for amino-terminus construct). 2, pcDNA3/FLAGWT1 (+/+) (dam-) was linearized with Cla I and filled-in with Klenow fragment, then cut with Xba I (for carboxy-terminus construct). Amino- and carboxy-terminus fragments were excised from the

pGEM7Zf(+)/WT1 (+/+) and (-/-). The pGEM7Zf(+)/WT1 (+/+) and (-/-) plasmids were first linearized with Afl III and filled in with Klenow fragment, then cut with Cla I for the amino-terminal fragment. The pGEM7Zf(+)/WT1 (+/+) and (-/-) was first linearized with Afl III and chewed back with Mung bean exonuclease, then cut with Xba I for the carboxy-terminal fragment. pcDNA3/FLAG backbones were ligated with these domain fragments with T4 DNA ligase to generate pcDNA3/WT1N (+ exon 5) and pcDNA3/WT1C (+ and - KTS insertion). In addition, the amino-terminus construct contained a SV40-derived nuclear localization sequence (PKKKRKV), since the putative WT1 NLS is located within the carboxy-terminal zinc-finger domain (Bruening et al., 1996). The NLS oligonucleotides used were;

```
    ClaI P  K  K  K  R  K  V  ClaI
5'-CGATCCCAAGAAGAAGCGGAAGGTCAT  -3'
3'-  TAGGGTTCTTCTTCGCCTTCCAGTAGC-5'
```

This NLS insertion was ligated into Cla I site of FLAG-WT1 domain constructs. All WT1 constants were verified by sequencing. Sp1 full-length and deletion constructs were kindly provided by Dr. R. Tjian (Courey and Tjian, 1988). Briefly, various Sp1 domains were made by using restriction endonucleases and exonuclease Bal31 and inserted into the pPac expression vector, which has an insect actin 5C gene promoter and polyadenylation signal to enhance expression in Schneider Drosophila line 2 (SL2) cells.

Construction of mutant and deletion WT1 plasmids

Parental human WT1 (+/+) and (-/-) cDNAs were cloned into pGEM7Zf(+) to generate pGEM7Zf(+)/WT1 (+/+) and (-/-). Mutant and deletion forms of WT1 were created by site-directed mutagenesis (Stratagene) of WT1 (+/+) and (-/-) cDNAs.

In the Denys-Drash syndrome-associated mutation (DDS), T^{1180} is replaced by cytosine, changing amino acid Arg^{394} to Trp (Coppes et al., 1993). In case of a WAGR-associated point mutation, G^{602} is changed to adenine, which alters Gly^{201} to Asp (Park et al., 1993). The substitution of a nucleotide T^{842} by cytosine (amino acid Leu^{280} to Pro) results from the editing of WT1 mRNA (Sharma et al., 1994). One of the proline-rich clusters located in the amino-terminal region was removed. The proline-rich domain (amino acid 54 to 68) contains 13 prolines out of 15 amino acids (N-PAPPPAPPPPPPPPP-C).

Mutagenesis oligonucleotides used are as follows;

```
DDS;              5'-GTTCTCCTGGTCCGACCACC-3'  (20-mer).
WAGR;             5'-TCTATGACTGCCACACCCCC-3'  (20-mer).
RNA editing;      5'-CCATCCCCTGCGGAGCCCAA-3'  (20-mer).
Proline-rich deletion;
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5'-CATACGGTTCCCTGGGTGGTCACTCCTTCATCAAACAGGA-3' (40-mer).

Bold letters indicate substituted nucleotides. For the proline-rich deletion mutant, the oligonucleotide consists of juxtaposed 5' and 3' sequences that flank the proline-rich domain in order to delete the corresponding 45 nucleotides with a loop-out. In vitro mutagenesis was confirmed with automated sequencing.

Reporter plasmid

A rat IGF-IR promoter sequence (-476 to +640) originally cloned by Werner et al. (1992) into p0Luc was excised with Hind III and inserted into the pGL3 luciferase reporter plasmid (Promega) for mammalian cell transfection, designated as pGL3/IGF-IR (-476/+640). The original p0Luc luciferase plasmid p0Luc/IGF-IR (-476/+640) was used for insect cell transfection.

Cell culture

CHO cells were seeded in F12 Nutrient Medium with 10% heat-inactivated FBS at 37°C with 5% CO_2. G401 cells are derived from a rhabdoid tumor (Garvin et al., 1993) and were maintained in McCoy's 5A medium supplemented with 10% heat-inactivated FBS. The Schneider Drosophila line 2 (SL2) is a insect cell line from Drosophila embryo (Schneider, 1972). SL2 cells were maintained in Schneider Drosophila medium (Life Technologies) with 10% heat-inactivated fetal bovine serum at room temperature.

Transient transfection study

Transient transfections were carried out as follows: CHO or G401 cells were seeded in 6-well plates at a density of $1x10^5$ a day before transfection. Next day, variable amounts of expression plasmids and 0.5 μg of reporter constructs were cotransfected along with 0.5 μg of pCMV/β-gal for an internal control using Lipofectin reagent (Life Technologies) in OPTI-MEM reduced-serum medium (Life Technologies). The total DNA amount transfected was adjusted to 6.0 μg/well with empty pcDNA3 expression plasmid. 16 hours later, medium was replaced with regular growing medium. 48 hour post-transfection, cells were lysed with 150 to 200 μl of luciferase assay lysis buffer (1% (v/v) Triton X-100, 25mM glycylglycine (pH7.8), 15mM $MgSO_4$, 4mM EGTA). Luciferase activity was measured by a luminometer (AutoLumat LB953, EG&G Berthold) with 10 μl of cell lysate, 300 μl of luciferase assay buffer (25mM glycylglycine (pH7.8), 15mM $MgSO_4$, 5mM ATP, 10mM NaOH), and 100 μl of luciferin solution (0.03% (w/v)). To correct for transfection efficiency, β-galactosidase assays were carried out. 30 μl of the

lysate from luciferase assay preparation was incubated with Z-buffer substrate (60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4$, 4mg/ml ONPG (o-nitrophenyl β-galactopyranoside), pH7.0) for 1 to 6 hours at 37°C. ONPG color conversion was determined on the basis of OD420.

SL2 cells were transfected using calcium phosphate (Di Nocera and Dawid, 1983). Briefly, SL2 cells were seeded in 6-well plates at a density of $1x10^6$ 3 to 4 hours prior to transfection in regular growing medium. Variable amounts (0 to 50 ng) of Sp1 expression plasmid (pPac/Sp1), 2.5 μg of WT1 expression vector (pcDNA3/WT1), and 0.5 μg of p0Luc reporter plasmid were dispensed into 100 μl of 250mM $CaCl_2$. Empty expression plasmids were used to adjust the total DNA amount to 3.0 μg. The DNA solutions were then added into 100 μl of 2xHBS buffer (280mM NaCl, 10mM KCl, 2.8mM Na_2HPO_4, 11mM Dextrose, and 42mM HEPES, pH 7.1) dropwise. After 30 min at room temperature, transfection mixtures were added into wells dropwise. At 44 to 48 hour post-transfection, the cells were lysed with 60 μl of luciferase assay lysis buffer (1% (v/v) Triton X-100, 25mM glycylglycine (pH7.8), 15mM $MgSO_4$, 4mM EGTA). Luciferase activity was measured in 20 μl of cell lysates as described above and normalized for total protein determined using the Bradford reagent (Bio-Rad).

The luciferase assay data from mammalian cell transfections was analyzed using two controls. One was transfection with empty pcDNA3 vector alone to measure background luciferase and β-gal activity. The other was to measure the basal luciferase activity of the reporter gene constructs using the pGL3 empty reporter plasmid. The background number was subtracted from all samples. Then, the luciferase numbers were normalized for β-galactosidase activity. The basal luciferase activity was subtracted from all other samples. The luciferase data is shown as the percentage of the IGF-IR promoter activity in the control. The SL2 cell transfection assays were normalized for protein amount since exogenous Sp1 protein affected β-galactosidase expression from the pCMV/β-gal plasmid (see Discussion section).

In order to verify WT1 and Sp1 expression in these experiments, transiently transfected cells were lysed with NP-40 lysis buffer (1% (w/v) NP-40, 0.2% SDS, 150mM NaCl, 20mM Tris-Cl, 1mM EDTA, 10% glycerol, 1mM PMSF, 1mM $NaVO_4$, 10 μg/ml leupeptin, 10 μg/ml aprotinin) and 50-100 μg of the lysates were separated on SDS-PAGE, followed by western immunoblotting with an anti-WT1 antibody (C19) and an anti-Sp1 antibody (PEP2) (both from Santa Cruz Biotechnology).

Yeast two-hybrid system

The yeast two-hybrid system was originally established by Fields and Song (1989) and modified by Vojtek et al. (Vojtek et al., 1993). In our version of this system, two proteins (X and Y) are fused to either the DNA-binding domain of the LexA bacterial regulatory protein or the acidic transcription activation domain of Herpesvirus protein VP16. When these two proteins are brought into proximity due to the association of proteins X and Y, a functional transcriptional activator is reconstituted that drives histidine synthase (HIS3) and β-galactosidase (lacZ) reporter genes. The two host strains used are of different mating types (MATa and MATα) and are histidine auxotrophs. Once fusion protein constructs are introduced into each strain separately, those strains can be mated to assess protein-protein association on the basis of histidine prototrophy and β-gal activity. In cases of high HIS3 background activity, the histidine synthase inhibitor 3-amino-1,2,4-triazole (3-AT) can be used.

Strains; L40 ; MATa trp1-901 leu2-3,112 his3Δ200 ade2 LYS2::(lexAop)4-HIS3 URA3::(lexAop)8-lacZ, and AMR70 ; MATα his3 lys2 trp1 leu2 URA3::(lexAop)8-lacZ.

Plasmids; WT1 amino- and carboxy-termini were inserted into the pBTM116 plasmid, which encodes the LexA DNA-binding domain and the TRP1 tryptophan synthase gene. In addition to WT1 amino- and carboxy-domains, Sp1 full-length, amino-, and carboxy-domains were inserted into pVP16, which encodes the VP16 transcription activation domain fused to a SV40-derived nuclear localization sequence and the LEU2 leucine synthase gene.

Transformation; Transformations of L40 with pBTM116 plasmids and AMR70 with pVP16 were carried out using lithium acetate as described by Schiestl and Gietz (1989). L40 and AMR70 transformants were selected by trptophan and leucine prototrophy, respectively.

Interaction analyses; Once transformants were mated, the conjugates were plated on histidine-lacking plates with or without 1-40mM 3-AT for 3-AT titration. Liquid β-gal assays were performed as described (Schneider et al., 1996).

Coimmunoprecipitation and Western blotting

CHO cells were transiently transfected with 5 μg of WT1 expression vector using Lipofectin reagent (Life Technologies). 48 hour after transfection, the cells were washed with PBS and harvested. The cells from one 100-mm dish were lysed by an addition of 200 μl of buffer A (50mM Tris-Cl, pH7.9, 0.5M KCl, 10% glycerol, 0.5mM EDTA, 1mM DTT, and 0.2% NP40) with proteinase inhibitors (1mM PMSF, 1mM $NaVO_4$, 10 μg/ml leupeptin, 10 μg/ml aprotinin), followed by three freeze-thaw cycles (Mengus et al.,

1995). To check expression levels, 50 µg of the lysate was separated on 8% SDS-PAGE. For coimmunoprecipitation, 200 µg of lysate was incubated with 1 µg of an anti-WT1 antibody (C19) in 500 µl of lysis buffer for 16 hours at 4 °C, followed by 1 hour of incubation with Protein A-Sepharose (Pharmacia). Immunoprecipitates were collected by centrifugation and washed four times with lysis buffer and then separated by 10 % SDS-PAGE. Separated proteins were transferred to Hybond ECL nitrocellulose membranes (Amersham). After blocking with Blotto (5% non-fat milk in TBS-T), the membranes were blotted with Sp1 (PEP2) (Santa Cruz Biotechnology). An anti-rabbit horseradish peroxydase-conjugated antibody was used as a secondary antibody (Amersham). Blots were processed for ECL detection (Amersham). Phosphorylated and unphosphorylated isoforms of the Sp1 protein migrated at 105 and 95 kD, respectively (Jackson et al., 1990).

Results

Section I. Differential repression activity of WT1 proteins in IGF-IR gene regulation

Construction of clinically-related mutants of WT1

The WT1 gene was identified as a transcriptional suppressor and subsequently shown to regulate several growth-regulating genes, including the IGF-IR gene (Werner et al., 1993). Transcriptional regulation by WT1 is of a particular interest since WT1 has four naturally occurring variants that result from two alternative splice sites and several altered forms, including clinically-related mutations. The potential differential effect of these various WT1 proteins in IGF-IR gene regulation has not been previously evaluated. In order to examine altered forms of WT1 for IGF-IR gene repression activity and effects on the self-oligomerization, mutated/deleted forms of WT1 were created by site-directed mutagenesis.

While Wilms' tumor-associated WT1 mutations are generally deletion or nonsense mutations, specific missense mutations are found in Denys-Drash syndrome, the most common being T^{1180} to cytosine (amino acid: Arg^{394} to Trp) in zinc finger 3 (Coppes et al., 1993). A G^{602} to adenine (amino acid: Gly^{201} to Asp) mutation was found in the non-deleted 11p13 allele of a WAGR patient (Park et al., 1993). The substitution of nucleotide T^{842} by cytosine (amino acid: Leu^{280} to Pro) corresponds to the RNA editing form of WT1 (Sharma et al., 1994). The amino-terminus of WT1 contains two proline-rich domains. The proline-rich domain is thought to play critical roles in transcription regulation and potentially in signal transduction through an association with src-homology 3 (SH3) domains. An internal deletion mutant of one of the proline-rich domains, which contains 13 proline residues out of 15 amino acids, was also created by loop-out mutagenesis. Schematic diagrams of these mutants are shown in Figure 4.

Differential repression of IGF-IR promoter by splice variants of WT1

Although transcriptional repression by the (-/-) form of WT1 has been demonstrated for many target genes, the relative specific repression activity of WT1 splice variants has not been examined in most cases. To measure a specific transcriptional repression of WT1 in IGF-IR gene regulation, luciferase reporter gene assays with transient transfection were employed. First, increasing amounts of a pcDNA3 mammalian expression plasmid encoding WT1 (-/-) were transiently transfected into CHO cells along with a luciferase

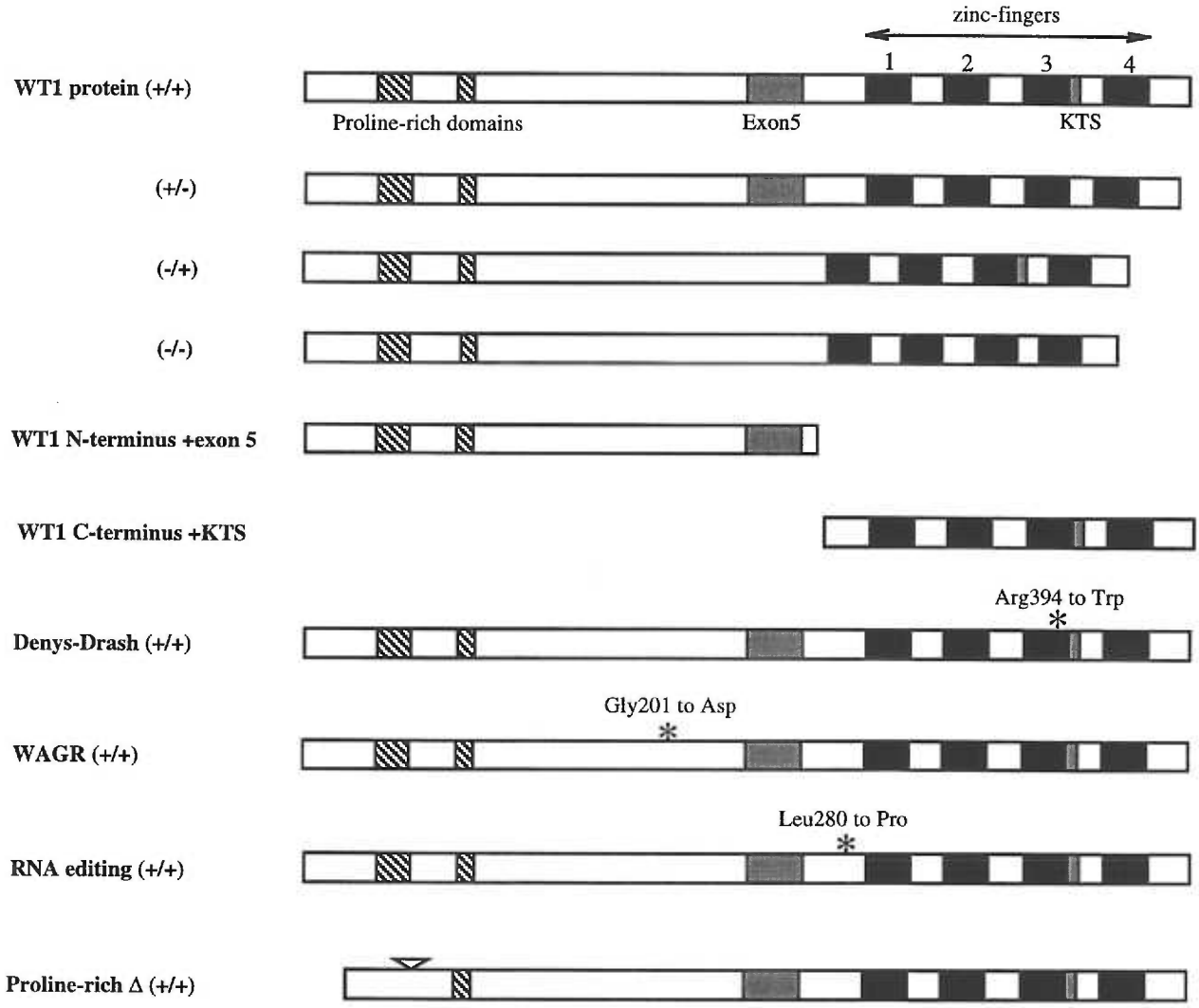

Figure 4. Schematic representations of wild-type and altered forms of WT1.

WT1 proteins contain two alternative splice sites (exon 5 and KTS insertion in exon 9), generating four variants. Top figure shows the WT1 protein with presence of both exon 5 and KTS insertion, designated as WT1 (+/+). Other naturally occurring variants are referred as WT1 (+/-); presence of exon 5 and absence of KTS insertion, WT1 (-/+); absence of exon 5 and presence of KTS insertion, and WT1 (-/-); absence of both sites. The amino-terminal domain contains first 300 amino acids including putative transcriptional repression domains. The carboxy terminus of WT1 consists of 150 amino acids containing zinc-finger DNA-binding domains. Altered forms of WT1 are as follows: Denys-Drash mutant changes Arg394 to Trp in zinc finger 3. WAGR-associated mutation is a Gly201 to Asp point mutation. RNA-editing form of WT1 changes Leu280 to Pro. Proline-rich deletion mutant (Proline-rich Δ) lacks 15 amino acids constituting the amino-terminal proline-rich domain.

reporter construct containing nt -476 to +640 of the rat IGF-IR promoter sequence (pGL3/IGF-IR (-476/+640)) and the pCMV/β-gal plasmid as an internal control for transfection efficiency. The results demonstrate that WT1 protein represses IGF-IR promoter activity in a dose-dependent fashion (Figure 5).

Next, the effect of naturally occurring splice variants of WT1 was examined. Four variants derived from the two alternative splice sites were transiently transfected into WT1-deficient G401 cells derived from a rhabdoid tumor (Figure 6, black bars), and CHO cells (Figure 6, dotted bars) in the same context as in Figure 5. In both CHO and G401 cells, the (+/-) and (-/-) variants of WT1 demonstrated nearly 80% repression of the IGF-IR promoter activity seen in cells transfected with pcDNA3 vector alone (defined as 100%); however, the (+/+) and (-/+) variants exhibited only ~40% repression activity. These results indicate that the presence or absence of the KTS insertion greatly affects the repression activity of WT1, presumably by disturbing the DNA-binding capability of WT1 since the KTS insertion is located between zinc fingers 3 and 4 that comprise the DNA-binding core. On the other hand, comparison of the repression activity of the (+/+) and (-/+) forms or the (+/-) and (-/-) forms showed no apparent differences, implying that exon 5-encoded sequences do not influence IGF-IR promoter regulation by WT1, at least in the context of transient transfection assays.

Partial repression of IGF-IR promoter activity by WT1 domains

The WT1 protein is comprised of putative transcriptional regulatory domains in its amino-terminus and zinc-finger DNA-binding domains at the carboxy-terminus. Although WT1 transcriptional repression is thought to be mediated by its binding to the target DNA sequences, DNA-independent actions, presumably through protein-protein association, are also possible. In order to elucidate the role of individual WT1 domains in IGF-IR regulation, amino- and carboxy-terminal expression constructs were examined by in reporter gene assays. The amino-terminal portion of WT1 consists of 300 amino acids containing putative transcription regulatory domains, and the carboxy-terminal WT1 fragment contains 150 amino acids including four zinc-finger domains (Figure 4). As shown in Figure 7, the carboxy-terminal WT1 domains demonstrated different repression activities. The +KTS carboxy terminus of WT1 (WT1C +KTS) exhibited only 20 to 25 % repression. However, the -KTS form (WT1C-KTS) exhibited nearly 60 % repression, mimicking the data obtained with the full-length constructs. Interestingly, the amino-terminal domain itself (WT1N+exon 5) exhibited 50 % repression activity, suggesting that putative protein-protein interactions are involved. The partial repression of both amino- and

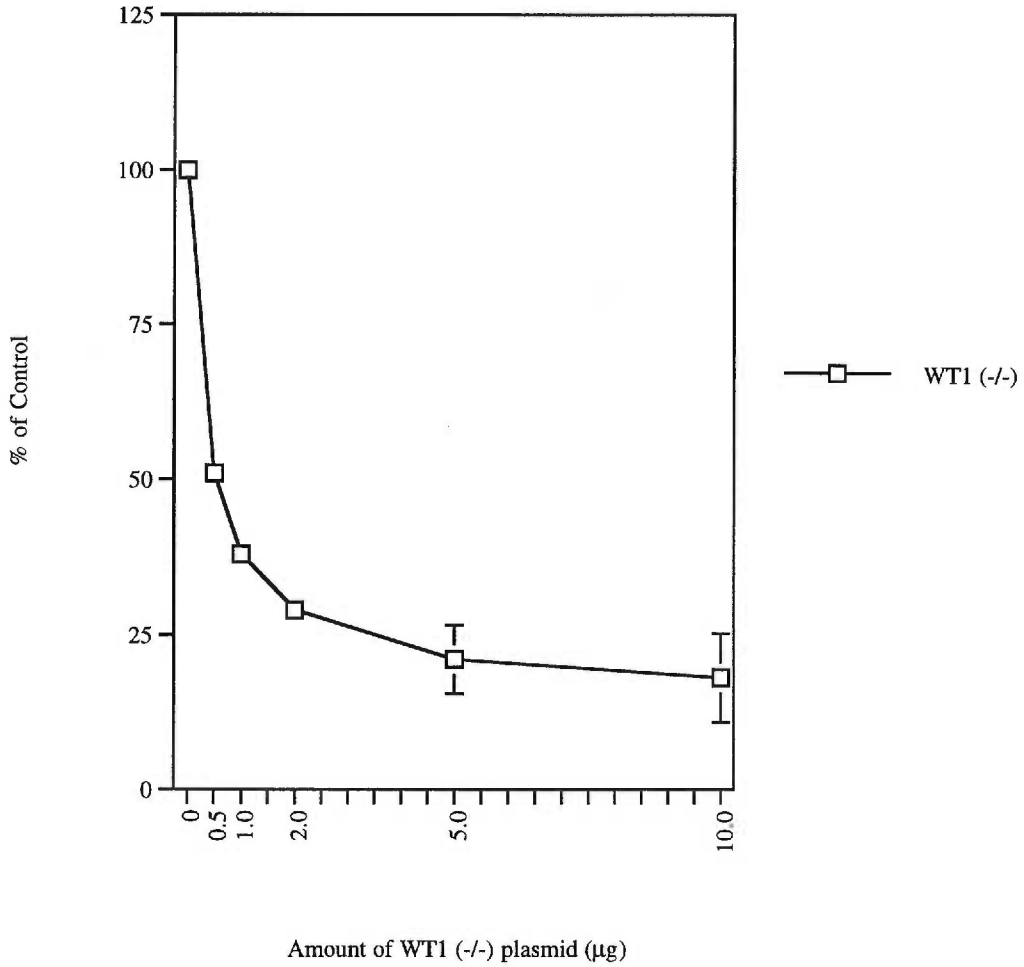

Figure 5. Dose-dependent IGF-IR promoter repression by WT1.

An increased amount of cytomegalovirus (CMV) promoter driven-WT1 (-/-) expression plasmid (pcDNA3/WT1 (-/-)) was transfected into CHO cells along with 0.5 μg of a luciferase reporter construct (pGL3) containing -476 to +640 nt of rat IGF-IR promoter sequence (pGL3/IGF-IR (-476/+640)) and 0.5 μg of the pCMV/β-gal plasmid as an internal control for transfection efficiency. Cells were seeded at $1x10^5$ in 6-well plates 16 hours before transfection. Transfection was carried out with Lipofectin reagent (Life Technologies) in OPTI-MEM reduced-serum medium (Life Technologies). 16 hours later, the medium was replaced with regular growing medium. At 44-48 hours of posttransfection, the cells were processed for luciferase and β-galactosidase assays. Luciferase data was normalized with β-galactosidase activity. The experiment was done in duplicate and each experiment was repeated three times. Error bars represent standard deviations (SD:n=2).

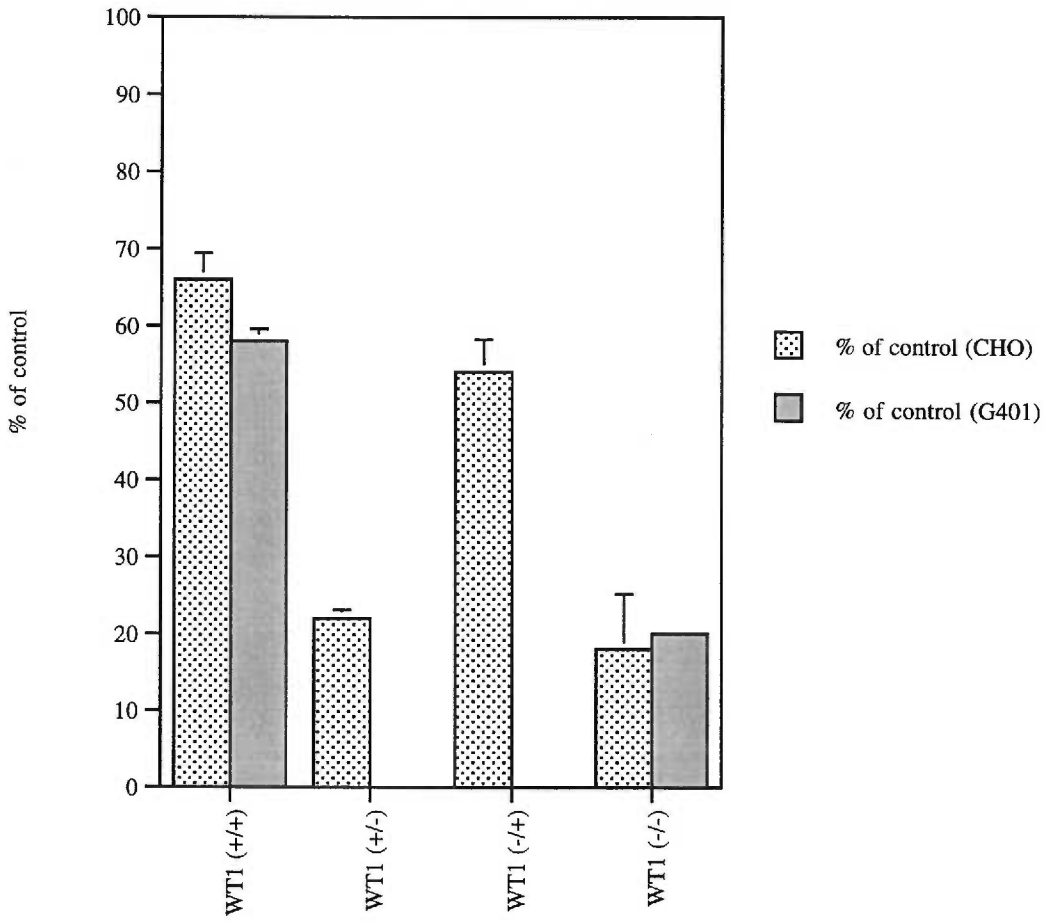

Figure 6. Differential IGF-IR repression by WT1 splice variants.

The WT1 gene encodes four naturally occurring splice variants; the presence or absence of exon 5 and the KTS insertion between zinc fingers 3 and 4. 5 μg of pcDNA3/WT1 expression vectors were transiently transfected into the rhabdoid tumor-derived cell line, G401 (black bars) and CHO cells (dotted bars) along with 0.5 μg of pGL3/IGF-IR (-476/+640) and 0.5 μg of pCMV/β-gal plasmid. Luciferase data was normalized for β-galactosidase activity. 100% represents the IGF-IR promoter-driven luciferase activity in CHO or G401 cells co-transfected with insertless pcDNA3 expression plasmid. The experiment was done in duplicate and each experiment was repeated three times. Error bars represent standard deviations (SD:n=2).

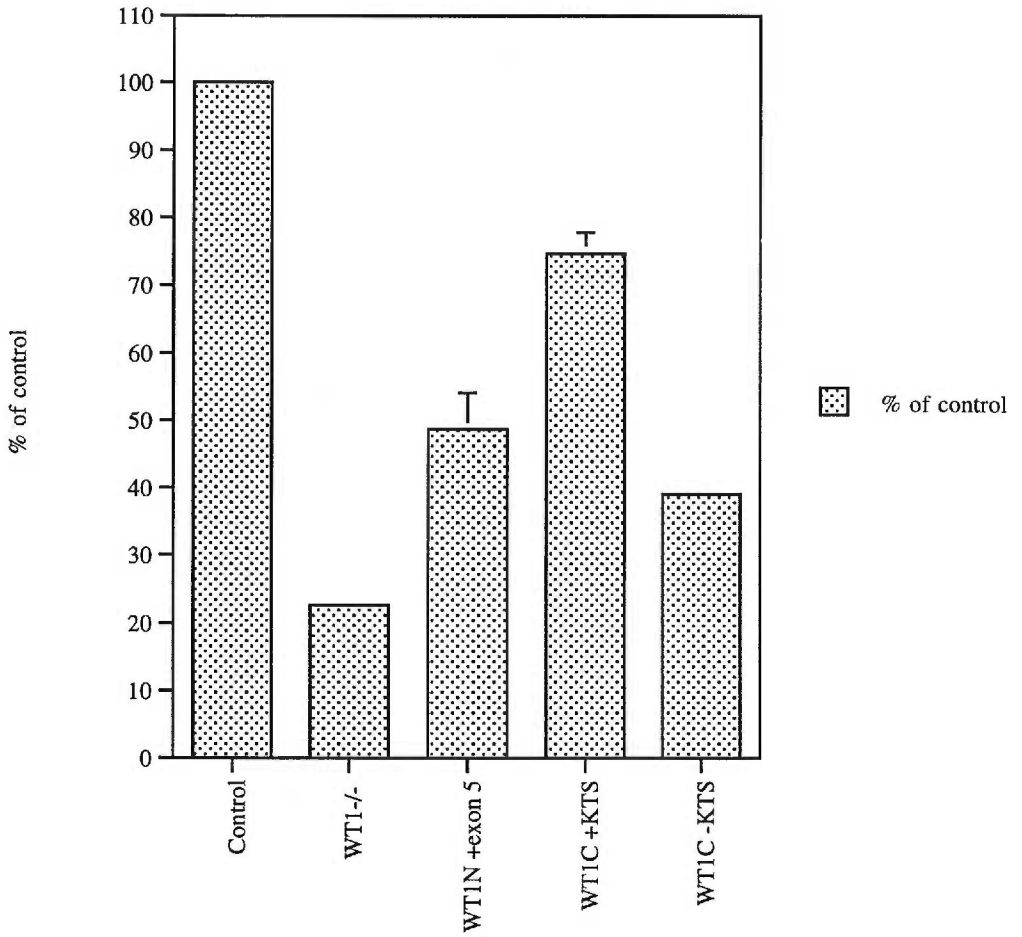

Figure 7. Partial repression of IGF-IR promoter activity by WT1 amino- and carboxy-domains.

Full-length WT1 lacking both exon 5 and KTS insertion (WT1 (-/-)), an amino-terminal domain containing exon 5 (WT1N +exon 5), and carboxy-terminal domains with or without KTS insertion (WT1C +KTS and WT1C -KTS) were examined for their transcriptional repression of the IGF-IR promoter. The amino portion of WT1 consists of 300 amino acids containing putative transcriptional regulation region and the carboxy terminus of WT1 contains 150 amino acids including zinc-finger domains. 5 μg of the expression plasmids and 0.5 μg of pGL3/IGF-IR (-476/+640) were used for each experiment. Control represents the IGF-IR promoter-driven luciferase activity in CHO cells co-transfected with insertless pcDNA3 expression plasmid. The experiment was done in duplicate and each experiment was repeated three times. Error bars represent standard deviations (SD:n=2).

carboxy-terminal domains as compared to full-length WT1 (WT1-/-) indicates that WT1 may exert its transcriptional suppression through both DNA binding and distinct protein-protein interactions involving the amino-terminal domain.

The effects of WAGR-associated, proline-rich domain-deletion, and the RNA-edited forms of WT1 in IGF-IR promoter repression

Besides wild-type splice variants, a handful of altered forms of WT1 have been reported in clinical settings, and a posttranscriptionally modified form has been described (Figure 4). To determine the possible repression activity of these altered forms of WT1, transient transfection assays were done with WAGR-associated, proline-rich domain-deleted, and RNA editing forms of WT1 (Figure 8). The WAGR-associated mutant and RNA editing forms of WT1 exhibited the same degree of repression as wild-type WT1 (-/-). In addition, the proline-rich deletion mutant exhibited even greater repression activity than wild-type WT1 (-/-) (Figure 8). The proline-rich domain is one of the distinct motifs found in WT1 and was originally speculated to play a role in gene regulation through protein-protein interaction.

The effect of Denys-Drash syndrome (DDS)-related mutant in IGF-IR promoter repression

In DDS patients, mutations are commonly found in the zinc-finger regions of WT1. In particular, a mutation of T^{1180} replaced by cytosine (amino acid Arg^{394} to Trp; R394W) in zinc finger 3 has been reported in 20 of 36 affected cases, making it one of the major missense mutations in Denys-Drash syndrome (Coppes et al., 1993). DDS mutations function in an autosomal dominant fashion consistent with a dominant-negative mode of action. The observations that WT1 could self-associate through its amino-terminus and that bacterially expressed DDS zinc finger domain lacks DNA-binding capability (Pelletier, et al., 1991 and Little, et al., 1995) led to the idea that the DDS mutant behaves in a dominant-negative fashion by interfering with the function of normal WT1 (Reddy et al., 1995 and Holmes et al., 1997). To address the effect of DDS mutant in IGF-IR regulation, the R394W mutant was examined.

Unexpectedly, the R394W DDS mutant exhibited repression activity itself in a dose-dependent manner (Figure 10). Both (+/+) and (-/-) forms of the DDS mutant were moderately active (~40 % repression at 5 μg), compared to wild-type (+/+) and (-/-) versions of WT1 (30 % and 70 %, respectively). Also, cotransfection of wild-type and

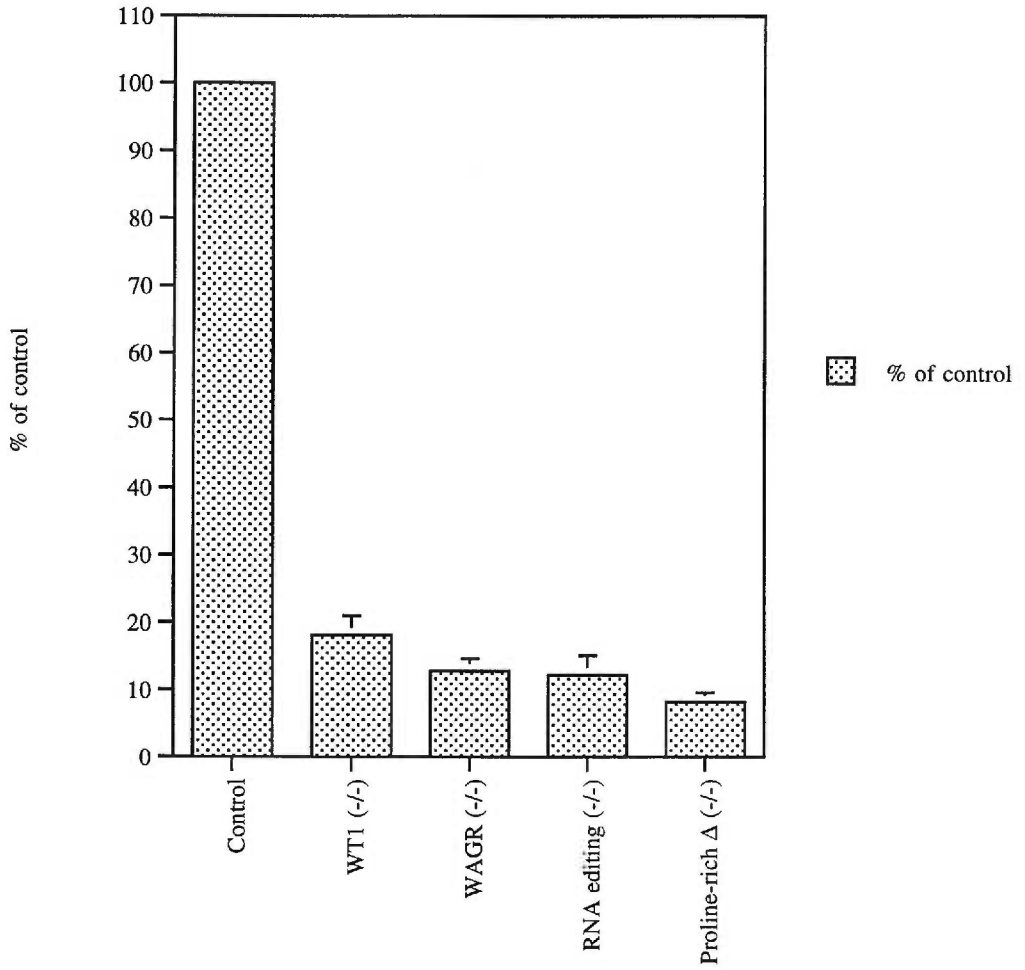

Figure 8. Effect of WAGR-associated mutant, RNA-editing, and proline-rich domain deletion forms of WT1 in IGF-IR promoter repression.

5 μg of wild-type or altered forms of WT1 (-/-) expression vectors were transiently transfected into CHO cells, along with 0.5 μg of pGL3/IGF-IR (-476/+640). Control is as in Figure 6. The experiment was done in duplicate and each experiment was repeated three times. Error bars represent standard deviations (SD:n=2).

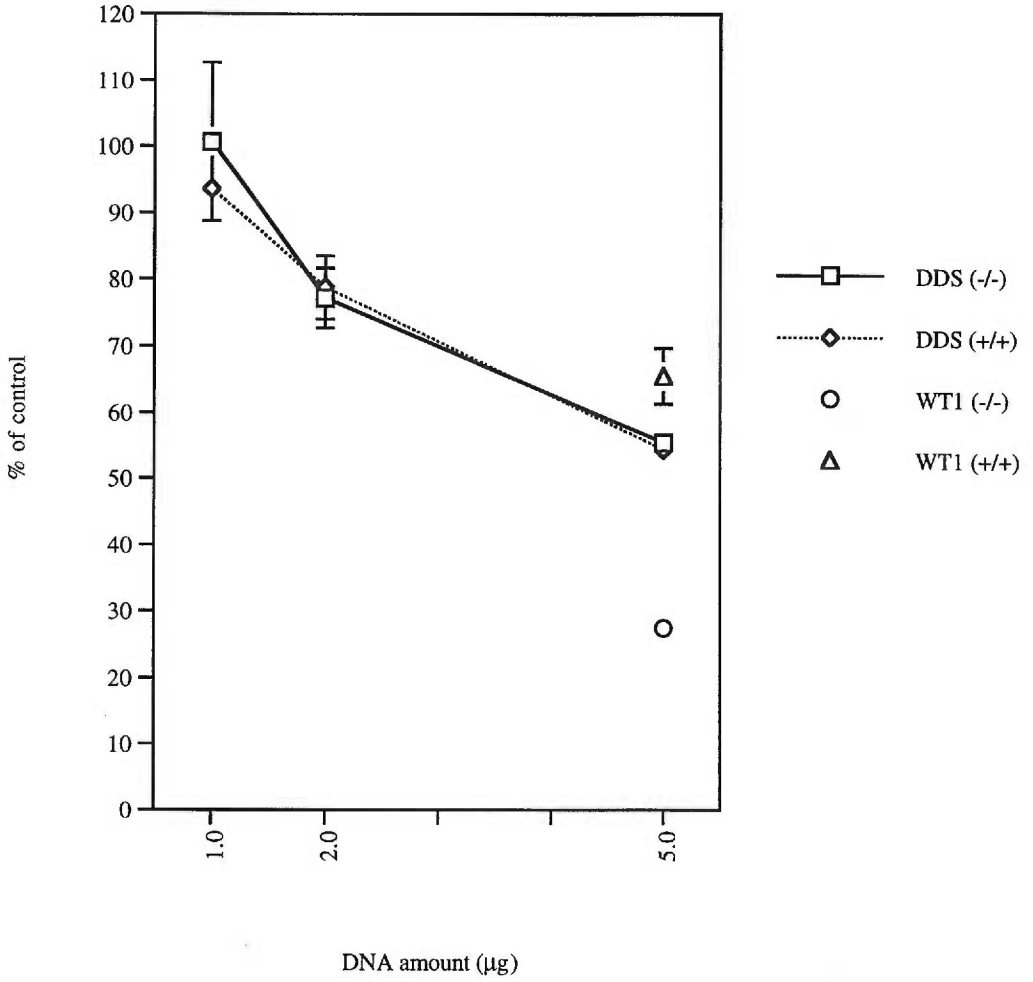

Figure 9. Dose-dependent repression of IGF-IR promoter by Denys-Drash syndrome-associated (DDS) form of WT1.

The DDS mutant contains a point mutation of T1180 to cytosine, changing amino acid Arg349 to Trp in zinc finger 3. Increased amounts (1 to 5 μg) of DDS plasmids (-/-) (square) and (+/+) (diamond) were transiently transfected into CHO cells along with 0.5 μg of pGL3/IGF-IR (-476 to +640) and 0.5 μg of the pCMV/β-gal plasmid as an internal control of transfection efficiency. Wild-type WT1 variants are shown as a circle (-/-) and a triangle (+/+) at 5 μg for comparison. Control is as in Figure 5. The experiment was done in duplicate and each experiment was repeated three times. Error bars represent standard deviations (SD:n=2).

DDS forms of WT1 showed that the DDS mutant exhibited an additive effect on IGF-IR promoter repression (Figure 10). Introduction of 2 μg of wild-type of WT1 (+/+) and (-/-) plasmids resulted in 20 % and 60 % repression, respectively. Cotransfection of increasing amounts of DDS mutant expression vectors (2 to 8 μg) with a fixed amount of wild-type WT1 plasmid (2 μg) resulted in additive effects on repression of IGF-IR promoter activity. These data suggest that the DDS mutant form of WT1 does not function in a dominant-negative manner, but potentially independently, through gain-of-function.

Expression of introduced wild-type and altered forms of WT1 in CHO cells

To verify that the differential activities seen in the transient transfection data described above reflected intrinsic difference in the activity of the various versions of WT1 protein, the expression level of various forms of WT1 in CHO cells was examined using western immunoblotting. Approximately 40 to 50 μg of all lysates were separated on a 10 % SDS-PAGE gel. The expected size for full-length WT1 is 53-55 kD with a variation of 2 to 3 kD, due to the presence or absence of the exon 5 and KTS insertions. As shown in Figure 11, all of the versions of WT1 protein analyzed were expressed to the same extent.

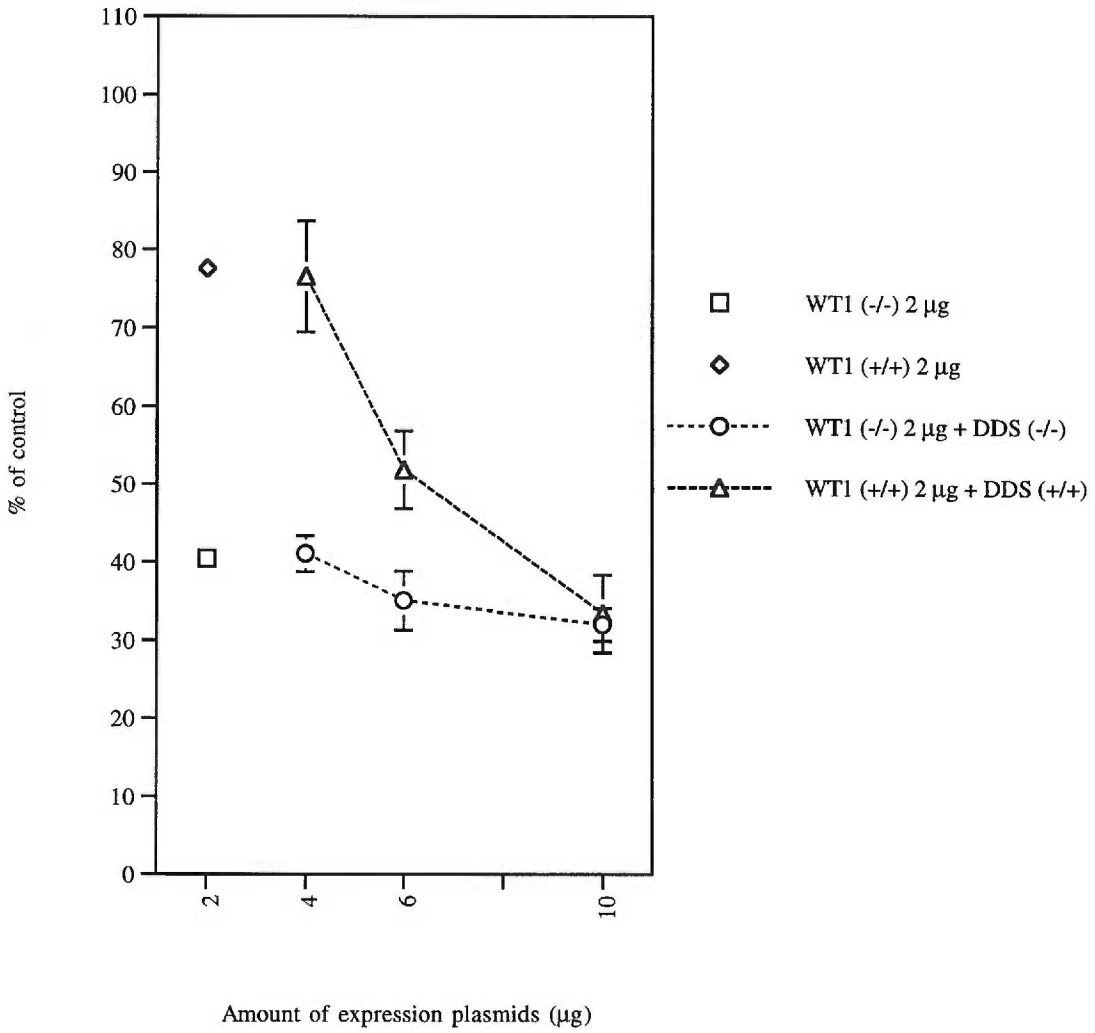

Figure 10. Additive effects of DDS mutants in IGF-IR regulation.

Wild-type and DDS mutant forms of WT1 constructs were transiently cotransfected into CHO cells along with pGL3/IGF-IR (-476/+640). Fixed amount (2 µg) of wild-type ((+/+) or (-/-)) and increased amounts (2 to 8 µg) of DDS expression plasmids ((+/+) (triangles) or (-/-) (circles)). Total DNA amount was adjusted to 10 µg with empty pcDNA3 plasmid. Wild-type WT1 variants are shown as a square (-/-) and a diamond (+/+) at 2 µg for comparison. Control is as in Figure 5. The experiment was done in duplicate and each experiment was repeated three times. Error bars represent standard deviations (SD:n=2).

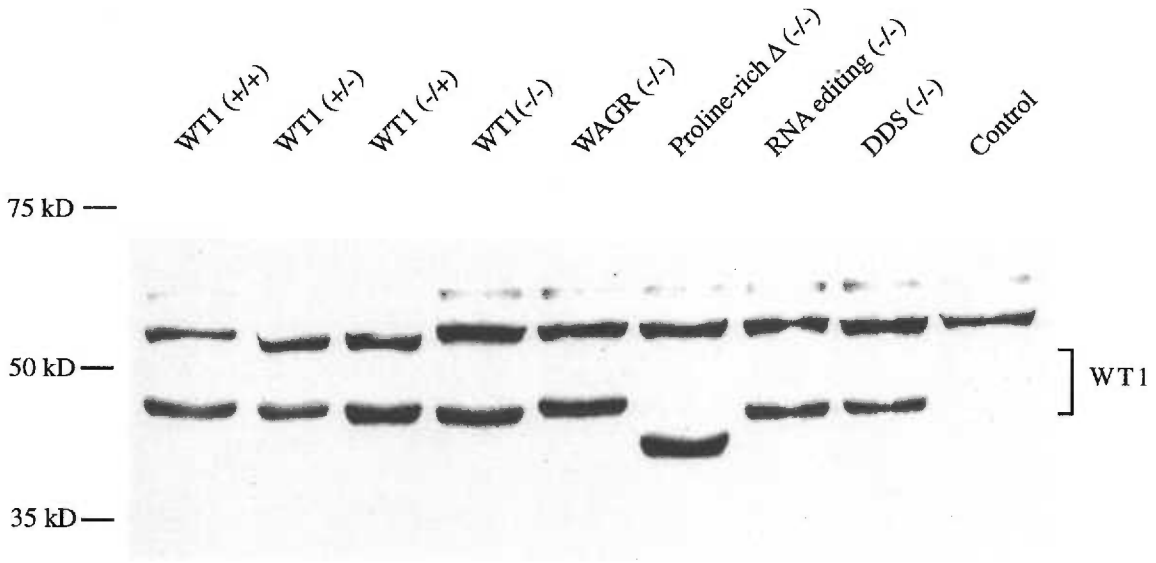

Figure 11. Expression of various exogenous WT1 proteins in CHO cells.

5 μg of wild-type and mutant WT1 expression plasmids were transiently transfected into CHO cells along with 0.5 μg of the pCMV/β-gal construct as an internal control. 44-48 hour posttransfection, cells were lysed. After normalized with β-galactosidase activity, which did not significantly differ among samples, approximately 40-50 μg of proteins was separated by 10 % SDS-PAGE. The proteins were transferred to nitrocellulose membrane (Amersham). WT1 proteins were detected by an anti-WT1 antibody, C19, that recognizes the carboxy-terminus of WT1. WT1 proteins were detected around 45 to 53 kD. Bands of 60 to 65 kD represent non-specific binding.

Section II. WT1 self-association study

Self-association capabilities of wild-type and altered forms of WT1

In previous studies, it was reported that WT1 self-associates through its amino-terminal region (Englert et al., 1995, Moffett et al., 1995, and Reddy et al., 1995), and it has been speculated that this interaction might play a role in WT1 function. To address the potential differential self-association capability of altered forms of WT1, the yeast two-hybrid system was employed (Figure 12). The yeast two-hybrid system is a suitable application for assessing protein-protein association *in vivo,* and is particularly appropriate for the study of WT1 self-association, as the interactions in the yeast two-hybrid system occur in the nucleus, and WT1 is a nuclear protein. Amino-terminal regions of WAGR-associated, RNA editing, and proline-rich deletion forms of WT1 were fused to LexA DNA-binding and VP16 transcriptional activation domains. When WT1 proteins interact with each other, the reconstituted transcription activator drives reporter genes and the strength of the interaction is measured by reporter gene activities.

First, yeast conjugants expressing various combinations of WT1 amino-terminal domains were examined for β-galactosidase activity (Figure 13). Wild-type conjugants showed the same degree of β-galactosidase activity regardless of the presence or absence of exon 5, indicating that exon 5 sequences do not appreciably influence protein-protein interaction. The conjugants of wild-type/mutant forms of WT1, including WAGR-associated, RNA editing, and proline-rich deletion forms exhibited β-gal activities similar to those of wild-type/wild-type conjugants. This result suggests the altered forms of WT1 do not disrupt self-association capability. However, conjugants expressing only mutant forms of WT1 exhibited significantly reduced β-galactosidase activity that was close to that of Lamin C negative control conjugants, indicating that self-association is disrupted by these alterations of the WT1 amino terminus.

Second, histidine synthase, the other reporter gene driven in this yeast two-hybrid system, was used to address WT1 self-association capability. 3-amino-1,2,4-triazole (3-AT) is a inhibitor of histidine synthase and the strength of histidine prototrophy can be titrated by adding increased concentrations of 3-AT. The conjugants used in the β-galactosidase assays above were examined using 3-AT titration (Figure 14). Yeast conjugants containing wild-type WT1 were viable at a concentration of 3-AT as high as 40mM, indicating WT1 self-association. However, conjugants expressing both wild-type and altered forms of WT1 (WAGR-associated and RNA-edited forms) grew in significantly decreased concentrations of 3-AT (10mM), implying disruption of self-association.

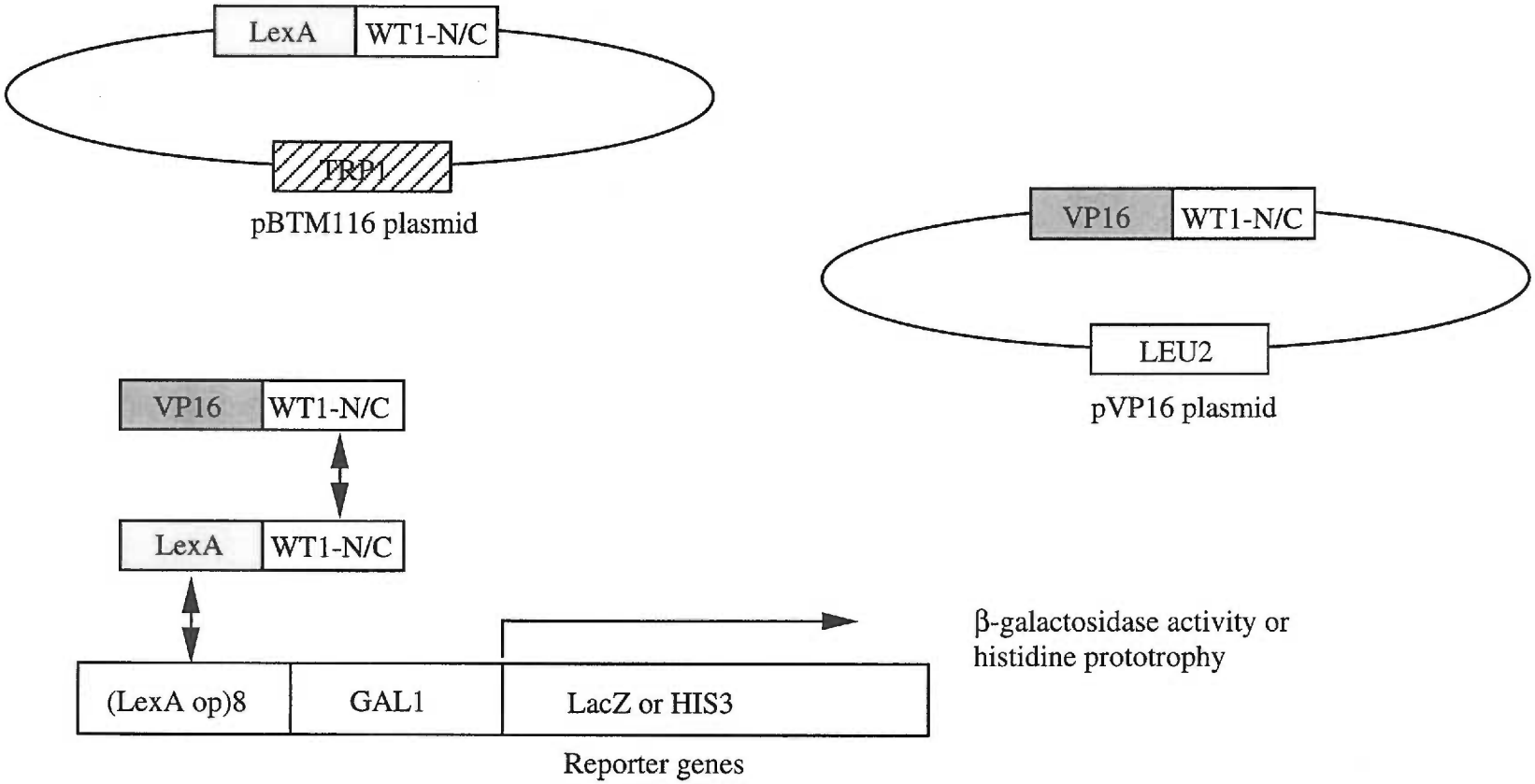

Figure 12. Schematic diagram of yeast two-hybrid system.

The pBTM116 plasmid encodes the LexA DNA-binding protein, which binds to LexA recognition sequences that are upstream of the GAL1 promoter driving reporter genes, β-galactosidase (β-gal) and histidine synthase (HIS3). pVP16 encodes the VP16 transcription activation domain. Amino- and carboxy-terminal domains of WT1 were fused to LexA and VP16 sequences in frame. The yeast expression plasmids were separately introduced into yeast strains that have different mating type, a and α. Transformants were selected for tryptophan and leucine prototrophy and then mated. When WT1 proteins interact with each other, fused LexA and VP16 proteins are placed in proximity at the LexA operator sequences and reconstitute a transcription activator that drives reporter genes. The physical interaction of WT1 proteins was assessed on the basis of β-galactosidase activity and growth capability in histidine-lacking plates supplemented with increased amounts of 3-amino triazole, which is a histidine synthase inhibitor.

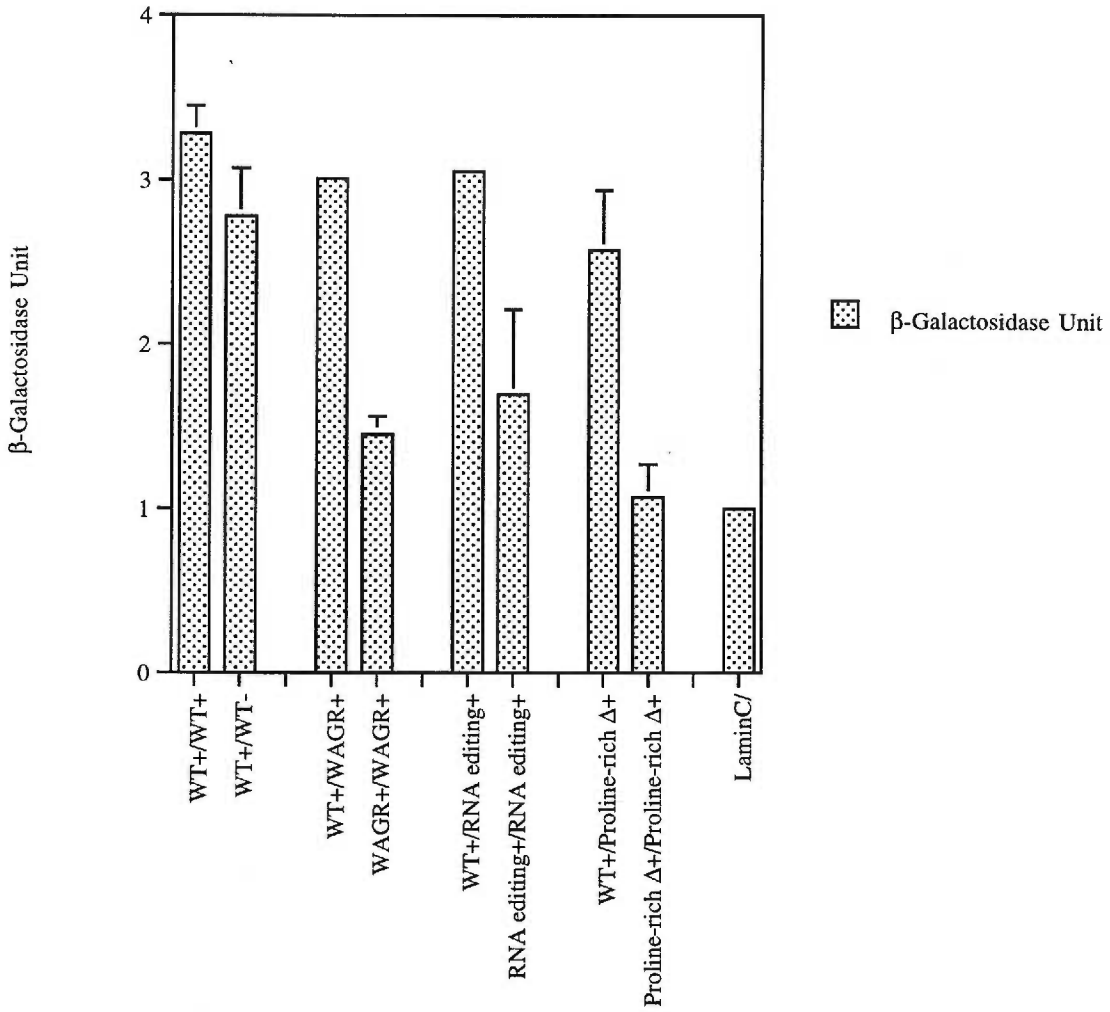

Figure 13. Quantitation of β-galactosidase activity produced in yeast conjugants expressing fusion proteins containing wild-type and altered forms of WT1 amino termini.

Amino-terminal domains of wild-type and altered forms of WT1 containing first 300 amino acids were fused to both the LexA DNA-binding domain and the VP16 transcriptional activation domain. Conjugants from various combinations were grown in histidine-lacking medium and subjected to β-galactosidase assays. Mixed fusions utilized WT+:LexA and WT-, WAGR+, RNA editing+, and Proline-rich Δ+:VP16. The activity was measured on the basis of color change of ONPG (o-nitrophenyl galactopyranoside) substrate. The basal level of β-galactosidase activity obtained in matings with lamin C is 1. A + or - indicates the presence or absence of exon 5. The experiment was repeated three times. Error bars represent the standard deviation (SD:n=2).

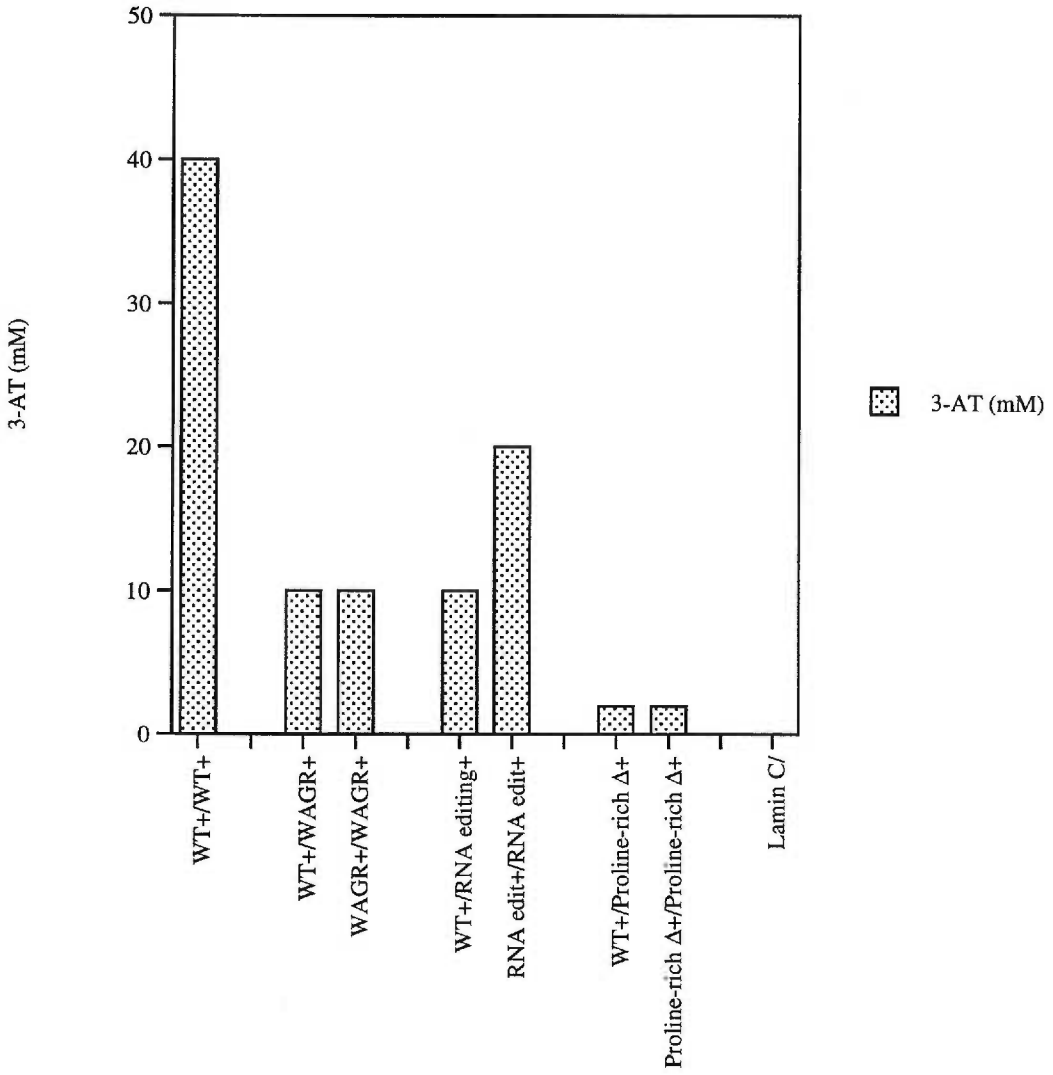

Figure 14. Titration of growth of yeast transformed with wild-type and altered forms of WT1 with increased concentrations of 3-amino-1,2,4-triazole.

Amino-terminal domains of wild-type and altered forms of WT1 containing the first 300 amino acids were fused to both the LexA DNA-binding domain and the VP16 transcriptional activation domain. Conjugants from various combinations were plated out on histidine lacking plates containing various concentrations of 3-amino-1,2,4-triazole (3-AT), which is a inhibitor of histidine synthase. Mixed fusions utilized WT+:LexA and WAGR+, RNA editing+, and Proline-rich Δ+:VP16. The values indicated are the highest concentration of 3-AT at which growth was detected after 2-3 days. A + or - indicates the presence or absence of exon 5. The experiment was repeated four times with identical results.

Conjugants transformed with WAGR-associated and RNA-edited forms of WT1 were only viable in 10 or 20mM 3-AT. The growth of proline-rich deletion mutant-containing conjugants was abrogated at a low concentration of 3-AT (2mM), indicating severely decreased self-association. No conjugates with Lamin C grew on plates supplemented with 3-AT and were used as a negative control. These results correlate well with the β-galactosidase assay data discribed above, and illustrate that mutations and deletions in the amino-terminal domain of WT1 severely reduce self-association ability when both interacting fusion proteins carry the same alteraction.

Section III. Functional interaction of WT1 and Sp1.

Specific activation of the IGF-IR promoter by full-length Sp1 and Sp1 domains.

In the regulation of IGF-IR gene expression, only two specific transcription factors have been directly implicated; WT1 and Sp1. Sp1 is a ubiquitously expressed transcriptional factor that activates many genes, including the IGF-IR gene (Beitner-Johnson et al., 1995).

In order to further explain the specific activation of the IGF-IR promoter by Sp1, luciferase reporter gene assays in Sp1-deficient SL2 cells were employed. First, increasing amounts of Sp1 were transiently transfected into SL2 cells along with a reporter construct, p0Luc/IGF-IR (-476/+640). As shown in Figure 15, up to 200-fold activation of IGF-IR promoter with 50 ng of Sp1 expression plasmid was achieved. Next, various Sp1 domain constructs (Figure 16) were examined in the same context to determine the domains responsible for IGF-IR promoter activation (Figure 17). Promoter activation was severely reduced by deletion of the Sp1 carboxy terminus (N636) and eliminated by additional deletion of the adjacent DNA-binding zinc-finger region (N539). Progressive deletion of the first and second pairs of glutamine- and serine/threonine-rich domains (as represented by constructs 516C, 327C, and 168C) proportionally reduced activation (120, 25, and 2-fold versus 300-fold for full-length Sp1). Deletions of an internal domain between the second serine/threonine-rich region and the zinc-finger domain (Δint349 and Δint162), which contains a high proportion of charged amino acids (Figure 2), increased IGF-IR promoter activation by 3-fold. These data demonstrate that (1) DNA binding by Sp1 is necessary for transcriptional activation, (2) the carboxy-terminal domain of Sp1 contributed significantly to transcriptional activation, (3) the two pairs of glutamine- and serine/threonine-rich domains in the amino terminus collectively contribute to transcriptional activation, and (4) an internal highly charged region can inhibit transcriptional activation.

WT1 domains responsible for the attenuation of Sp1 activation in IGF-IR promoter regulation

Transcriptional repression activity of WT1 in IGF-IR regulation was also examined in SL2 cells. Four splice variants and amino- and carboxy-terminal domains were transiently cotransfected along with the p0Luc/IGF-IR (-476/+640) reporter construct. The results are shown in Figure 18. All splice variants of WT1 are able to significantly reduce

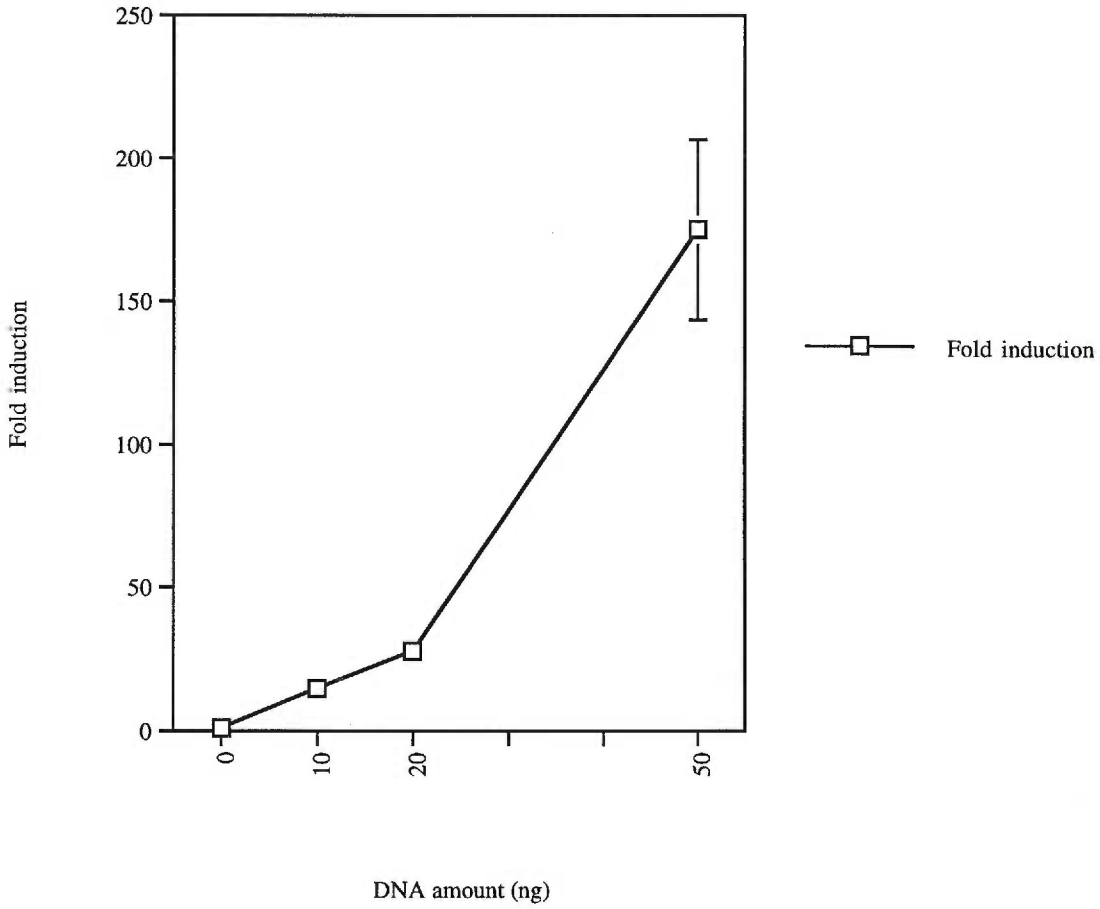

Figure 15. Dose-dependent activation of IGF-IR promoter by Sp1.

SL2 cells were placed in 6-well plates at $1x10^6$ in regular growing medium 3-4 hours prior to transfection. Increased amounts (10 to 200 ng) of pPacSp1 were transiently transfected along with 0.5 μg of a p0Luc luciferase reporter gene plasmid containing -476 to +640 nt of rat IGF-IR promoter sequence (p0Luc/IGF-IR (-476/+640)) and 2.5 μg of empty pcDNA3 plasmid as a carrier by calcium phosphate precipitation method. At 44-48 hours of posttransfection, the cells were processed for luciferase assays. Luciferase data was normalized with protein amounts. The experiment was done in triplicate and each experiment was repeated three times. Error bars represent standard deviations (SD:n=3).

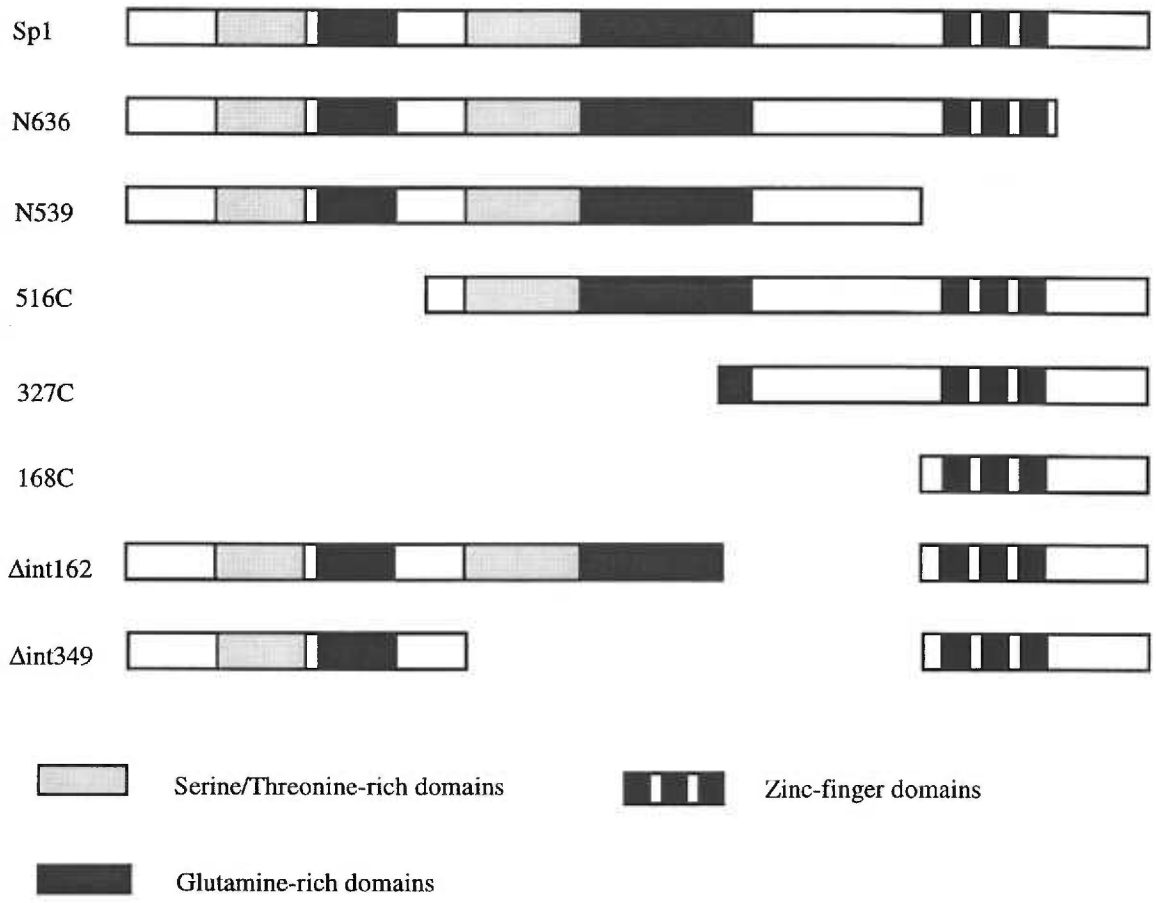

Figure 16. Sp1 full-length and deletion constructs.

Human Sp1 cDNA is cloned into the pPac expression vector, which contains actin 5C gene promoter and polyadenylation sites for enhanced expression in Drosophila SL2 cells. Deletion constructs were generated by using restriction sites and Bal31 exonuclease. N636 contains first 636 amino acids from amino-terminus. N539 lacks the zinc-finger region in carboxy terminus. 516C lacks first Serine/Threonine- and Glutamine-rich domains. 327C lacks most of the Serine/Threonine- and Glutamine-rich domains. 168C consists of only zinc-finger and carboxy-terminal regions. Internal deletion constructs are Δint162 and Δint349. Δint162 lacks a region between second Glutamine-rich and the zinc-finger domains. Δint349 is deleted from the second Serine/Threonine-rich domain to before the zinc-finger domain.

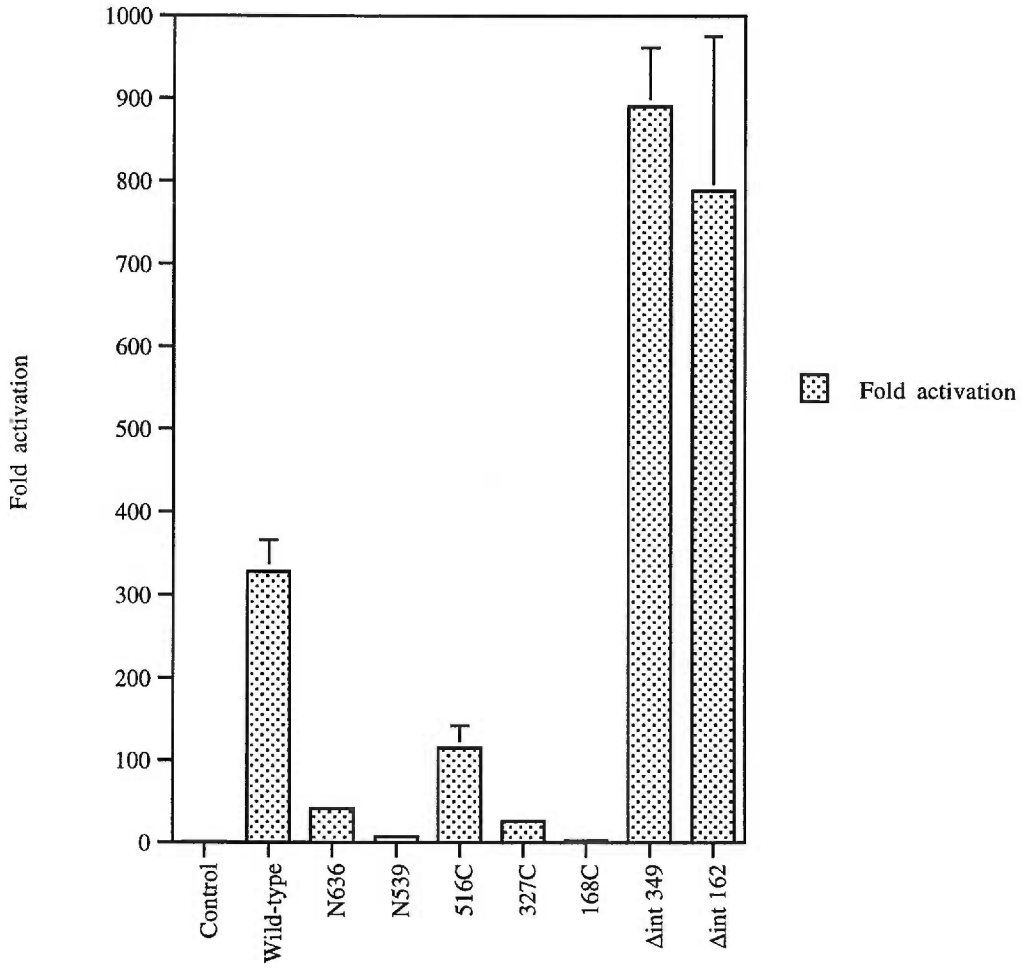

Figure 17. Sp1 domain requirement in IGF-IR activation.

SL2 cells were transiently transfected with 60 ng of wild-type and various deletion constructs of Sp1 (Figure 12) in 6-well plates along with 0.5 μg of the p0Luc/IGF-IR (-476/+640) and 2.5 μg of empty pcDNA3 plasmid as a carrier by the calcium phosphate precipitation method. Luciferase data was normalized with protein amounts. The experiment was done in triplicate and each experiment was repeated three times. Error bars represent standard deviations (SD:n=3).

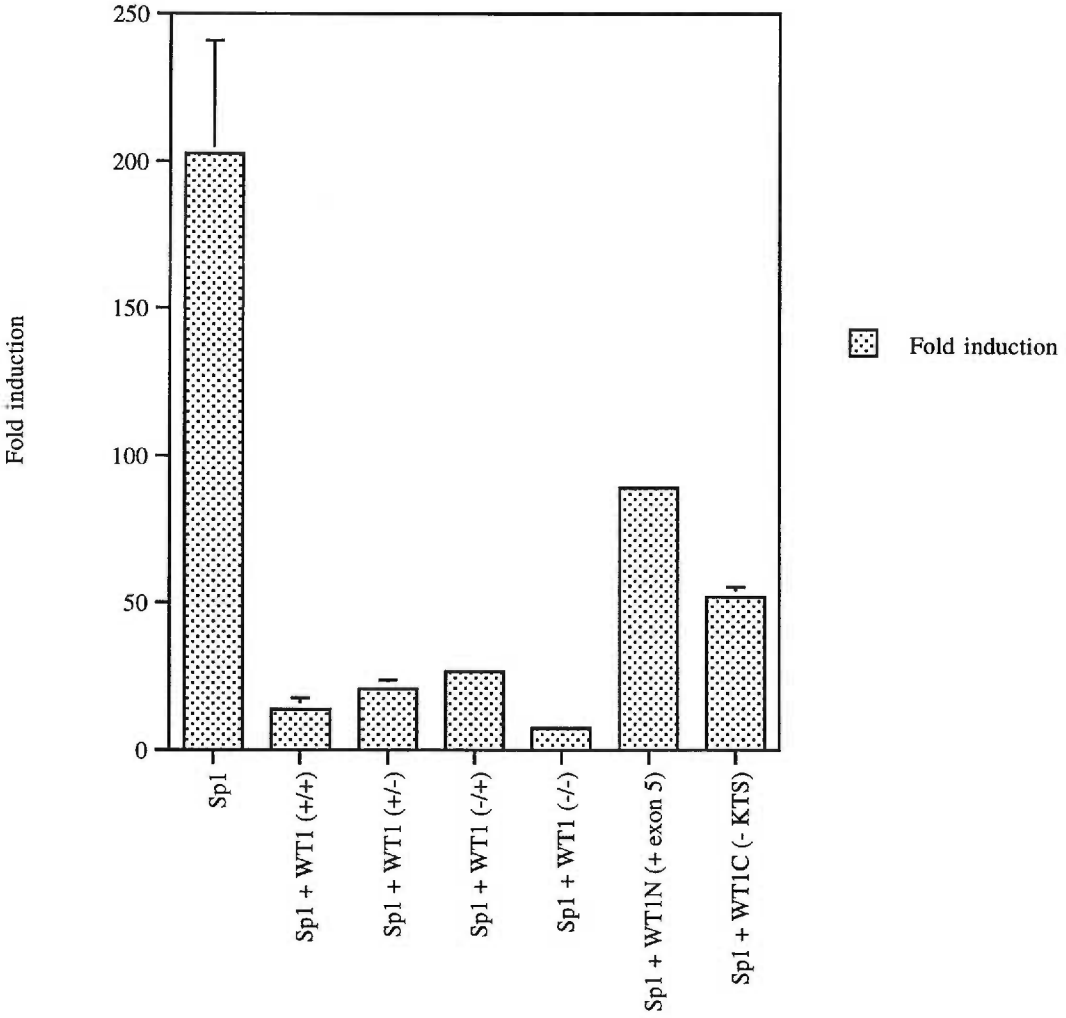

Figure 18. Attenuation of Sp1 activation by full-length and truncated WT1.

SL2 cells were transiently cotransfected with 50 ng of pPac/Sp1 and 2.5 µg of full-lengths and domains of pcDNA3/WT1 expression plasmids in 6-well plates along with 0.5 µg of a p0Luc/IGF-IR (-476/+640). + and - in the parenthesis indicate the presence or absence of exon 5 and KTS insertion in order. The amino-terminal domain contains the first 300 amino acids including putative transcriptional repression domain. The carboxy-terminus of WT1 consists of 150 amino acids containing zinc-finger DNA-binding domains. Luciferase data was normalized for protein amounts. The experiment was done in triplicate and each experiment was repeated three times. Error bars represent standard deviations (SD:n=3). The p value of WT1 domain co-transfections is $p<0.0001$ and considered significant.

activation of p0Luc/IGF-IR by Sp1. In the absence of exon 5, the KTS insertion increases repression, analogous to the data obtained in CHO cells (Figure 6). In the presence of exon 5, however, the presence or absence of the KTS sequence had little effect. As in CHO cells, the WT1 domain constructs exhibited partial repression. The amino-terminal (+ exon 5) and carboxy-terminal domain (-KTS) constructs exhibited 50 and 70 % repression, respectively.

In addition, the Sp1 domain(s) responsible for the WT1 attenuation were investigated by using four Sp1 constructs (N636, 516C, Δint349, and Δint162) (Figure 19). If domain(s) responsible for the functional interaction of WT1 and Sp1 are deleted, WT1 should not repress the Sp1 activation of the IGF-IR promoter. However, WT1 (-/-) exhibited ≈ 80 % repression for all Sp1 deletions and no apparent differential susceptibility of these mutants to the WT1 inhibition was observed.

Expression of Sp1 proteins introduced into SL2

As a control experiment, the expression level of introduced Sp1 in SL2 cells was examined by western immunoblotting (Figure 20). 1.5 μg of Sp1 expression plasmids was transiently transfected into SL2 cells seeded in 6-well plates. 100 μg of lysate was separated on 10 % SDS-PAGE gels. Transferred proteins were probed with an anti-Sp1 antibody, PEP-2 (Santa Cruz Biotechnology). Full-length, N636, N539, 516C, and 327C proteins contain an epitope recognized by the antibody. As shown, the relative expression levels were equal, except for the full-length form, which was slightly less expressed. The apparent lesser expression of the full-length Sp1 may mean that the residual activation of the amino- and carboxy-terminal deletion constructs shown in Figure 20 was over-estimated. The conclusion that these domains contribute to transcriptional activation is unaffected, however. No comparison of the relative level of expression of the full-length and internal deletion constructs was possible with the Sp1 antibody used. The proposed inhibitory function of the C domain of Sp1 in IGF-IR promoter regulation will require further confirmation.

Physical interaction of WT1 and Sp1

Since recognition sites for WT1 and Sp1 in the IGF-IR promoter sequence are adjacent and in some cases overlapping, it is feasible that WT1 and Sp1 placed in proximity may physically interact. To investigate this possibility, the yeast two-hybrid system was employed to examine the potential direct interaction of WT1 and Sp1.

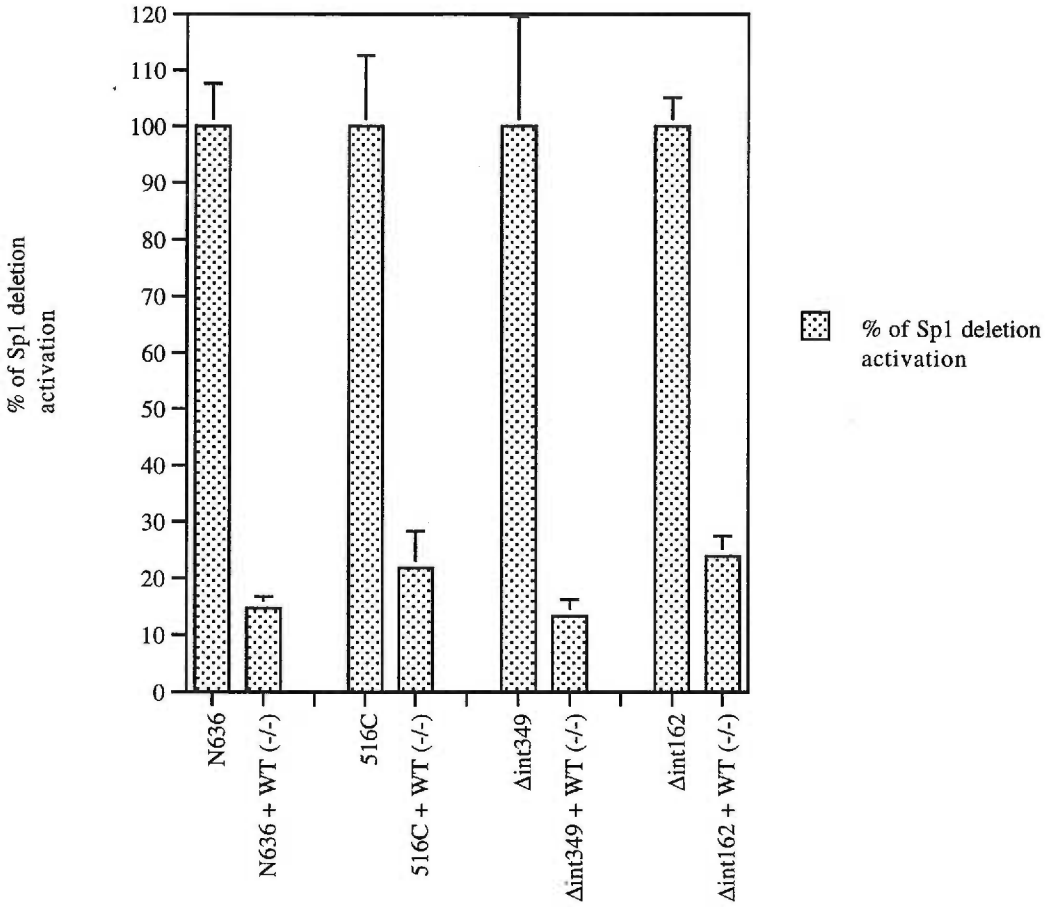

Figure 19. Functional interaction of WT1 with Sp1 domain mutants.

SL2 cells were transiently cotransfected with 60ng of various Sp1 deletion constructs and 2.5 μg of either an empty pcDNA vector or pcDNA3/WT1 (-/-) along with 0.5 μg of p0Luc/IGF-IR (-476/+640) in 6-well plates. Luciferase data was normalized for protein concentration. The experiment was done in triplicate and each experiment was repeated three times. Values shown for WT1 (-/-) are the precentage of the value obtained with the corresponding Sp1 construct alone, although the relative activity of these Sp1 constructs varied as shown in Figure 17. Error bars represent standard deviations (SD:n=3).

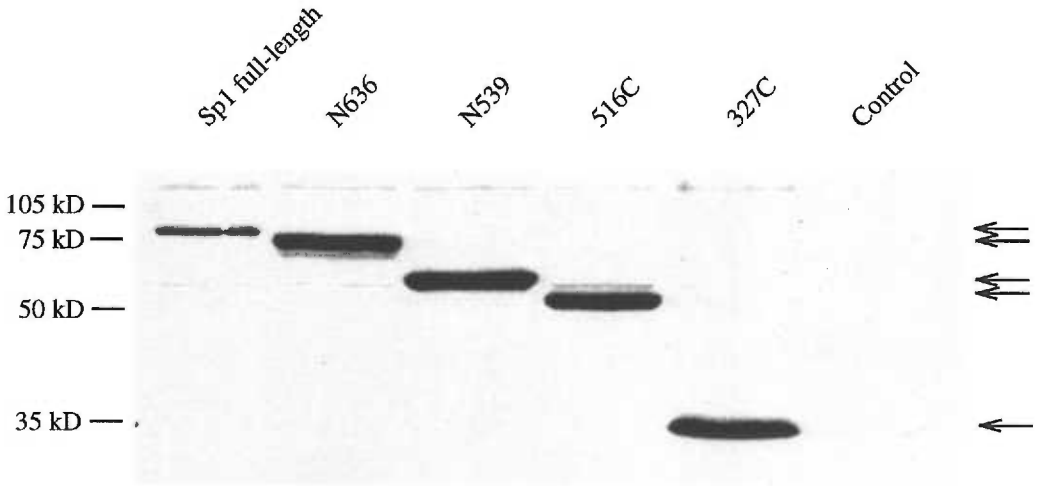

Figure 20. Expression of transfected Sp1 constructs in SL2 cells.

Expression level of exogenous Sp1 proteins were examined by western immunoblotting. 1.5 μg of various Sp1 expression plasmids were transfected into SL2 cells. Control sample was transfected with empty pPac expression plasmid. 72 hour posttransfection, cells were harvested, and 100 μg of lysates were separated on a 10 % SDS-PAGE gel. The proteins were transferred to nitrocellulose membrane and blotted with an anti-Sp1 antibody (PEP2). The arrows indicate full-length and deletion Sp1 proteins.

The amino- and carboxy-terminal domains of WT1 and full-length Sp1 were fused to the LexA DNA-binding domain. The amino- and carboxy-domains and full-length Sp1 were ligated to VP16 transcription activation domain in frame. These fusion constructs were separately introduced into different mating type yeast strains, L40 and AMR70. Transformants were then mated in the combinations described in Table I. Interaction of fusion proteins was assessed on a basis of blue (positive) and white (negative) color by the filter colorimetric assays of the conjugants (Vojtek et al., 1993). As shown, none of WT1 and Sp1 combinations exhibited blue color, indicating that there is no direct interaction between WT1 and Sp1. The detected interaction of full-length and amino-terminal domains of Sp1 is consistent with reports of self-oligomerization of Sp1 through an amino-terminal domain (Courey et al., 1989 and Pascal and Tjian, 1991).

As a second approach, coimmunoprecipitation of WT1 and Sp1 from CHO cells was performed. Sp1 is ubiquitously expressed in many cell types, including CHO cells. WT1 expression was induced by transient transfection using pcDNA3/WT1 (-/-). Lysate harvested from transfected CHO cells was incubated with an anti-WT1 antibody (C19) and subsequently with Protein-A conjugated Sepharose to form an immune complex. The immune complex was separated on a 10 % SDS-PAGE gel. Proteins in the complex were transferred to nitrocellulose and blotted with an anti-Sp1 antibody (PEP2). As shown in Figure 21, Sp1 protein was detected in the WT1 immune complex (lane 2) but not in lysate from transfected cells precipitated with protein A-Sepharose alone. Compared to the control lysate, in which both phosphorylated (p105) and unphosphorylated (p95) forms of Sp1 were detected (lane 4), only p95 Sp1 was coimmunoprecipitated with WT1. The small amount of Sp1 coimmunoprecipitated from untransfected cells presumably resulted from association with endogenous WT1 present at low levels in CHO cells. The Sp1 immunoprecipitated with endogenous WT1 was also the p95 form (lane 3). A reverse coimmunoprecipitation was performed using an anti-Sp1 antibody (PEP2) as a primary antibody to form a immune complex. No clear presence of WT1 in the Sp1 immune complex was discernible, in part due to the presence of primary Sp1 antibody heavy chain that migrated at the position of WT1, and which reacted with the secondary antibody used for the ECL detection of WT1.

Since the yeast two-hybrid assay data demonstrated no direct interaction, the coimmunoprecipitation results suggest that WT1 and Sp1 may indirectly physically interact with each other through an unknown protein(s). In addition, since only p95 form of Sp1 was precipitated by WT1, the phosphorylation state of Sp1 may affect its association with WT1.

Table I

WT1-Sp1 association using yeast two-hybrid system

Yeast strains, L40 and AMR70, were tranformed with plasmids encoding the indicated chimeric fusion proteins. Transformed L40 and AMR70 cells were then mated to form conjugants containing the reporter LexAop-LacZ. Interaction between fusion proteins was assessed on a basis of blue (positive) and white (negative) color of the conjugants transfered to membranes soaked with X-gal substrate solution (Vojtek et al., 1993).

DNA-binding domain (L40)	Transcription activation domain (AMR70)	Conjugant color
LexA-Lamin C	VP16	White
LexA-WT1(1-300)	VP16-Sp1(1-560)	White
LexA-WT1(1-300)	VP16-Sp1(561-778)	White
LexA-WT1(301-445)	VP16-Sp1(1-560)	White
LexA-WT1(301-445)	VP16-Sp1(561-778)	White
LexA-WT1(301-445)	VP16-Sp1(1-778)	White
LexA-Sp1(1-778)	VP16-Sp1(1-560)	Blue
LexA-Sp1(1-778)	VP16-Sp1(561-778)	White
LexA-Sp1(1-778)	VP16-Sp1(1-778)	Blue

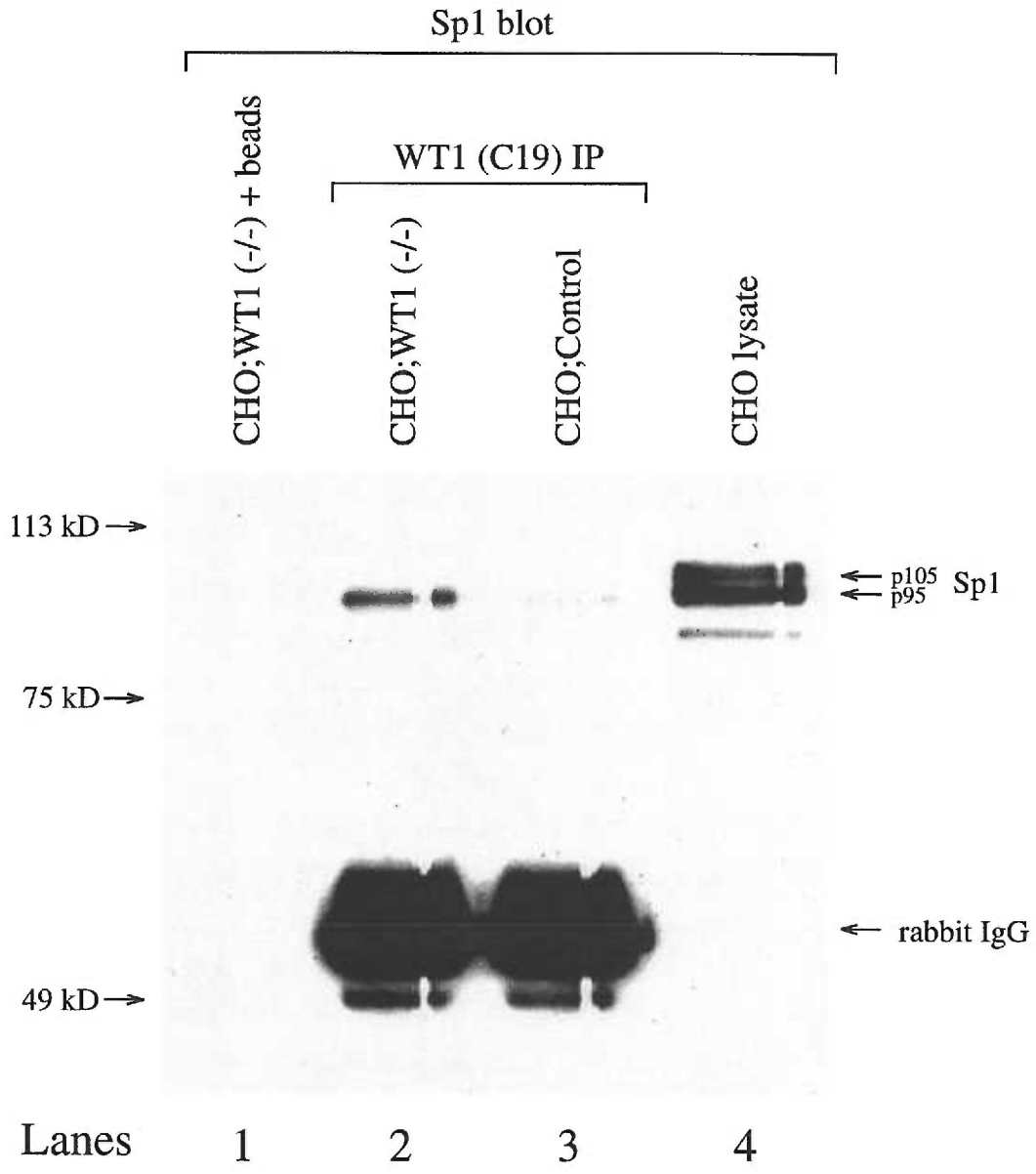

Figure 21. Coimmunoprecipitation of WT1 and Sp1.

500 µg of lysates from CHO cells transfected with WT1 (-/-) construct or empty pcDNA3 plasmid (control) were incubated with an anti-WT1 antibody (C19) overnight. The immune complex was incubated with Protein A-conjugated sepharose for 2 hours. The immune complex was separated on a 10 % SDS-PAGE gel. Proteins were transferred to nitrocellulose membrane and probed with an anti-Sp1 antibody (PEP2). Sp1 p95 was detected in the WT1 immune complex (lane 2). Control lysate exhibited slight Sp1 precipitation (lane 3). Protein A-conjugated sepharose did not precipitate WT1 or Sp1 (lane 1). CHO lysate transfected with WT1 (-/-) (20 µg) contains both p105 and p95 forms of Sp1 (lane 4). Lower bands are rabbit IgG from C19 antibody used for immunoprecipitation that was detected by secondary anti-rabbit IgG antibody.

Discussion and Conclusions

Differential repression activity of naturally occurring WT1 variants in the the regulation of IGF-IR gene expression

In this study, I examined the potential differential activity of WT1 variants in IGF-IR promoter repression. Transient transfection reporter gene assays demonstrated that the (+/-) and (-/-) forms of WT1 repressed IGF-IR promoter activity stronger than (+/+) and (-/+) forms in CHO and G401 cells (Figure 6), suggesting that the presence or absence of the KTS insertion between zinc fingers 3 and 4 influences transcriptional activity. This observation was expected, since the differential repression activity of WT1 variants has been demonstrated with other gene promoters, including RAR-α (Goodyer et al., 1995), TGF-β1 (Dey et al., 1994), PDGF A-chain (Wang et al., 1995) and Pax-2 (Ryan et al., 1995). These reports illustrate that -KTS forms of WT1 have higher repression activity regardless of the presence or absence of the exon 5 sequence, presumably by disturbing DNA-binding capability. To support this notion, DNA-binding studies with these WT1 variants with the presence or absence of the KTS insertion demonstrate that the +KTS form is less capable of binding DNA using synthetic oligonucleotides (Rauscher et al.,1990 and Bickmore., 1992) and native sequences found in the PDGF A-chain gene promoter (Wang et al.,1993) and in the Pax-2 gene promoter (Ryan et al., 1995). Another type of naturally occurring WT1 variant results from RNA editing (Sharma et al., 1994). The RNA edited form of WT1 (conversion of amino acid leucine 280 to proline) was shown to exhibit reduced repression of the Egr-1 gene promoter. However, the RNA-edited form of WT1 exhibited even better repression of IGF-IR promoter activity (Figure 8). Since the amino-terminal domain affected by RNA editing is thought to be involved in protein-protein interaction, this higher repression activity might result from changes in its protein association. However, the role of the RNA editing form of WT1 in IGF-IR gene regulation will require further study.

In addition, WT1 domains were examined for IGF-IR promoter repression. Interestingly, the amino- and carboxy-terminal domains of WT1 exhibited partial repression activity (Figure 7). Repression activity was 50 % with amino-terminal and 30 to 60 % with carboxy-terminal domains in the presence or absence of the KTS insertion, respectively. These results raise two possible mechanisms for WT1 function. 1, Requirement of zinc-finger domains that most likely interfere with other DNA-binding factor(s) critical for IGF-IR expression. 2, An involvement of the amino-terminal domain of WT1, which may be through an interaction with factors required for IGF-IR expression and an inhibition of their function. This observation was unexpected since the same amino- and carboxy

-ternimal domains of WT1 appeared to retain no repression activity with many target genes, including PDGF A-chain (Wang et al., 1992 and Wang et al., 1995), Egr-1 (Madden et al., 1991), RAR-α (Goodyer et al., 1995), and WT1 itself (Rupprecht et al., 1994).

IGF-IR promoter repression by mutant forms of WT1

An examination of WT1 repression in IGF-IR gene promoter activity was extended to clinically relevant and deletion forms of WT1. The WAGR mutation (substitution of glycine 201 with aspartic acid) exhibited ≈ 80 % repression, which is stronger than wild-type WT1 (Figure 8). This is a intriguing observation since Park et al. (1993) demonstrated that this substitution converts the WT1 protein from a transcriptional repressor to an activator in the context of Egr-1 regulation. In addition, a WT1 construct deleted in one of the proline-rich clusters (proline-rich deletion) was examined in the same context (Figure 8). The proline-rich deletion showed maximum repression activity among the WT1 constructs examined in this study. A similar proline-rich deletion construct (deletion of amino acids 56 to 67) was examined for Egr-1 promoter activity (Madden et al., 1993). The construct exhibited partial, but reduced repression function. These results demonstrate that these alterations in the amino-terminal region do not disrupt, but rather enhance WT1 transcriptional repression potential in the context of IGF-IR gene regulation through an unknown mechanism.

Self-association is not required for IGF-IR repression by WT1

Previous reports of WT1 self-association defined regions responsible for the interaction to the first 182 amino acids (Reddy et al., 1995 and Moffett et al .,1995). To determine effects of mutant forms of WT1 on self-association activity, WT1 self-association assays were performed with amino-terminal domains of wild-type and altered forms using the yeast two-hybrid system (Figures 13 and 14). Homologous combinations of WAGR-associated, RNA editing, and proline-rich deletion forms of WT1 amino-terminal domains exhibited severely reduced self-association capability. This might be due to a redistribution of charge (WAGR-associated: glycine to aspartic acid) or spatial hindrance (RNA editing: leucine to proline). The proline-rich deletion may lack a critical domain required for self-association. Alternatively, it might be potentially due to a conformational change caused by the large deletion. Since these mutations did not affect IGF-IR promoter repression as shown above, these data strongly suggest that WT1 self-association is not absolutely required for IGF-IR gene regulation. However, the role of

self-association in WT1 gene regulation has been suggested in another context by Reddy et al. (1995). In that report, Reddy et al. concluded that self-association was critical in WT1 function since: 1, WT1 transcriptional activation using an artificial promoter construct, EGR3tkCAT, which contains 3 Egr-1 binding sites fused to the human tymidine kinase promoter and the CAT gene, was disrupted by mutant WT1 proteins that lack DNA-binding capability. 2, These mutant WT1 proteins still could self-associate. However, since this observation was based on a synthetic promoter and this transcriptional activation by WT1 is not seen with native promoters of WT1 target genes, the biological relevance of this study is not clear. Also, Moffett et al. (1995) demonstrated that the WAGR-associated mutation had no effect on the self-association capability of WT1 by assessing wild-type and WAGR mutant WT1 proteins as a heterodimer using far-western blotting. However, in the clinical situation, the WAGR mutation was found in a patient that has one allele of 11p13 deleted with the other containing the mutation; thus only the mutant allele is functional. Therefore, examination of WAGR-WAGR self-association would be more appropriate.

In this self-association study, differential behavior of heterodimers with wild-type/mutant forms of WT1 was observed. The self-association capability of heterodimers was drastically disrupted by 3-AT titration, but not in β-galactosidase assays. Physical interaction between wild-type and mutant forms of WT1 may occur, and this interaction may be strong enough to drive the β-galactosidase reporter gene, but not the histidine synthase gene.

Dominant function of DDS mutant in regulation of the IGF-IR gene

DDS mutations function in a autosomal dominant fashion (Coppes et al., 1993). Moffett et al. (1995) demonstrated that the most common DDS mutant (R394W) itself does not have repression activity and interfered with the function of wild-type WT1 in a cotransfection study with RAR-α promoter constructs. More recently, Holmes et al. (1997) reported that a short amino-terminal fragment of WT1 inhibits transcriptional activity of the wild-type protein, but retains self-association capability. These findings suggested that the DNA binding-deficient DDS mutant likely functions in a dominant-negative fashion. To evaluate that notion, the DDS mutant (R394W) was examined for regulation of IGF-IR promoter activity (Figure 9). Unexpectedly, the DDS mutant protein functions in this context. Both (+/+) and (-/-) forms of DDS showed the same degree of repression (≈ 50 %). The DDS mutant in conjunction with wild-type WT1 exhibited an additive effect (Figure 10), further supporting the dominant mode of the DDS mutant protein in the context of IGF-IR gene regulation. Therefore, I propose here that the DDS mutant protein

regulates the IGF-IR gene differently from wild-type WT1, and potentially recognizes novel target genes. Differential regulation of target genes by wild-type WT1 and the DDS mutant protein is consistent with the clinical phenotypes, in which Wilms' tumor and DDS are significantly different.

This dominant function of DDS mutant may require DNA binding, with the DDS mutant binding to novel target sequences (gain-of-function). Previously, the DDS mutant (R394W) protein produced in bacteria as a fusion protein or in an *in vitro* translation system using a rabbit reticulocyte lysate was unable to bind to the EGR consensus binding sequence (Pelletier et al., 1991 and Little et al., 1995). In addition, it was demonstrated that the bacterially produced zinc-finger domain protein of the DDS mutant did not identify obvious DNA-binding motifs using modified EGR consensus recognition sequences, even though it might have slightly different binding properties from wild-type WT1: 5' GCG-(G/T)-(G/A)-(G/T)-GCG 3' (middle three nucleotides are recognized by zinc finger 3) (Borel et al., 1996). However, since these studies employed the bacterially produced protein, it might not reflect the biological activity of the full-length protein because of an aberrant conformation of the protein resulting from renaturing and refolding procedures in preparation and a lack of posttranslational modifications, e.g. phosphorylation and glycosylation. In addition, potential interaction of the DDS mutant protein with another recognition sequence $(TCC)_n$ has not been addressed. Therefore, it is still possible that the DDS mutant protein could bind to $(TCC)_n$ elements or other target sequences.

Alternatively, although the DDS mutant protein does not retain DNA-binding capability, as described in previous studies, it might exert transcriptional repression through its amino-terminal domains as demonstrated in Figure 6. The same degree of transcriptional activity of (+/+) and (-/-) of the DDS mutant is consistent with this idea.

Sp1 domains required for the activation of IGF-IR promoter

To identify domain(s) responsible for IGF-IR activation, various Sp1 deletion constructs were examined in IGF-IR promoter activation (Figure 17). The results reveal that (1) DNA-binding by Sp1 is necessary for transcriptional activation, (2) the carboxy-terminal end of Sp1, domain D, contributed significantly to transcription activation, (3) the two pairs of glutamine- and serine/threonine-rich domains in the amino terminus collectively contribute to transcritptional activation, and (4) an internal highly charged region, domain C, can inhibit transcriptional activation. These results are consistent with previous observations that domains A, B, and D are involved in synergistic transcriptional

activation (Courey et al., 1989 and Pascal and Tjian, 1991) that has been demonstrated in many contexts.

The enhanced activation exhibited by these internal deletion constructs could be explained by two possible mechanisms. One would be that the internal deletion constructs place the transcriptional activation domains (Domain A and B) and the DNA-binding domains in closer proximity, making transcriptional activation more efficient. However, this possibility is unlikely because the large deletion in the intΔ349 construct exhibited the same activation as the shorter deletion mutant Δint162. Additionally, the successful use of the yeast two-hybrid system is, in fact, based on the lack of specific requirement for spacing of transcriptional activation and DNA-binding domains.

An alternative possibility is that domain C functions simply as an inhibitory domain. An inhibitory function for domain C is supported by the study of Sp3, a Sp1 family transcription factor that has now been shown to have dual activities carried out by defined transcriptional activation and inhibitory domains. Dennig et al. (1996) reported that a highly charged amino acid domain found in Sp3 that is homologous to domain C of Sp1 exhibits transcriptional inhibitory activity. In fact, inhibitory domains have been described in several transcriptional activators, including c-Jun (Baichwal and Tjian, 1990), Egr-1 (Gashler et at., 1993), C/EBP (Nerlov and Ziff, 1994), and ATF-2 (Li and Green, 1996). The function of this inhibitory domain in Sp1 might be modulation of its activational function. This was demonstrated in the case of c-Jun (Baichwal et al., 1991) and ATF-2 (Li and Green, 1996). There are several possible mechanisms for this transcriptional inhibition of Sp1. First, the inhibitory domain masks the activation domain intramolecularly. This was shown for the ATF-2 inhibitory domain (Li and Green, 1996). Second, the inhibitory domain may interact with transcriptional inhibitory protein(s). Third, the inhibitory domain interacts with and sequesters other transcription activator proteins. Fourth, the inhibitory domain directly associates with the basal transcriptional machinery. This was demonstrated by the association of the Drosophila zinc-finger protein Krüppel with $TF_{II}E\beta$, which resulted in transcriptional repression (Sauer et al., 1995).

Functional interaction of WT1 and Sp1 in regulation of the IGF-IR gene

The GC-box and $(TCC)_n$ motif recognition sites of WT1 and Sp1 are closely related to each other. The presence of adjacent and overlapping target sequences for WT1 and Sp1 in the IGF-IR promoter (summarized in Figure 3) indicated possible functional interactions of WT1 and Sp1. In this study, the functional interaction between WT1 and Sp1 in the regulation of IGF-IR was examined by using reporter gene assays in SL2 cells

(Figure 18). Robust Sp1 activation of the IGF-IR promoter was significantly reduced by co-expression of WT1. In the absence of exon 5, the KTS insertion increased repression, which correlated with the data obtained in CHO cells (Figure 6). In the presence of exon 5, repression activity was similar in the presence or absence of the KTS insertion; which is different from the results in CHO cells. This KTS-independent repression may reflect cell type-specific mechanisms.

The potential role of specific WT1 domains was also examined (Figure 18). As seen in CHO cells, both amino- and carboxy-terminal domains exhibited partial repression. These results suggest several possible mechanisms for the functional interaction of WT1 and Sp1; 1, WT1 interferes with Sp1 for target DNA sequences through its zinc-finger domains. 2, The amino-terminal domain of WT1 associates with Sp1 domains that involve transcriptional activation. 3, The amino-terminal domain of WT1 sequesters factor(s) required for Sp1 activation. 4, The amino-terminal domain of WT1 interacts with transcriptional inhibitory protein(s).

In addition, the localization of Sp1 domain(s) responsible for the functional interaction with WT1 was examined using four Sp1 constructs (N636, 516C, Δint349, and Δint162) that exhibited IGF-IR promoter activation (Figure 19). These studies did not identify a specific domain of Sp1 involved in WT1 inhibition of Sp1 activation. These data indicate that the functional interaction of WT1 and Sp1 involves either DNA-binding domain or a small region between the first glutamine- and second serine/threonine-rich domains that is conserved in these deletion mutants. In fact, the DNA-binding domain of Sp1 is involved in association with other proteins (summarized in Figure 2) and may also be necessary for WT1 interaction, although deleting the DNA-binding domain abolishes activation completely, making it difficult to assess functional interaction in cotransfection studies.

Physical interaction between WT1 and Sp1

The functional interactions between WT1 and Sp1 observed in IGF-IR regulation might involve their physical interaction since WT1 and Sp1 are placed in proximity by binding to closely apposed target DNA sequences in the IGF-IR promoter. The possibility of WT1 and Sp1 interaction was examined by coimmunoprecipitation (Figure 21). The physical interaction of Sp1 with WT1 (-/-) was detected using an anti-WT1 antibody, C19 (Figure 21, lane 2). Sp1 protein migrates as two bands on SDS-PAGE, p95 and p105, depending upon phosphorylation state (Jackson et al., 1990). Interestingly, only the p95 non-phosphorylated form of Sp1 coimmunoprecipitated with WT1 (Figure 21, lane 2).

Although the role of phosphorylation in Sp1 function is not fully understood, phosphorylation modifies the DNA-binding capability of Sp1; DNA binding activity of Sp1 is reduced by casein kinase II (Armstrong et al., 1997) and is increased by cAMP-dependent protein kinase (PKA) (Rohlff et al., 1997), which results in modulation of transcriptional activity. In addition, phosphorylation of Sp1 might function in subcellular localization, by analogy with signal transducers and activators of transcription (STATs) (Darnell et al., 1997). The fact that WT1 could bind to unphosphorylated Sp1 suggests a possible mechanism for the functional interaction of WT1 and Sp1; WT1 associates with the unphosphorylated form of Sp1 to inhibit Sp1 activation of gene expression. When Sp1 is phosphorylated, Sp1 is free from sequestration by WT1 in nucleus. Indeed, functional data obtained from transient transfection studies support this notion. In CHO cells, both phosphorylated and unphosphorylated Sp1 proteins are present with the unphosphorylated form slightly more abundant (Figure 21, lane 4). The WT1 (-/-) form exhibited maximum 80 % inhibition of the IGF-IR promoter in these cells (Figure 6) that is significantly reduced compared to the repression seen in SL2 cells, in which the WT1 (-/-) repressed more than 90 % (Figure 18). Since the introduced Sp1 in SL2 cells was presumably the unphosphorylated and unglycosylated form based on its size (≈ 80 kD; Figure 20) that is significantly smaller than endogenous Sp1 proteins in CHO cells, cotransfected WT1 may associate with this unphosphorylated Sp1 protein better, thereby inhibiting Sp1 function stronger in SL2 cells than in CHO cells.

WT1 and Sp1 interaction was also assessed using the yeast two-hybrid system (Table I). In these assays, an attempt to detect direct interaction of WT1 and Sp1 was not successful using amino- and carboxy-terminal domains of WT1 and full-length and domains of Sp1. Although it is possible that the junction of the amino- and carboxy-terminal regions of WT1 is critically involved in WT1 and Sp1 interaction, the yeast two-hybrid data may imply the involvement a third protein(s) in the physical interaction of WT1 and Sp1.

Taken together, the functional and physical interactions of WT1 and Sp1 suggest several possible transcriptional mechanisms for IGF-IR gene regulation: 1, WT1 interferes with Sp1 for binding to target DNA sequences through its zinc-finger domains. This is supported by the fact that carboxy-terminal domain of WT1 retains partial repression activity. 2, The amino-terminal domain of WT1 sequesters factor(s) required for Sp1 activation. 3, Physical interaction of WT1 and Sp1 inhibits other factor(s) required for Sp1 activation to associate with Sp1. 4, Physical interaction of WT1 and Sp1 in the absence of WT1 DNA-binding could prevent DNA binding by Sp1. Partial repression by the amino-terminal domain of WT1 might be explained by either mechanism 2 or 4.

Considerations of the functional and physical interaction between WT1 and Sp1 in IGF-IR gene regulation

To date, only two transcription factors have been identified to interact with WT1; the p53 tumor suppressor (Maheswaran et al., 1993 and 1995), and a prostate apoptosis response protein, par-4 (Johnstone et al., 1996). An association of WT1 with p53 modifies both WT1 and p53 transcriptional function, decreasing WT1 repression activity and increasing p53 transactivation. Also, par-4 modulates WT1 repression; inhibition of WT1 transcription activation and augmentation of WT1 transcriptional activation using synthetic EGRtkCAT and GAL4tk promoter constructs, respectively. On the other hand, physical interaction between Sp1 and other transcription factors has been demonstrated; these include the zinc-finger protein YY1 (Lee et al., 1993), GATA-1 (Merika et al., 1995), and the RelA (p65) NFκB subunit (Perkins et al., 1994). These associations require the zinc-finger region of Sp1 (summarized in Figure 2). In addition, a TATA association factor, $TAF_{II}110$, that is one component of the basal transcriptional machinery binds to a region between the B and C domains (Hoey et al., 1993). Therefore, in the large scheme of IGF-IR gene regulation, these factors possibly modulate the interaction of WT1 and Sp1. Additionally, the interaction of WT1 and Sp1 might influence the activity of these factors.

The functional interaction of Egr-1 and Sp1 has been demonstrated in several contexts; 1, Synergistic activation of the Tissue factor gene promoter (Cui et al., 1996). 2, Egr-1 displacement of Sp1 binding to recognition sequences in the PDGF A-chain (Khachigian et al., 1995) and Interleukin 2 promoters (Skerka et al., 1995). 3, Differential activation in phenylethanolamine N-methyltransferase (PNMT) gene promoter (Ebert and Wong, 1995). 4, Activation by Sp1 and repression by Egr-1 in the adenosine deaminase promoter (Ackerman et al., 1991). As would have been predicted from the results of these studies, this functional interaction between WT1 and Sp1 was demonstrated in the regulation of p75 neurotrophin receptor gene (Poukka et al., 1996). This functional interaction also potentially applies to other genes that were shown to be regulated by WT1 and Sp1, including CSF-1, TGF-β1, c-myc, $p21^{WAF1/CIP1}$, RAR-α, and WT1. In particular, the IGF-II gene is an interesting target to be examined since the IGF-II and IGF-IR gene products comprise an autocrine axis for IGF action. If this is the case, transcriptional regulation by WT1 and Sp1 is critical in IGF function and its aberrant regulation significantly contributes to Wilms' tumor development and other clinical manifestations.

Considerations of gene transfections

In the transient transfection assays in CHO and G401 cells, a pCMV/β-gal construct was cotransfected with other expression plasmids for correction of transfection efficiency. This CMV promoter-driven β-gal construct is commonly used for normalization of transfection efficiency. However, we routinely observed lower β-galactosidase activity in WT1 transfectants and relatively high activity (> 2-fold) in control cells transfected with empty pcDNA3 expression plasmid. Initially, I considered this as a reflection of transfection efficiency, but this observation was consistent over many experiments. Also, β-galactosidase assays in SL2 cell transient transfections produced intriguing results. Sp1 transfectants and control experiments exhibited high and low β-galactosidase activities, respectively, with four-fold differences commonly seen.

These observations imply that exogenous WT1 and Sp1 might regulate the CMV promoter in the β-galactosidase construct in a negative and a positive manner, respectively. This is one of the potential problems in transfection studies. If the transfection efficiency is normalized with β-galactosidase activity from an internal control plasmid, the results will underestimate the transcriptional activity of WT1 and Sp1, which means that the apparent repression activity of WT1 and transactivation of Sp1 are less than actual activity. In order to avoid misinterpretation of data, normalization with protein amount was employed in the case of SL2 transfections, and experiments were repeated enough times to obtain consistent results. On the other hand, the repression activity of WT1 in mammalian cell transfections was probably underestimated since these data were normalized for β-galactosidase activity. Alternative approaches would include the use of a β-galactosidase expression plasmid driven by a different promoter (which could still, however, be regulated by cotransfected expression vectors), or by cotransfection of the β-galactosidase protein itself (Howcroft et al., 1997).

Future directions

In this study, a -476/+640 fragment of the IGF-IR promoter segment was used to assess the transcriptional regulatory effects of WT1 and Sp1. This region was previously demonstrated to exhibit maximum promoter activity and contains all of the WT1 and Sp1 sites defined by both gel-shift and DNase I footprinting analyses. The data obtained using this construct should thus reflect the maximum range of WT1 and Sp1 actions and

interactions analyzable in a transient transfection approach, and provides the foundation for several future lines of investigation.

1. The use of individual 5'-flanking and 5'-UTR sequences (including the initiator region) as well as specific motifs (in particular the 5'-flanking and 5'-UTR regions that contain overlapping WT1 and Sp1 sites) in transient transfection assays would provide a more detailed picture of specific WT1-Sp1 interactions and their contribution to overall IGF-IR promoter activity.

2. The IGF-IR promoter reporter plasmid used in transient transfection is unlikely to exhibit authentic chromatin architecture (Smith and Hager, 1997). Transfection of WT1 and Sp1 expression plasmids into cell lines containing stably integrated copies of IGF-IR promoter fragments fused to a suitable reporter gene would allow the analysis of the IGF-IR promoter by these factors in a chromatin context that may closely approximate that of the endogenous IGF-IR gene.

3. The establishment of inducible cell lines expressing regulatable WT1 would allow the verification of WT1 regulation of the endogenous IGF-IR gene through the use of nuclear run-on assays or analysis of IGF-IR pre-mRNA.

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