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Regulation of Insulin-Like Growth Factor I Receptor Gene Expression by the Wilms' Tumor Supressor WT1

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Abstract

The insulin-like growth factor I receptor (IGF-I-R) has been implicated in the etiology and/or progression of Wilms' tumor, or nephroblastoma, a pediatric neoplasm of the kidney that is often associated with deletion or mutation of the WT1 tumor suppressor gene. The levels of IGF-I-R mRNA in the tumors were sixfold higher than in normal adjacent kidney tissue and were inversely correlated to the levels of WT1 mRNA, suggesting that the expression of the IGF-I-R gene is under inhibitory control by WT1. Cotransfection of an IGF-I-R promoter-luciferase reporter construct together with a WT1 expression vector resulted in a dose-dependent suppression of promoter activity. Multiple WT1 binding sites were mapped in the 5'-flanking and 5'-untranslated regions of the IGF-I-R gene using gel retardation and DNaseI footprinting assays. Thus, suppression of the IGF-I-R promoter by WT1 involves multiple interactions of its zinc finger domain with sites located both upstream and downstream of the transcription initiation site. Finally, we showed that expression of the endogenous IGF-I-R gene is decreased in G401 cells stably transfected with a WT1 expression vector. Reduction in expression of the IGF-I-R gene is associated with a decrease in a number of IGF-I-mediated biological effects. Thus, deletion or mutation of the WT1 gene in Wilms' tumor and other malignancies can result in overexpression of the receptor, with enhanced autocrine/paracrine activation by locally produced or circulating IGFs.

Index Entries: IGF-I receptor; WT1; tumor suppressors; Wilms' tumor; transcription; gene expression.

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Introduction

The insulin-like growth factor I receptor (IGF-I-R) is a membrane-bound tyrosine kinase that mediates most of the biological actions of the IGFs, a family of mitogenic peptides with important roles in growth and differentiation (Daughaday and Rotwein, 1989; LeRoith et al., 1991; Cohick and Clemmons, 1993; Werner et al., 1994a). The receptor is composed of two extracellular α subunits that are involved in ligand binding and two transmembrane β subunits. Alpha and β subunits are generated by proteolytic cleavage of a precursor protein that is the product of a single gene located at the distal end of chromosome 15 (Ullrich et al., 1986; Nissley and Lopaczynski, 1991; Werner et al., 1991; Abbott et al., 1992). The IGF-I-R is expressed by virtually every cell and tissue, consistent with the role of the IGFs as progression factors during the cell cycle (Lowe, 1991).

In addition, the IGF-I-R fulfills a central role in transformation and proliferation events (Baserga and Rubin, 1993; Baserga et al., 1994). Thus, fibroblast cell lines derived from mouse embryos in which the receptor gene has been disrupted cannot be transformed by simian virus 40 T antigen or by an activated Ras (Sell et al., 1993). Reintroduction of a functional receptor renders the cells susceptible to transformation by these and other oncogenes. The expression of the IGF-I-R gene has been shown to be regulated by a number of growth factors and oncogenes, suggesting that activation of this receptor may be a common pathway in tumorigenesis (Kaleko et al., 1990; Coppola et al., 1994; Rubini et al., 1994).

In tumors of the central nervous system (CNS) in particular, the IGF-I-R has been shown to mediate the autocrine and paracrine effects of the IGFs (Glick et al., 1993). Thus, in rat C6 and human T98G glioblastoma cells introduction of antisense RNA to the IGF-I receptor resulted in the inhibition of IGF-I-mediated growth (Ambrose et al., 1994; Resnicoff et al., 1994).

To understand the molecular mechanisms involved in regulation of IGF-I-R gene expression, we characterized its regulatory region (Werner et al., 1990, 1992). The promoter region of the IGF-I-R gene lacks TATA and CAAT elements and is especially GC rich (Cooke et al., 1991; Mamula and Goldfine, 1992). Transcription from this gene is initiated from a unique "initiator" motif that acts in concert with upstream Sp1 sites (Smale and Baltimore, 1989).

The promoter region of the IGF-I-R gene contains, in addition, multiple putative binding sites for members of the early growth response (EGR) family of transcriptional activators, including the tumor suppressor WT1 (Sukhatme, 1992; Werner et al., 1994b). WT1 is a DNA-binding protein with a glutamine- and proline-rich *N*-terminus and four zinc fingers in its C-terminus (Morris et al., 1991; Van Heyningen and Hastie, 1992; Rauscher, 1993). WT1 has been shown to repress the activity of a number of promoters containing the consensus sequence 5'-GCGGGGGCG-3', including the IGF-II, PDGF-A chain, TGF-β, CSF-1, and Pax-2 genes (Madden et al., 1991; Drummond et al., 1992; Gashler et al., 1992; Harrington et al., 1993). The WT1 gene is expressed in the kidney, gonadal ridge, spleen, brain, and spinal cord during embryonic development, and in the kidney, gonads, and uterus in the adult (Buckler et al., 1991; Pelletier et al., 1991). Inactivation or deletion of the WT1 gene was postulated to be a key event in the etiology of a subset of Wilms' tumors, a pediatric kidney neoplasm (Call et al., 1990; Gessler et al., 1900; Rose et al., 1990).

A role for the IGF-I-R in the etiology of Wilms' tumor was inferred from the observation that an antibody to the human receptor inhibited ^{125}I-IGF-I binding and IGF-I-stimulated thymidine uptake by Wilms' tumor cells in culture (Gansler et al., 1989). Furthermore, ip injection of this antibody to nude mice carrying Wilms' tumor heterotransplants prevented tumor growth and resulted in partial tumor remission. The present study was therefore designed to gain further insight into the role of the IGF-I-R in Wilms' tumor and to define the molecular mechanisms involved in the regulation of IGF-I-R gene expression by WT1.

Materials and Methods

Tissue Samples

Twenty-five samples of Wilms' tumors and seven samples of normal adjacent kidney tissue were dissected from surgical specimens obtained

at the Department of Pathology and Laboratory Medicine of the Medical University of South Carolina. Samples were frozen in liquid nitrogen and stored at –80°C until RNA preparation. Tumors were divided in two groups: those containing differentiated heterologous elements, including striated muscle, cartilage, and bone, and those tumors lacking these elements, i.e., composed primarily of blastema with epithelial differentiation.

Cell Cultures

Chinese hamster ovary (CHO), G401, and 293 cells were obtained from the American Type Culture Collection (Rockville, MD). G401 is a human kidney sarcoma-derived cell line that was originated from a tumor explant obtained from a 3-mo-old male, and 293 is a transformed human embryonic kidney cell line. CHO cells were maintained in Ham's F12 nutrient mixture with 10% fetal bovine serum (FBS), G401 in McCoys' 5A medium with 10% FBS, and 293 in Dulbecco's modified Eagle's medium with 10% FBS.

RNA Preparation

RNA was prepared from tumor samples using the RNazol kit. For RNA isolation from G401 and 293 cultures, cells were lysed in 4*M* guanidinium isothiocyanate containing 0.01% mercaptoethanol and ultracentrifuged on a cesium chloride gradient as described (Chirgwin et al., 1979). RNA was quantitated by measuring its A_{260} and its integrity was confirmed by ethidium bromide staining of the 28S and 18S rRNA bands after gel electrophoresis.

Northern Blot Analysis of WT1 mRNA

Poly(A)$^+$ RNA was prepared from G401 and 293 cells by oligo(dT)-cellulose chromatography. Poly(A)$^+$ RNA was electrophoresed through a 1.2% agarose–2.2*M* formaldehyde gel, transferred to a nylon membrane, and baked for 2 h at 80°C under reduced pressure. Blots were hybridized with an ~1.8-kb *Eco*RI fragment of the human WT33 cDNA clone (Call et al., 1990), labeled by a modified random priming technique. Blots were hybridized and washed following standard protocols.

Solution Hybridization-RNase Protection Assay of IGF-I-R mRNA

Levels of IGF-I-R mRNA were determined by a solution hybridization-RNase protection assay with an antisense RNA probe that was generated by subcloning a 379-bp *Eco*RI-*Xho*I fragment of the human IGF-I-R cDNA into pGEM3 (Ullrich et al., 1986; Ota et al., 1989). The resulting construct was linearized with *Hin*dIII and transcribed with T7 RNA polymerase in the presence of [^{32}P]UTP. Ten micrograms of total RNA from tumor samples, normal adjacent tissue, and G401 and 293 cells were hybridized with 2×10^5 dpm of labeled probe at 45°C for 16 h in a buffer containing 75% formamide. After hybridization, RNA samples were digested with RNases A and T1 and the protected hybrids were extracted with phenol-chloroform, precipitated with ethanol, and electrophoresed on 8% polyacrylamide/8*M* urea denaturing gels. Hybridization of this probe to human RNA results in two protected bands that may correspond to alternatively spliced variants of the human IGF-I-R mRNA. Both bands in the autoradiograms were scanned using a laser densitometer.

Plasmids and DNA Transfections

The following fragments of the IGF-I-R gene promoter were fused to a promoterless firefly luciferase reporter gene in the p0LUC vector (Brassier et al., 1989) and used in transient cotransfection experiments: –2350/+640, –476/+640, –416/+232, –455/+30, and –40/+640 (nucleotide 1 corresponds to the transcription initiation site). The construction of the above plasmids, as well as their relative basal promoter activities, have been previously described (Werner et al., 1992, 1994b).

The following WT1 expression vectors were employed in cotransfection experiments: pCMVhWT, pCMVhWT-TTL, and pCMVhWT-17AA-KTS. These vectors were constructed by inserting human WT1 cDNAs downstream of the cytomegalovirus promoter in the vector pCB6+ (Madden et al., 1991). Expression vector pCMVhWT-17AA-KTS contains a 51-bp insert encoding a 17-amino acid fragment following residue 248 and a 9-bp insert encoding a 3-amino acid fragment (Lys-Thr-Ser) following residue 390. The pCMVhWT expression vector encodes a WT1 protein lacking both inserts.

pCMVhWT-TTL was used as a negative control since it contains a stop codon 5' of the zinc-finger coding sequence, thus abolishing the DNA-binding capacity of the expressed protein.

Cells were transfected using the Lipofectin reagent in a reduced-serum medium (Opti-MEM, Life Technologies, Inc., Gaithersburg, MD). Each 60-mm dish received 1 μg of reporter plasmid and variable amounts of expression vector. The total amount of DNA transfected was kept constant using pCB6+ DNA. In addition, 5 μg of a β-galactosidase expression vector was used with each dish. Twenty-four hours after transfection, DNA-containing medium was changed to serum-containing medium and the plates were incubated for an additional 48 h, at which time the cells were harvested and luciferase and β-galactosidase activities were measured as previously described (Werner et al., 1992).

For stable transfections, G401 cells were plated in 35-mm dishes and transfected with 20 μg pCMVhWT using the Lipofectin reagent. After 24 h, selection by 500 μg of geneticin was started. Following 2 wk of geneticin selection, independent colonies were picked using cloning cylinders. Clones overexpressing WT1 were selected by Northern blot analysis as described above.

Gel Retardation Assays

The following fragments of the proximal 5'-flanking and 5'-untranslated regions of the IGF-I-R gene were employed in gel retardation assays: −494/−331, −331/−135, −135/−26, −29/+185, +115/+341, and +341/+640. Fragments were labeled by either filling in the 5'-protruding end with ^{32}P-labeled dCTP using Klenow enzyme, or by exchanging the 3'-terminal phosphate with [γ-^{32}P]ATP using T4 polynucleotide kinase. Labeled probes were separated from unincorporated nucleotides and purified on 5% nondenaturing polyacrylamide gels, as previously described (Werner et al., 1994b).

Binding assays were performed by preincubating 0, 200, and 500 ng of the bacterially expressed zinc finger domain of WT1 (WTZF) in 9 μL of 20 m*M* HEPES, pH 7.5, 70 m*M* KCl, 12% glycerol, 0.05% Nonidet P-40, 100 μ*M* $ZnSO_4$, 0.5 m*M* dithiothreitol, 1 mg/mL bovine serum albumin (BSA), and 0.1 mg/mL poly (dI·dC), for 15 min at 4°C. Seventy-five thousand dpm (0.2–1 ng) of the labeled fragment were then added, and the reaction was incubated for an additional 10 min. The reaction products were electrophoresed through a 5% polyacrylamide gel that was run at 250 V for 2 h at 4°C. Gels were fixed in 10% acetic acid and autoradiographed at −70°C.

DNase I Footprinting

The following DNA fragments were used in DNase I footprinting assays: −331/+115, −39/+341, +115/+341, and +341/+640. Supercoiled DNAs were linearized at the 5' end of the fragment and labeled with ^{32}P-labeled deoxynucleotide triphosphates using Klenow enzyme. The inserts were excised using a restriction enzyme that cleaves at the other end of the insert and purified by agarose gel electrophoresis.

Binding assays were performed essentially as described for the gel retardation assays, except that poly (dI·dC) was omitted from the reaction mixture. After the binding reaction 10 μL of a 20 m*M* $MgCl_2$ and 0.2 m*M* $CaCl_2$ solution were added to the tubes, which were then incubated for 10 min at 4°C, following which DNaseI (1.25–10 ng, Worthington Bichemical Co., Freehold, NJ) was added for 2 min. Reactions were stopped by adding 20 μL of 20 m*M* EDTA, pH 8.0, 1% SDS, 0.2*M* NaCl, and 2 μg tRNA, extracted with phenol/chloroform, precipitated with ethanol, and loaded on an 8% polyacrylamide gel. Maxam-Gilbert A and G sequencing reactions were run as markers.

Soft Agar Assay

Anchorage-independent growth was assessed by counting the number of colonies formed in 0.25% agarose (with a 0.5% agarose underlay) (Werner et al., 1995). Cells were seeded in McCoys' 5A medium-10% FBS with or without IGF-I (26 n*M*) at a density of 1000 cells/cm^2, and the number of colonies was measured after 3 wk of incubation at 37°C under a humid atmosphere containing 5% CO_2.

Results and Discussion

Expression of the IGF-I-R Gene in Wilms' Tumor

The level of expression of the IGF-I-R gene in Wilms' tumor was determined with a sensitive solution hybridization-RNase protection assay with

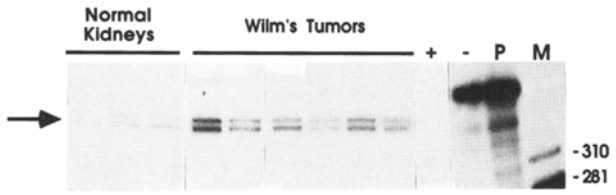

Fig. 1. Expression of the IGF-I-R gene in Wilms' tumor and normal adjacent kidney tissue. The levels of IGF-I-R mRNA were measured by solution hybridization-RNase protection assay using 10 μg of total RNA from six individual Wilms' tumors and three normal kidney samples. The probe employed was a human IGF-I-R antisense RNA probe labeled with ^{32}P-UTP. Lanes: +, probe alone with RNase; –, probe alone without RNase; P, native probe; M, molecular weight marker. Reproduced from Werner et al. (1993).

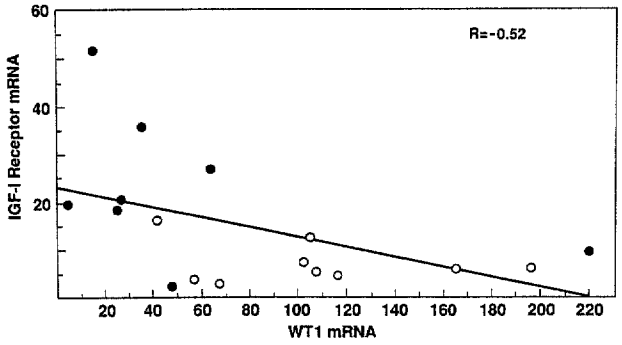

Fig. 2. Multiple regression analysis of IGF-I-R mRNA and WT1 mRNA in 17 Wilms' tumor samples. ○, tumor samples without heterologous elements; ●, tumor samples that include heterologous elements. Tumor #38 is not included *(see text)*. Reproduced from Werner et al. (1993).

total RNA obtained from a collection of previously described tumors (Gerald et al., 1992; Werner et al., 1993) and a specific 32P-labeled human IGF-I-R antisense RNA probe. Scanning densitometry of the two protected bands showed that the levels of IGF-I-R mRNA in the tumors were ~5.8-fold higher than in normal adjacent kidney tissue (Fig. 1). Comparison of the levels of IGF-I-R mRNA to those of WT1 mRNA in individual tumors showed an interesting correlation: With the exception of one sample (tumor #38) that showed very high levels of both IGF-I-R and WT1 mRNAs, multiple regression analysis showed a significant inverse correlation between these two parameters (R: –0.52; $p < 0.05$) (Fig. 2). This finding is consistent with the action of WT1 as a negative regulator of IGF-I-R gene expression.

Coexpression Studies

To characterize the molecular mechanisms responsible for the reciprocal pattern of IGF-I-R and WT1 gene expression in Wilms' tumor samples, coexpression studies were performed using an IGF-I-R gene promoter-containing reporter plasmid [p(–2350/+640)LUC] and WT1 expression vectors (pCMVhWT and pCMVhWT-TTL) encoding full-length and truncated WT1 proteins, respectively. We performed these studies in CHO cells since we have previously demonstrated that the IGF-I-R promoter is very active in this cell line. As shown in Fig. 3A, there was a dose-dependent repression of IGF-I-R promoter activity by the WT1 gene product. Thus, the luciferase activity in extracts of CHO cells cotransfected with 1 μg of the IGF-I-R promoter-reporter plasmid and 20 μg of the active WT1 expression vector was only ~15% of the activity elicited in the absence of WT1 expression. When the reporter plasmid was cotransfected with a truncated expression vector lacking the zinc-finger domain (pCMVhWT-TTL), no reduction in promoter activity was seen (Fig. 3A).

To determine the involvement of the different WT1 binding sites in the IGF-I-R promoter in transcriptional regulation by WT1, coexpression studies were performed using IGF-I-R promoter/reporter plasmids containing different portions of 5'-flanking and 5'-untranslated sequences, together with the WT1 expression vector. As shown in Fig. 3B, the promoter activity of constructs containing a smaller number of WT1 sites was inhibited to a lower extent, and it appeared that the inhibitory effect of WT1 was proportional to the number of potential sites and less dependent on the location of those sites. Thus, constructs p(–2350/+640)LUC and p(–476/+640)LUC, containing twelve putative WT1 sites each, were inhibited by 82–87%, whereas constructs p(–416/+232)LUC and p(–40/+640)LUC, containing seven putative WT1 sites each, were inhibited by 59 and 64%, respectively. Construct p(–455/+30)LUC, which contains six putative WT1 sites, was inhibited by 46%.

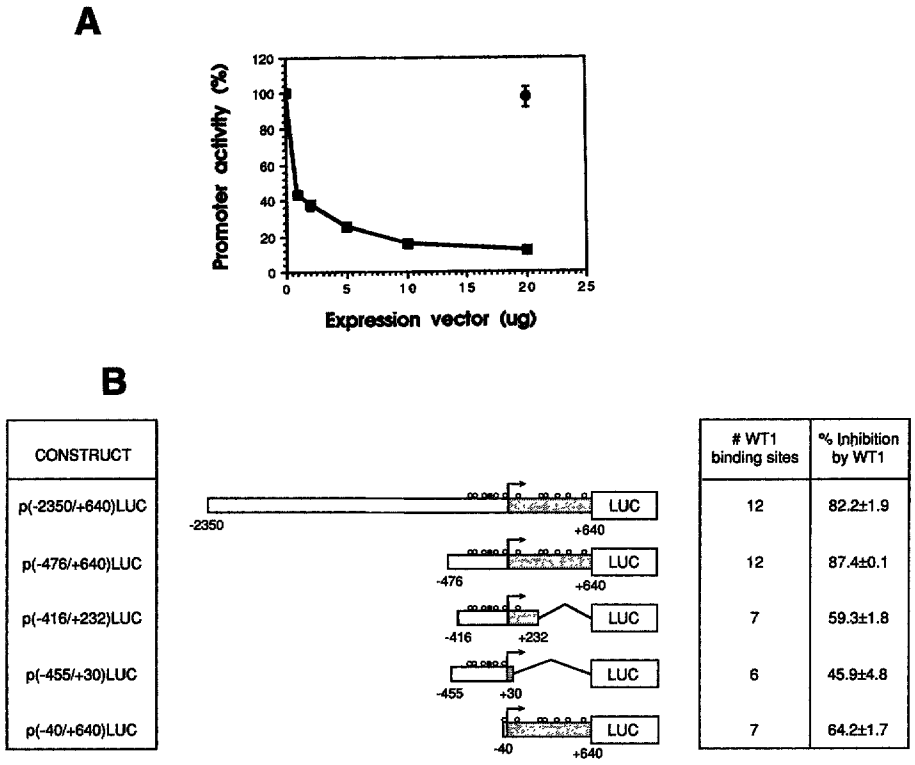

Fig. 3. Transcriptional regulation of the IGF-I-R promoter by WT1. **(A)** One microgram of the reporter plasmid p(-2350/+640)LUC was cotransfected into CHO cells with increasing amounts of the WT1 expression vector, pCMVhWT (closed squares), or with 20 μg of a mutant WT1 expression vector, pCMVhWT-TTL, lacking the DNA-binding domain (closed circle). The values of luciferase shown are normalized per β-galactosidase. Where not shown, SEM bars are smaller than the size of the symbol. **(B)** Deletional analysis of IGF-I-R promoter regulation by WT1. Reporter plasmids containing different portions of 5'-flanking and 5'-untranslated regions were cotransfected in CHO cells with expression vector pCMVhWT. The circles represent WT1 binding sites footprinted by WTZF (*see* Fig. 5 and Table 1). The closed circle is a perfectly conserved WT1 consensus site.

Interaction of the Zinc Finger Domain of WT1 with the IGF-I-R Promoter

To analyze the interactions between WT1 and the IGF-I-R promoter, gel retardation assays were performed using the purified zinc finger domain of WT1. For this purpose, the region extending from –494 to +640 was dissected into six fragments that were individually end-labeled and employed in binding reactions with WTZF (Fig. 4). Fragments lacking putative WT1 binding sites (–494/–331 and –135/–26) did not generate any retarded bands. Fragment –331/–135, which exhibits five putative binding sites, generated four shifted bands. Fragment –29/+185, which encompasses the initiator, and fragment +115/+341 generated two retarded bands each and fragment +341/+640 generated only one retarded band. The formation of DNA-protein complexes was prevented when gel shift assays were performed in the presence of an excess of the unlabeled probe (data not shown). Thus, there was in general a very good agreement between the number of putative sites and the number of retarded bands.

To more accurately map the WT1 binding sites in the IGF-I-R promoter, DNaseI footprinting assays were performed (Table 1 and Fig. 5). Five footprints were generated in the 5'-flanking region, only one of which is identical to the GCGGGGGCG consensus sequence. The other four sites conform to this sequence at eight out of nine nucleotides. The initia-

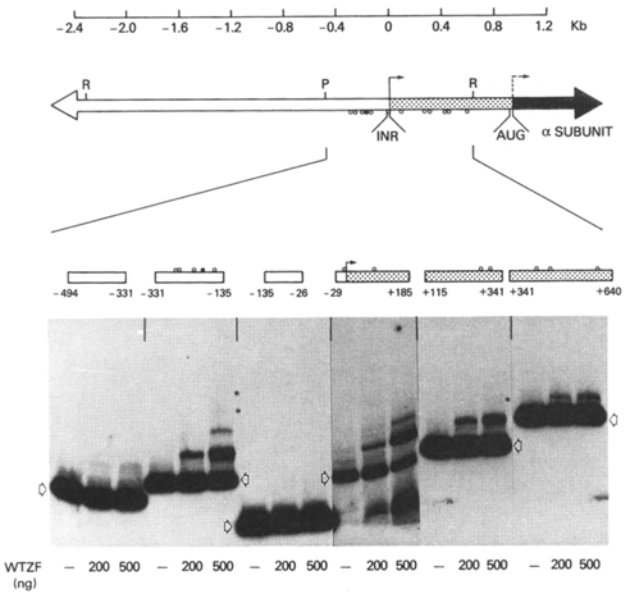

Fig. 4. Gel retardation analysis of the proximal IGF-I-R promoter with WTZF. The region extending from –494 to +640 was dissected into six fragments that were end-labeled with ^{32}P-labeled nucleotides and used in binding reactions with 0, 200, and 500 ng of purified WTZF. Circles denote the location of sites that were footprinted by WTZF (*see* Fig. 5 and Table 1). The closed circle shows the consensus GCGGGGGCG site. The initiator is denoted by an arrow. 5'-flanking sequences are shown as open bars and 5'-untranslated sequences are shown as dotted bars. Open arrows in the gel indicate free probe and asterisks denote bands that appear on long exposures. Reproduced from Werner et al. (1994b).

tor element itself includes an AGCCCCCAG sequence between nucleotides –7 and +2 that binds WTZF with low affinity. Six WT1 sites were footprinted in the 5'-untranslated region, although none of these sites was identical to the consensus sequence. For example, the sequence GAGGGGGAA, located at positions +78 to +86 and +453 to +461, binds WTZF with medium affinity, which may suggest that the core GGGGG sequence is essential for binding activity.

The results of gel retardation and DNaseI footprinting assays, when combined with those of functional studies, indicate that tumor suppressor WT1 can suppress the activity of the IGF-I-R promoter through physical interaction with multiple sites located both upstream and downstream of the transcription start site. This interaction may result in a stringent inhibitory control of the IGF-I-R promoter. In addition, some of the effects of WT1 may be achieved by direct binding to the initiator, thus impairing the ability of this element to assemble a functional transcription complex.

Table 1
Sequences of the IGF-I-R
Gene Promoter Footprinted by WTZF

Location	Sequence	Relative binding[a]
–262/–254	**G**T**GGGGGCG**	+++
–250/–242	**GCG**T**GGGCG**	+++
–220/–212	**GCGGGGGC**C	+++
–196/–188	**GCGGGGGCG**	+++
–163/–155	**CGCCC**G**CGC**	++
–7/+2	A**GCCCCC**AG	+
+78/+86	**GA**G**GGGG**AA	++
+276/+284	A**GCCCCCGC**	+++
+303/+311	**GCGGGGGC**C	++
+412/+420	**GCGGC**G**GCG**	+
+453/+461	**GA**G**GGGG**AA	++
+590/+598	T**CGGGGGCG**	+++

[a]The affinity of WT1 binding sites for WTZF was arbitrarily determined as follows: +++, footprints generated by 60 ng or less of WTZF; ++, footprints generated by 125 ng or more WTZF; +, footprints generated by 250 ng of WTZF. Nucleotides in **bold** and underlined letters correspond to the consensus sequence.

Regulation of IGF-I-R Gene Expression by WT1 in Intact Cells

To establish whether WT1 is involved in regulation of the endogenous IGF-I-R gene, in addition to the transfected promoter, we measured the levels of IGF-I-R mRNA in two kidney-derived cell lines expressing different levels of endogenous WT1. Northern blot hybridization of 10-μg aliquots of poly (A)+ RNA from G401 and 293 cells with a WT1 cDNA probe showed that WT1 was expressed by 293 cells, whereas no expression was seen in G401 cells, even after long exposure times (Fig. 6A). Solution hybridization-RNase protection assay with a human antisense IGF-I-R RNA probe revealed an opposite pattern of expression, i.e., levels of IGF-I-R mRNA in G401 cells were ~2.5-fold higher than in 293 cells (Fig. 6B).

To determine whether the different levels of IGF-I-R mRNA could be caused by differences in basal promoter activity, cells were transiently transfected

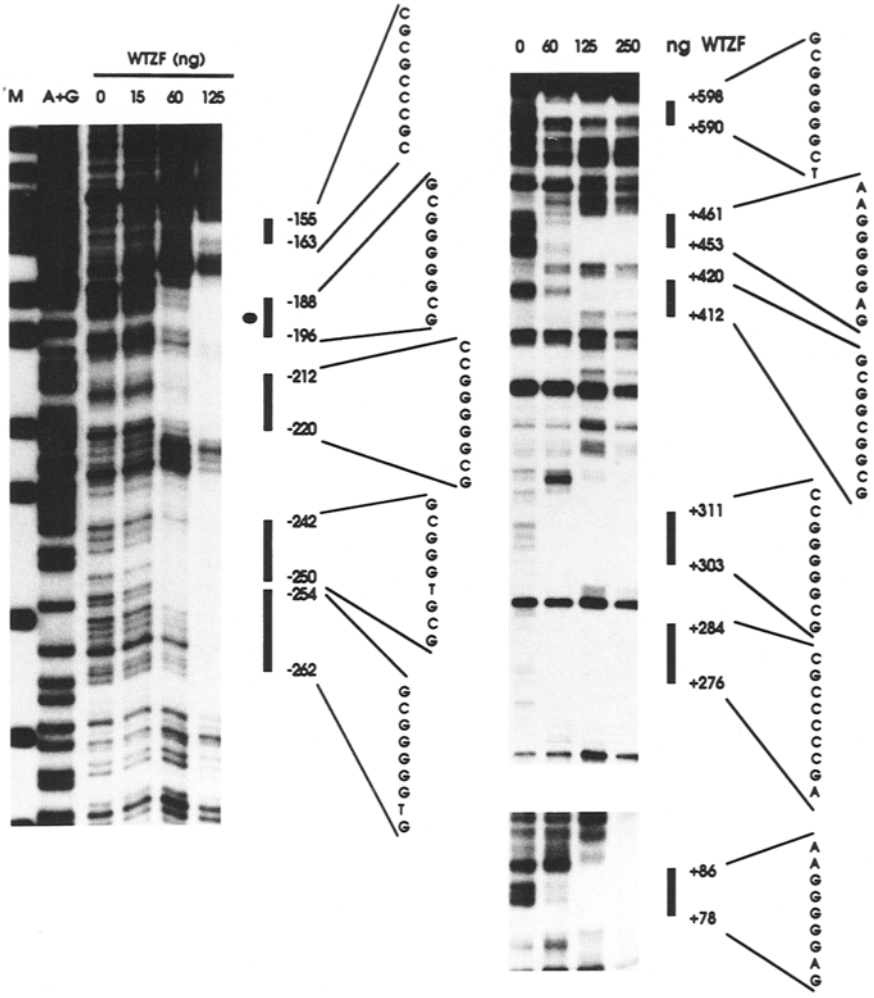

Fig. 5. DNase I footprinting analysis of the 5'-flanking **(left)** and 5'-untranslated **(right)** regions of the IGF-I-R gene with WTZF. The location of footprinted WT1-like binding sequences is denoted by thick bars. The sequences shown at the right correspond to the sense strand. The site indicated with a dot is the consensus GCGGGGGCG sequence. M, molecular weight marker; A + G, Maxam-Gilbert sequencing ladder. Adapted from Werner et al. (1994b).

with the IGF-I-R reporter p(–476/+640)LUC. The activity of the IGF-I-R promoter in G401 cells was ~70-fold higher than the activity exhibited by the promoterless p0LUC vector (Fig. 6C). On the other hand, basal promoter activity in 293 cells was only ~10-fold higher than that of the p0LUC control. These results suggest that high levels of endogenous WT1 in 293 cells can suppress the activity of the IGF-I-R promoter, with ensuing decrease in the levels of mRNA and binding. On the other hand, undetectable levels of WT1 mRNA in G401 cells correlate with a very high basal promoter activity and with higher levels of receptor transcript.

Expression of WT1 in G401 Cells

To further corroborate the functional role of the IGF-I-R promoter as a target for tumor suppressor WT1 we stably transfected WT1-negative G401 cells with a WT1 expression vector. G418-resistant colonies were isolated and the expression of WT1 was assessed by Northern blot analysis. A number of colonies were found that expressed a 2.1-kb WT1 mRNA (Fig. 7A). The proliferation rate in serum-containing media of G401 cells expressing WT1 (G401/WT1+) was significantly reduced in comparison to control G418-resistant clones that

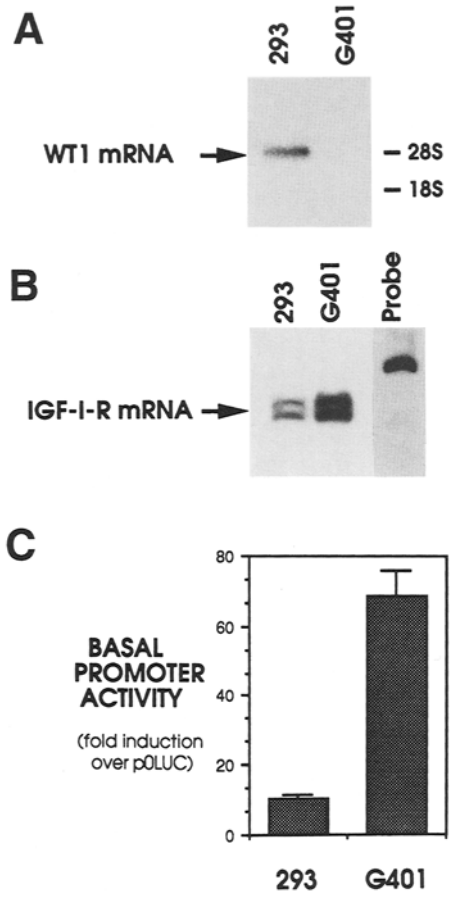

Fig. 6. Regulation of IGF-I-R gene expression by WT1. **(A)** Northern blot analysis of WT1 mRNA using 10 μg of poly(A)$^+$ mRNA from 293 and G401 cells and a ^{32}P-labeled fragment of the human WT33 cDNA clone as probe. **(B)** Solution hybridization-RNase protection assay of IGF-I-R mRNA. Ten micrograms of total RNA from 293 and G401 were hybridized with a ^{32}P-labeled human IGF-I-R antisense RNA probe, digested with RNases A and T1, and electrophoresed on an 8% polyacrylamide-8*M* urea gel. **(C)** Basal IGF-I-R promoter activity. Confluent cultures of 293 and G401 cells were transiently transfected with 10 μg of the p(–476/+640)LUC reporter plasmid (or 10 μg of the promoterless vector pOLUC) and 10 μg of the β-galactosidase control plasmid. The luciferase values were normalized with respect to the β-galactosidase levels and the results are expressed as the fold increase over the value for pOLUC.

did not express the WT1 transfectant. As shown in Fig. 7B, the number of G401/WT1+ cells per plate was ~44% of the number of G401/WT1– cells.

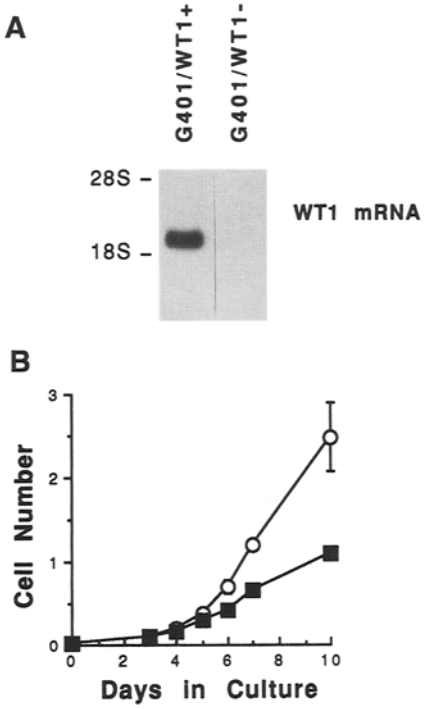

Figure 7. Expression of WT1 in G401 cells. A WT1 expression vector containing a neomycin resistance gene was transfected into WT1-negative G401 cells and positive clones were selected using G418. **(A)** Northern blot hybridization of WT1 mRNA using 20 μg of total RNA from a WT1-expressing clone (G401/WT1+) and a G418-resistant nonexpressing clone (G401/WT1–). **(B)** Cellular proliferation of G401/WT1+ (closed squares) and G401/WT1– (open circles) in complete medium containing 10% FBS. Reproduced from Werner et al. (1995).

We next examined whether this decrease in cellular proliferation in G401/WT1+ cells was associated with a reduction in the expression of the endogenous IGF-I-R gene. Results of solution hybridization-RNase protection assay showed that the levels of IGF-I-R mRNA in WT1-expressing G401 cells were now ~60% of the values in control G401/WT1– (Fig. 8A). These reduced levels were correlated with a significant decrease in the activity of a transfected IGF-I-R promoter (Fig. 8B). These data, thus, suggests that the mechanism of action of tumor suppressor WT1 involves suppression of the IGF-I-R promoter.

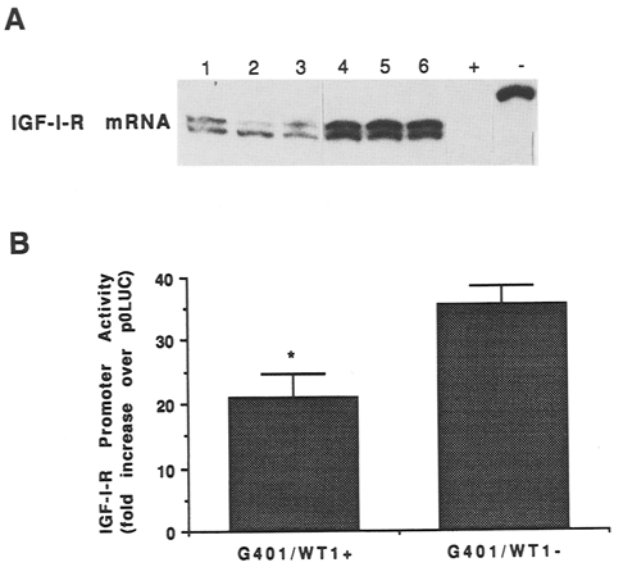

Fig. 8. Regulation of endogenous IGF-I-R gene expression by WT1. **(A)** Solution hybridization-RNase protection assay of IGF-I-R mRNA in G401/WT1+ (lanes 1–3) and G401/WT1– (lanes 4–6) cells. Lane +, probe alone with RNases A and T1; lane –, probe alone without RNases. **(B)** Basal IGF-I-R promoter activity in G401/WT1+ and G401/WT1– cells. Cells were transiently transfected with 10 μg of the p(–2350/+640)LUC reporter plasmid and 10 μg of β galactosidase vector. Luciferase values, normalized per β galactosidase activity, are expressed as fold increase over the value for pOLUC.

Biological Effects of IGF-I in WT1-Expressing G401 Cells

Binding assays using ^{125}I-labeled IGF-I, as well as two analogs of IGF-I with reduced affinity to the IGF-binding proteins [des(1–3)IGF-I and long R^3 IGF-I], were correspondingly reduced in WT1-expressing G401 cells. We examined whether this decrease in IGF-I-R transcription and cell-surface receptors was correlated with a reduction in a number of IGF-I-mediated biological effects. We showed that in serum-free medium IGF-I (2.6 n*M*) in combination with EGF and PDGF-BB, as well as alone (Fig. 9A and data not shown), stimulated the proliferation of WT1-expressing cells to an extent that was, in separate experiments, only ~25–50% of the effect seen in G401 control cells. Additionally, the capacity of IGF-I to stimulate [^{3}H]thymidine incorporation by G401/WT1+ cells was ~23–81% of its capacity in G401/WT1– cells (Fig. 9B). Finally, IGF-I-stimulated anchorage-independent growth (as measured by soft agar assay) was also significantly different between WT1-positive and -negative G401 clones (Fig. 10). Addition of IGF-I (26 n*M*) to the soft agar resulted in a 40% increment in the number of colonies generated by G401/WT1+ cells, whereas it induced a 129% increase in G401/WT1– cells.

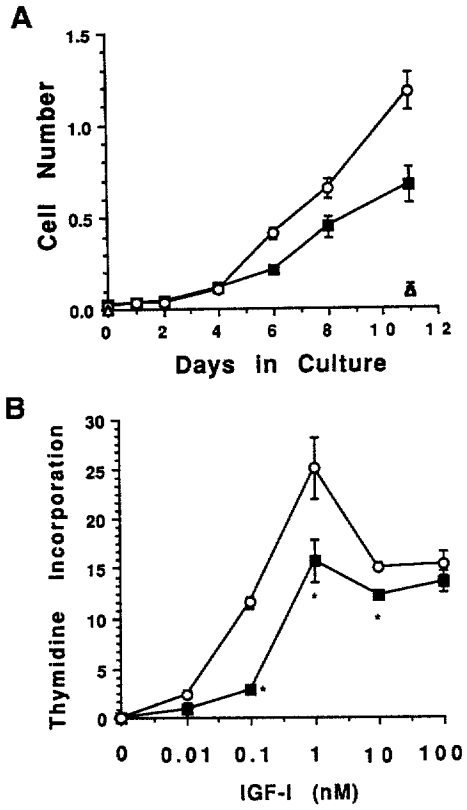

Fig. 9. Biological effects of IGF-I in WT1-expressing G401 cells. **(A)** Time-course of IGF-I-stimulated cellular proliferation in serum-free medium containing IGF-I (2.6 n*M*), EGF (3.2 n*M*), and PDGF (0.2 n*M*). G401/WT1+ cells are denoted by closed squares and G401/WT1– cells are denoted by open circles. The triangles represent cells grown in the absence of supplements. **(B)** IGF-I-stimulated [^{3}H]thymidine incorporation in G401/WT1+ (closed squares) and G401/WT1– (open circles) cells. Confluent cultures were kept for 24 h in serum-free medium, following which cells were incubated for 16 h with different concentrations of IGF-I. [^{3}H]thymidine was then added for 1 h and incorporation was measured.

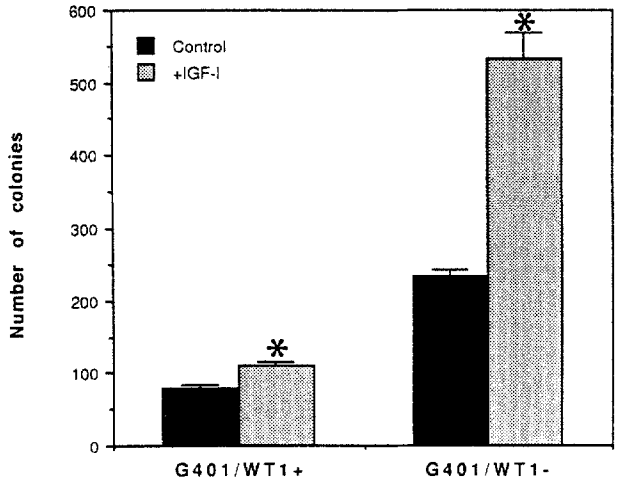

Fig. 10. Anchorage-independent growth of WT1-positive and -negative G401 cells. Cells were plated in 0.25% agarose (with a 0.5% agarose underlay) in McCoy's 5A medium with 10% FBS, with or without IGF-I (26 n*M*). The number of colonies was measured after 3 wk of incubation. *Significantly different from the same clone in the absence of IGF-I ($p < 0.01$). Reproduced from Werner et al. (1995).

Conclusions and Implications for Tumors of the CNS

The results of our study indicate that, in addition to being a target for positive growth stimulators, such as oncogenes and growth factors, the IGF-I-R gene can also be regulated by negative factors, i.e., tumor-suppressor gene products. A number of mechanisms have been described in Wilms' tumor that result in loss of activity of WT1. These mechanisms include constitutional deletions, missense or nonsense mutations often affecting the DNA-binding domain, or alternative splicing involving exon 2 that produces a WT1 variant that acts as a transcriptional activator (Call et al., 1990; Gessler et al., 1990; Rose et al., 1990; Little et al., 1992; Haber et al., 1993). Inability of the inactive WT1 in Wilms' tumor to suppress transcription from the IGF-I-R promoter and from the IGF-II P3 promoter, which also contains a number of WT1 sites, may result in overexpression of both ligand and receptor. Activation of the IGF-I-R by locally produced IGF-II may be an important mechanism for the progression of Wilms' tumor.

Overexpression of the IGF-I-R has been postulated to be an important mechanism in the progression of neurally derived tumors, including glioblastomas, meningiomas, and others (Glick et al., 1989). A number of tumor suppressors, including p16, NF-1, Rb, and p53, have been shown to be deleted or mutated in brain tumors (Pomeroy, 1994). Future studies will establish whether the IGF-I-R promoter is a target for neurally expressed tumor suppressors and whether loss of suppression of the receptor promoter by inactive tumor suppressors constitute a general theme in brain oncogenesis.

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