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Inhibition of Cellular Proliferation by the Wilms' Tumor Suppressor WT1 Is Associated with Suppression of Insulin-Like Growth Factor I Receptor Gene Expression

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We have investigated the regulation of the insulin-like growth factor I receptor (IGF-I-R) gene promoter by the Wilms' tumor suppressor WT1 in intact cells. The levels of endogenous IGF-I-R mRNA and the activity of IGF-I-R gene promoter fragments in luciferase reporter constructs were found to be significantly higher in G401 cells (a Wilms' tumor-derived cell line lacking detectable WT1 mRNA) than in 293 cells (a human embryonic kidney cell line which expresses significant levels of WT1 mRNA). To study whether WT1 could suppress the expression of the endogenous IGF-I-R gene, WT1-negative G401 cells were stably transfected with a WT1 expression vector. Expression of WT1 mRNA in G401 cells resulted in a significant decrease in the rate of cellular proliferation, which was associated with a reduction in the levels of IGF-I-R mRNA, promoter activity, and ligand binding and with a reduction in IGF-I-stimulated cellular proliferation, thymidine incorporation, and anchorage-independent growth. These data suggest that a major aspect of the action of the WT1 tumor suppressor is the repression of IGF-I-R gene expression.

Several lines of evidence suggest that there is a basic requirement for insulin-like growth factor I receptor (IGF-I-R) action during the cell cycle: (i) the IGF-I-R is constitutively expressed by most tissues, consistent with the role of IGF-I as a progression factor (2, 44, 45); (ii) overexpression of the IGF-I-R in BALB/c3T3 cells abrogates all requirements for exogenous growth factors (28); and (iii) deletion of the IGF-I-R gene in mice by homologous recombination is incompatible with postnatal life (1, 21). The IGF-I-R is also implicated in malignant transformation, as shown by its high level of expression in many tumors and cancer cell lines (45). Fibroblast cell lines established from mouse embryos homozygous for a targeted disruption of the IGF-I-R gene cannot be transformed by the simian virus 40 large tumor antigen (36).

We have previously characterized the regulatory region of the IGF-I-R gene and shown that it contains 12 binding sites for the product of the WT1 tumor suppressor gene, a transcription factor whose deletion or mutation has been implicated in the etiology of a subset of Wilms' tumors (4, 12, 34, 40, 41, 43). Expression of WT1 protein in Chinese hamster ovary cells results in a dose-dependent decrease in the activity of a cotransfected IGF-I-R promoter-luciferase reporter construct. This effect of WT1 involves the interaction of its zinc finger domain with multiple consensus binding sites in both the 5′ flanking region and the 5′ untranslated region (UTR) of the IGF-I-R gene (41).

In addition, we have previously shown that the IGF-I-R gene is overexpressed in Wilms' tumor in comparison with normal kidney tissue, a finding that is consistent with the IGF-I-R gene promoter being a target for the inhibitory action of WT1 (42). The IGF-II gene, whose P3 promoter is also negatively regulated by WT1, is similarly overexpressed in Wilms' tumor (8, 14, 26, 32). It is therefore possible that the elevated levels of locally produced IGF-II bind and activate the IGF-I-R, resulting in a mitogenic loop which may constitute a potential mechanism for the progression of the tumor.

To better understand the molecular mechanisms responsible for the transcriptional regulation of the IGF-I-R gene by WT1 in vivo, we have studied the expression of the IGF-I-R gene in cell lines expressing different levels of endogenous WT1. Our results indicate that transcription factor WT1 can suppress the expression of the IGF-I-R gene in intact cells. Reduced levels of IGF-I-R mRNA and protein are associated with a decrease in the magnitude of several IGF-I-mediated biological effects, suggesting that the antiproliferative effect of WT1 may be mediated, at least in part, by repression of the IGF-I-R gene promoter.

MATERIALS AND METHODS

Cell cultures, plasmids, and DNA transfections. G401 and 293 cells were obtained from the American Type Culture Collection (Rockville, Md.). The G401 cell line was originally established from a Wilms' tumor explant obtained from a 3-month-old male (27), though it has been recently proposed that G401 is, in fact, a sarcoma-derived cell line (11). 293 is a transformed human embryonic kidney cell line. G401 cells were grown in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS), and 293 cells were grown in Dulbecco's modified Eagle medium containing 10% FBS.

For transient transfection and cotransfection experiments, a genomic DNA fragment extending from nucleotides −476 to +640 (nucleotide 1 corresponds to the transcription start site of the IGF-I-R gene) was subcloned upstream of a promoterless firefly luciferase reporter gene (pOLUC). The promoter activity of this fragment, which includes most of the proximal promoter region, has been previously described (41). In addition, the following fragments were used in some transient transfection experiments: −2350 to +640, −455 to +30, and −40 to +640.

A WT1 expression vector (pCMVhWT) was constructed by inserting an ~2.1-kb human WT1 cDNA downstream of the cytomegalovirus promoter in the pCB6+ vector (23). This vector contains a selectable neomycin gene conferring G418 resistance.

Cells were transfected by using the Lipofectin reagent (Life Technologies,

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Gaithersburg, Md.) as previously described. In transient transfection experiments, each 60-mm-diameter dish received 10 μg of reporter plasmid and 10 μg of a β-galactosidase expression vector (pCMV β; Clontech). In cotransfection experiments, each dish received 1 μg of reporter plasmid, 5 μg of pCMV β, and variable amounts of expression vector. The total amount of DNA transfected was kept constant by using pCB6+ DNA. Twenty-four hours after transfection or cotransfection, the 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 (40).

For stable transfections, parental G401 cells were plated in 35-mm-diameter dishes and transfected with pCMVhWT (20 μg) by using the Lipofectin reagent. After 24 h, selection by 500 μg of G418 (Geneticin; Life Technologies) per ml was started. Following 2 weeks of G418 selection, independent colonies were picked by using cloning cylinders (Specialty Media Inc., Lavallette, N.J.). Clones overexpressing WT1 were selected by Northern (RNA) blot analysis as described below.

Northern blot analysis of WT1 mRNA. G401 and 293 cells were lysed in 4 M guanidinium isothiocyanate containing 0.01% β-mercaptoethanol, and total RNA was isolated by ultracentrifugation of the cell lysates on a cesium chloride gradient as described previously (5). The 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. Poly(A)$^+$ RNA was prepared 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 by using a pressure blotter (Stratagene, La Jolla, Calif.), 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 (4), labeled by a modified random priming technique. Blots were hybridized and washed as described previously (44).

Solution hybridization-RNase protection assay of IGF-I-R mRNA. Ten micrograms of total RNA from G401 and 293 cells was hybridized with 2×10^5 dpm of a human antisense RNA probe that was generated by subcloning a 379-bp *Eco*RI-*Xho*I fragment of the human IGF-I-R cDNA (38) into pGEM-3 (25). Solution hybridization-RNase protection assays were performed as previously described (44). Hybridization of this probe to human RNA results in two protected bands, which may correspond to alternatively spliced variants of the human IGF-I-R mRNA (15, 25).

IGF-I binding. Binding of ^{125}I-IGF-I, ^{125}I-des(1-3)IGF-I, and ^{125}I-long R^3 IGF-I analogs to the IGF-I-R in G401 and 293 cells was performed in confluent monolayers in 12-well plates as previously described (25). ^{125}I-IGF-I was obtained from Amersham Corp. (Arlington Heights, Ill.), and ^{125}I-des(1-3)IGF-I was from Lofstrand Laboratories (Gaithersburg, Md.). ^{125}I-long R^3 was a gift from S. Peter Nissley (National Institutes of Health).

Cellular proliferation. Cells were seeded in 12-well plates (2,500 cells per cm^2), using McCoy's 5A medium–10% FBS, and the proliferation rate was determined by cell counting using a hemocytometer. To measure IGF-I-stimulated cellular proliferation, cells were seeded in serum-containing medium. After 24 h, cells were washed twice with Hanks' balanced salt solution, and McCoy's 5A medium containing the following supplements was added: 1% bovine serum albumin (BSA), transferrin (15 μg/ml), IGF-I (2.6 nM), platelet-derived growth factor BB (PDGF-BB; 0.2 nM), and epidermal growth factor (EGF; 3.2 nM). In some experiments, cells were incubated with medium containing only BSA, transferrin, and IGF-I.

Thymidine incorporation. Confluent cells in 12-well plates were starved for 24 h in serum-free medium, following which IGF-I was added and the cells were incubated for an additional 16 h. [*methyl*-^{3}H]thymidine (1 μCi per well; Amersham Corp.) was then added for 1 h. The cells were rinsed twice with ice-cold phosphate-buffered saline, twice with ice-cold 5% trichloroacetic acid, and twice with ethanol. The cells were dissolved in 0.3 ml of 1 N NaOH, neutralized with 0.3 ml of 1 N HCl, and counted in a liquid scintillation counter.

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). Cells were seeded in McCoy's 5A medium–10% FBS with or without IGF-I (26 nM) at a density of 1,000 cells per cm^2, and the number of colonies was measured after 3 weeks of incubation at 37°C under a humid atmosphere containing 5% CO_2.

RESULTS

Expression of the endogenous IGF-I-R gene in G401 and 293 cells. To begin to understand the molecular mechanisms responsible for the transcriptional regulation of the IGF-I-R gene by the WT1 gene product, we measured the expression of the IGF-I-R gene in two kidney-derived cell lines. Northern blot hybridization of 10-μg aliquots of poly(A)$^+$ RNA from 293 and G401 cells with a 1.8-kb *Eco*RI fragment of the human WT33 cDNA showed that the WT1 gene was expressed by 293 cells, whereas no expression was detected in G401 cells, even after long exposure times (Fig. 1A). These results corroborate similar findings by Huang et al. (16). The levels of IGF-I-R mRNA in G401 and 293 cells were measured by a solution hybridization-RNase protection assay using 10 μg of total RNA and a ^{32}P-labeled IGF-I-R antisense RNA probe. Scanning densitometry of the two protected bands showed that the levels of IGF-I-R mRNA in G401 cells were ~2.5-fold higher than in 293 cells (Fig. 1B). Consistent with these results, the specific binding of ^{125}I-IGF-I to G401 cells in culture was ~2-fold higher than the binding exhibited by 293 cells (47.8% ± 4.4% specific binding per mg of protein in G401 cells versus 22.6% ± 0.6% in 293 cells).

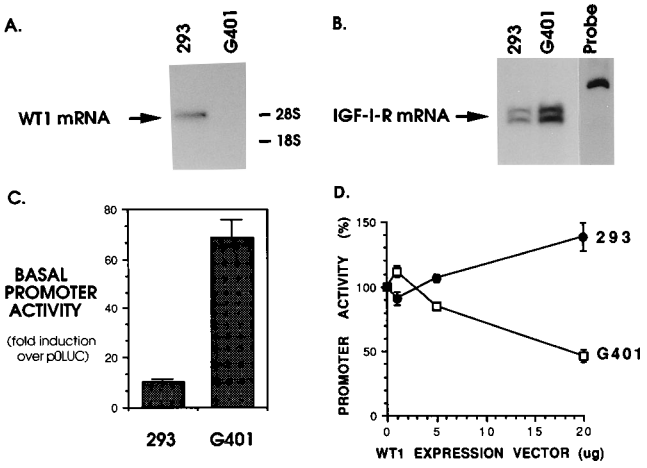

FIG. 1. Regulation of IGF-I-R gene expression by WT1. (A) Northern blot hybridization of WT1 mRNA, using 10 μg of poly(A)$^+$ mRNA from 293 and G401 cells and a ^{32}P-labeled fragment of the human WT33 cDNA clone as probe. The positions of the 28S and 18S rRNA bands are indicated. The autoradiogram was exposed for 24 h at −70°C. (B) Solution hybridization-RNase protection assay of IGF-I-R mRNA. Ten micrograms of total RNA from 293 and G401 cells was hybridized with a ^{32}P-labeled human IGF-I-R antisense RNA probe, digested with RNases A and T_1, and electrophoresed on an 8% polyacrylamide–8 M urea gel. The autoradiogram was exposed for 96 h at −70°C. (C) Basal IGF-I-R promoter activity. Confluent cultures of 293 and G401 cells were transiently transfected with 10 μg of the p(−476/+640LUC) reporter plasmid (or 10 μg of pOLUC) and 10 μg of the β-galactosidase control plasmid, using the Lipofectin reagent. 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. Results represent means ± SEM of two independent experiments, each performed in triplicate. (D) Effect of WT1 expression on IGF-I-R promoter activity. One microgram of the reporter plasmid p(−476/+640LUC) was cotransfected into 293 (●) and G401 (□) cells with increasing amounts of the WT1 expression vector, pCMVhWT. Luciferase activity was normalized for β-galactosidase activity, and the results are expressed as percentages of the levels seen in the absence of pCMVhWT. Results are means ± SEM of two independent experiments, each performed in triplicate. Where not shown, the SEM bars are smaller than the symbol size.

Basal IGF-I-R promoter activity in G401 and 293 cells. To determine whether the different levels of IGF-I-R mRNA in G401 and 293 cells could be ascribed to differences in basal promoter activity, cells were transiently transfected with an IGF-I-R promoter construct containing 476 bp of the 5′ flanking region and 640 bp of the 5′ UTR cloned upstream of a luciferase reporter gene [p(−476/+640)LUC]. As shown in Fig. 1C, IGF-I-R promoter activity in G401 cells was ~70-fold higher than the activity exhibited by the promoterless pOLUC vector. On the other hand, basal promoter activity in 293 cells was only ~10-fold higher than that of the pOLUC control.

Coexpression studies. To establish whether the reciprocal pattern of WT1 and IGF-I-R gene expression in G401 and 293 cells could be due, in part, to transcriptional repression of the IGF-I-R promoter by endogenous WT1, cotransfection exper-

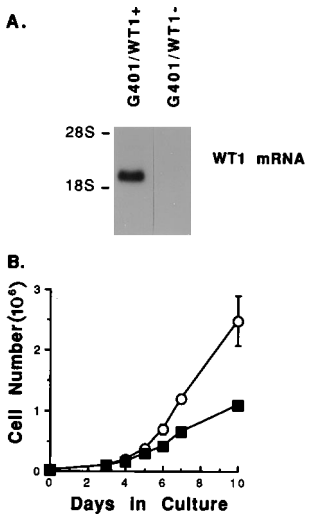

FIG. 2. Expression of WT1 in G401 cells. A WT1 expression vector containing a neomycin resistance gene was transfected into G401 cells by using the Lipofectin reagent, and positive clones were selected by using G418. (A) Northern blot hybridization of WT1 mRNA, using 20 μg of total RNA from a WT1-expressing clone (G401/WT1+) and a G418-resistant nonexpressing clone (G401/WT1−). Blots were hybridized with a ^{32}P-labeled fragment of the human WT33 cDNA clone and exposed for 72 h. The size of the WT1 mRNA (~2.1 kb) corresponds to the size of the insert in the pCMVhWT vector. (B) Cellular proliferation of G401/WT1+ (■) and G401/WT1− (○) clones. A total of 25,000 cells were seeded in each well of two 12-well plates, using McCoy's 5A medium containing 10% FBS, and the number of cells was measured at different times with a hemocytometer. Each point represents the average of two determinations. Where not shown, the SEM bars are smaller than the symbol size. The *y* axis represents number of cells per well.

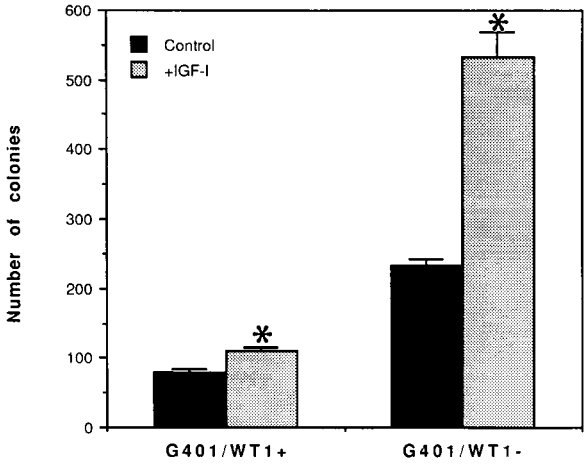

FIG. 3. Anchorage-independent growth of WT1-positive and -negative G401 cells. Cells were plated in 0.25% agarose (with a 0.5% agarose underlay) in McCoy's 5A medium–10% FBS with or without IGF-I (26 nM) at a density of 1,000 cells per cm^2. The number of colonies was measured after 3 weeks of incubation. Results are means ± SEM of three dishes per group. *, significantly different from same clone in the absence of IGF-I ($P < 0.01$).

iments were performed with 1 μg of the p(−476/+640)LUC reporter plasmid and increasing amounts of a WT1 expression vector (pCMVhWT). In G401 cells, the cotransfected WT1 gene product was able to repress the activity of the p(−476/+640)LUC construct in a dose-dependent manner. Maximal repression was seen with 20 μg of pCMVhWT, at which concentration the activity of the promoter was inhibited by ~53% (Fig. 1D). No further reduction was seen with up to 40 μg of pCMVhWT (data not shown).

Interestingly, cotransfection of 293 cells with p(−476/+640)LUC and increasing amounts of pCMVhWT resulted in an increment in promoter activity (~38%) above the basal level at 20 μg of WT1 expression vector (Fig. 1D).

Expression of WT1 in G401 cells. We next studied whether WT1 could suppress the expression of the endogenous IGF-I-R gene. For that purpose, G401 cells were stably transfected with a WT1 expression vector. Total RNA was prepared from confluent cultures of cells derived from 63 isolated colonies, and the expression of WT1 was assessed by Northern blot analysis using 20 μg of total RNA. We found eight clones (G401/WT1+) that expressed a 2.1-kb WT1 mRNA (Fig. 2A). The expression of WT1 protein was confirmed by Western blotting (immunoblotting) using a polyclonal rabbit serum against WT1 (data not shown). G418-resistant clones that did not express the WT1 transfectant (G401/WT1− clones) were used as negative controls.

Growth characteristics of WT1-expressing G401 cells. As shown in Fig. 2B, the proliferation rate in serum-containing medium of G401 cells expressing WT1 was significantly reduced. Thus, after 10 days in culture, the number of G401/WT1+ cells per plate was ~44% of the number of G401/WT1− cells. To assess anchorage-independent growth of the G401 clones, cells were plated in soft agar with 10% FBS, and the number of colonies was determined after 3 weeks. As seen in Fig. 3, the number of colonies generated by G401/WT1+ cells was 34% of the number of colonies formed by G401/WT1− cells (79 ± 5 versus 232 ± 10 colonies per 10,000 cells, respectively; results are means ± standard errors of the means [SEM] of three dishes per group).

Expression of the endogenous IGF-I-R gene in G401/WT1+ cells. The level of IGF-I-R mRNA in G401/WT1+ clones was measured by solution hybridization-RNase protection assay and found to be ~60% of the level seen in control G401/WT1− cells (Fig. 4A). To determine whether the decreased level of IGF-I-R mRNA in WT1-expressing G401 clones was associated with a reduction in IGF-I-R promoter activity, G401/WT1+ and G401/WT1− cells were transiently transfected with four previously described IGF-I-R promoter-luciferase reporter constructs (41). As shown in Fig. 4B, the activity in G401/WT1+ cells of p(−2350/+640)LUC and p(−476/+640)LUC, two constructs containing 12 WT1 binding sites each, was 50% of the activity seen in G401/WT1− cells (bars 1 and 2). The activity in G401/WT1+ cells of construct p(−455/+30)LUC, containing six WT1 binding sites in the 5′ flanking region, was 60% of the activity seen in G401/WT1− cells (bar 3). Finally, the activity in G401/WT1+ cells of construct p(−40/+640)LUC, containing one WT1 site in the 5′ flanking region and six sites in the 5′ UTR, was 44% of the activity seen in G401/WT1− cells (bar 4). For control purposes, cells were also transfected with luciferase reporter constructs under the control of IGF-binding protein 1 (IGFBP-1) and IGFBP-2 promoters (kindly provided by D.-S. Suh and Y. Boisclair, National Institutes of Health). The IGFBP-1 promoter was totally inactive in these cells, whereas the activity of the IGFBP-2 promoter in G401/WT1+ cells was 52% of that in G401/WT1− cells (data not shown). These results are in agreement with the presence of potential WT1 sites in the IGFBP-2 promoter (31a). However, since IGFBP-2 is not expressed by these cells (as measured by Northern and Western blotting), the different growth effects of IGF-I (as described below) cannot be attributed to this binding protein.

To determine whether the decreased expression of the IGF-I-R gene is associated with reduction in cell surface receptors,

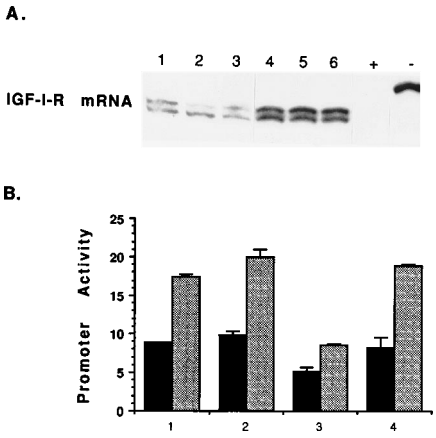

FIG. 4. Regulation of endogenous IGF-I-R gene expression by WT1. (A) Solution hybridization-RNase protection assay of IGF-I-R mRNA in G401/WT1+ and G401/WT1− cells. Twenty-microgram samples of total RNA from three individual G401/WT1+ plates (lanes 1 to 3) and three G401/WT1− plates (lanes 4 to 6) were hybridized with a human IGF-I-R antisense RNA probe as described in the text. Lane +, probe alone with RNases A and T_1; lane −, probe alone without RNases. The autoradiogram was exposed for 24 h. (B) Activity of the IGF-I-R promoter in G401/WT1+ (black bars) and G401/WT1− (dotted bars) cells transiently transfected with 10 μg of four IGF-I-R promoter constructs (or pOLUC) and 10 μg of β-galactosidase vector. The constructs used were p(−2350/+640)LUC (bar 1), p(−476/+640)LUC (bar 2), p(−455/+30)LUC (bar 3), and p(−40/+640)LUC (bar 4). Luciferase values, normalized for β-galactosidase activity, are expressed as fold increase over the value for pOLUC. The activities of all four constructs in G401/WT1+ cells were significantly different ($P < 0.01$) from the activities in G401/WT1− cells.

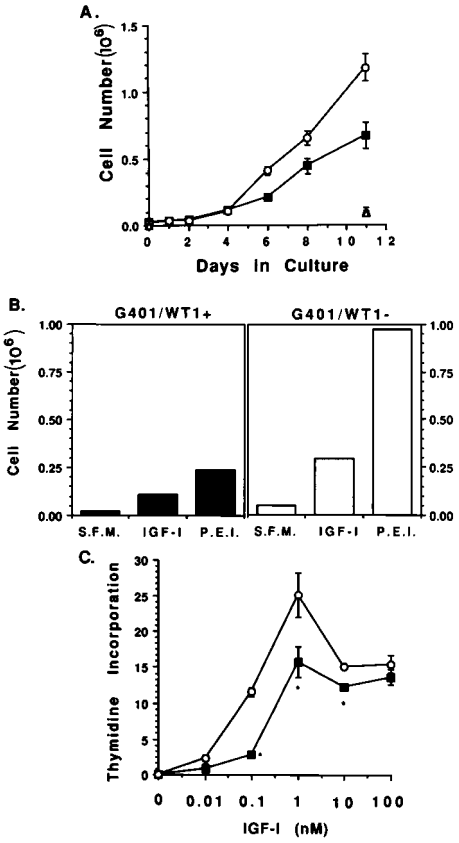

FIG. 5. Biological effects of IGF-I in WT1-expressing G401 cells. (A) Time course of IGF-I-stimulated cellular proliferation. A total of 25,000 cells were seeded in each well of 12-well plates, using McCoy's 5A medium containing 10% FBS. After 24 h, cells were washed with Hanks' balanced salt solution and further incubated in serum-free medium containing IGF-I (2.6 nM), EGF (3.2 nM), and PDGF (0.2 nM). ■, G401/WT1+ cells, grown in serum-free medium with supplements; ○, G401/WT1− cells, grown in medium with supplements; △, G401/WT1− cells, grown in serum-free medium without supplements (the open triangle for day 11 is superimposed over a datum point for G401/WT1+). The *y* axis represents number of cells per well. (B) IGF-I effect on cellular proliferation. Cells were plated as described above. After 24 h, cells were washed and incubated in serum-free medium without (S.F.M.) or with IGF-I alone (IGF-1) or IGF-I, PDGF, and EGF (P.E.I.). Bars represent number of cells per well, determined after 10 days in culture. (C) IGF-I-stimulated [^{3}H]thymidine incorporation in G401/WT1+ (■) and G401/WT1− (○) 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 as described in Materials and Methods. *, significantly different from the respective control ($P < 0.05$). Thymidine incorporation is expressed as cpm per 10^3 cells.

binding of labeled ^{125}I-IGF-I tracer was measured. Since G401 cells express some IGFBPs (39a), binding assays were performed in the presence of excess unlabeled insulin, which does not bind to the IGFBPs. Specific IGF-I-R binding was obtained by subtracting the binding that was not inhibited by insulin, and that therefore represented binding to IGFBPs, from the maximal binding. The results obtained indicate that ^{125}I-IGF-I binding to G401/WT1+ cells was significantly less than binding to G401/WT1− cells (14.16% ± 0.35% versus 22.05% ± 0.33% specific binding per mg of protein, means ± SEM of triplicate wells, $P < 0.001$). Similar differences were obtained by using tracer ^{125}I-des(1-3)IGF-I (29.84% ± 1.88% versus 44.08% ± 0.28%, $P < 0.02$) and ^{125}I-long R^3 IGF-I (data not shown), two analogs of IGF-I which recognize IGFBPs with 500-fold-reduced affinity.

In addition, we were unable to detect by Northern blot analysis any expression of IGF-II and PDGF A-chain mRNAs, two genes previously shown to be regulated by WT1, in G401 clones.

Biological effects of IGF-I in WT1-expressing G401 cells. To determine if the reduced rate of cellular proliferation in G401/WT1+ clones was due, at least partially, to the decreased expression of the IGF-I-R, we examined the effect of IGF-I on cellular proliferation and thymidine incorporation. IGF-I (2.6 nM), in combination with EGF and PDGF-BB as well as alone (Fig. 5A and B), stimulated the proliferation of WT1-expressing G401 cells to an extent which was, in separate experiments, only ~25 to 50% of the effect seen in G401 control cells. In a control experiment, EGF in combination with PDGF (but without IGF-I) had a modest effect on proliferation. The stimulation of G401/WT1+ cells by PDGF and EGF alone was 50% less than in G401/WT1− cells (data not shown). Additionally, the capacity of IGF-I to stimulate [^{3}H]thymidine incorporation by G401/WT1+ cells was ~23 to 81% of its capacity in G401/WT1− cells. This differential effect of IGF-I was seen at concentrations of ligand ranging from 0.1 to 10 nM (Fig. 5C). Additionally, 10% FBS stimulated thymidine incorporation in G401/WT1+ cells threefold less than in G401/WT1− cells.

IGF-I-stimulated anchorage-independent growth was also found to differ significantly between WT1-positive and -negative G401 clones (Fig. 3). Addition of IGF-I (26 nM) to the soft agar assay resulted in a 40% increment in the number of colonies generated by G401/WT1+ cells (111 ± 4 versus 79 ± 5 colonies) and a 129% increase in G401/WT1− cells (532 ± 37 versus 232 ± 10 colonies).

DISCUSSION

Wilms' tumor, or nephroblastoma, is the most common abdominal malignancy in children, with an incidence of 1 in 8,000

live births. Tumors arise from embryonic metanephric blastemal cells that would normally differentiate into the various components of the kidney. Wilms' tumor, therefore, constitutes a paradigm for the relationship between malignancy and aberrant differentiation (31, 39).

A Wilms' tumor predisposition gene, or tumor suppressor gene, WT1, has been isolated, and its inactivation was shown to be a key step in the etiology of a subset of Wilms' tumors (4, 12, 34). The IGF-I-R has also been implicated in the etiology and/or development of Wilms' tumor. Intraperitoneal administration of a monoclonal antibody against the human IGF-I-R to nude mice bearing Wilms' tumor heterotransplants prevented tumor growth and resulted in partial tumor remission (10). Furthermore, we have recently demonstrated that the IGF-I-R gene is overexpressed in Wilms' tumor and that the levels of IGF-I-R mRNA in individual tumors are inversely correlated with the levels of WT1 mRNA (42). These data are therefore in agreement with our in vitro finding that the IGF-I-R promoter is negatively regulated by WT1 (41).

During ontogeny, the WT1 gene is expressed by the kidney and urogenital system at defined stages, suggesting a role for this transcription factor in kidney differentiation (3, 30). Furthermore, inactivation of the WT1 gene by targeting in embryonic stem cells resulted in apoptosis of the metanephric blastema at day 11 of gestation and failure of the ureteric bud to grow out from the Wolffian duct (18). Since recent studies using antisense oligonucleotides against the IGF-I-R in embryonic kidneys in culture showed that inactivation of the receptor resulted in inhibition of kidney growth, reduction in nephron population, and disorganization of the ureteric bud branches (22), we investigated in this study whether WT1 could suppress the expression of the IGF-I-R gene in intact cells through repression of the IGF-I-R promoter.

For this purpose, we first analyzed the expression of the IGF-I-R gene in two kidney-derived cell lines that express different levels of endogenous WT1. The levels of IGF-I-R mRNA in 293 cells, a cell line with relatively high levels of WT1 mRNA, were low and were correlated with a very low activity of a transfected IGF-I-R promoter construct. On the other hand, the levels of IGF-I-R mRNA in G401, a cell line devoid of endogenous WT1, were much higher and were correlated with a very high level of IGF-I-R promoter activity. These results support our previous hypothesis that the promoter of the IGF-I-R gene is a target for the inhibitory action of WT1 (41, 42). This conclusion was further substantiated by the results of coexpression studies in which the IGF-I-R promoter-luciferase reporter construct was transiently cotransfected into G401 and 293 cells together with increasing amounts of a WT1 expression vector. In G401 cells, expression of WT1 induced a dose-dependent decrease in the activity of the IGF-I-R promoter. On the other hand, overexpression of WT1 in 293 cells produced an increase in promoter activity. The lack of inhibition by exogenously added WT1 may be due to the high level of endogenous WT1 in 293 cells and its already maximal suppression of IGF-I-R promoter activity. The mechanism for the observed increase in promoter activity is as yet unclear. It has been shown, however, that under certain circumstances, WT1 is able to activate transcription, and this effect appears to depend on the endogenous level of p53, a tumor suppressor gene product which may interact with WT1 (24).

The mechanism of action of WT1 has not yet been clarified. It is clear that WT1 modulates transcription of target promoters following binding of its zinc finger domain to consensus binding sites. Unlike p53, however, for which a potential mediator of tumor suppression, WAF1−, was isolated (9), no such mediators have been so far identified in the case of WT1. In this study, we investigated the functional role of the IGF-I-R gene promoter as a target for tumor suppressor WT1. For this purpose, we stably transfected WT1-negative G401 cells with a WT1 expression vector. The results obtained clearly demonstrate that the antiproliferative effect of WT1 in G401 cells is associated with a decrease in the levels of IGF-I-R mRNA, promoter activity, and ligand binding and with a reduction in IGF-I-stimulated cellular proliferation, thymidine incorporation, and anchorage-independent growth.

The data presented here suggest that the mechanism of action of WT1 involves suppression of the IGF-I-R gene promoter. The finding that a construct containing WT1 sites exclusively in the 5′ flanking region [p(−455/+30)LUC] was less suppressed than a construct containing WT1 sites mainly in the 5′ UTR [p(−40/+640)LUC] (Fig. 4B, bars 3 and 4) may suggest that in the intact cell, WT1 binding sites in the 5′ UTR are more sensitive to the inhibitory effect of WT1 than sites in the 5′ flanking region. Reduced transcription of the IGF-I-R gene results in decreased cell surface IGF-I binding sites and in a concomitant decrease in the magnitude of some IGF-I-mediated biological effects. The fact that PDGF and EGF, in the absence of IGF-I, had a smaller growth effect in G401/WT1+ cells than in G401/WT1− cells is consistent with the results of studies which showed that EGF is able to increase the levels of IGF-I in BALB/c3T3 cells (29).

The potent antiproliferative effect of WT1 may explain the fact that we were able to obtain only a small number of WT1 mRNA-expressing G401 clones. Similar attempts to introduce a WT1 cDNA in a kidney epithelial cell line (CV-1) by Dey et al. resulted in extremely low levels of WT1 expression, which could only be detected by reverse transcription PCR (7). The difficulty in obtaining cell lines stably transfected with antimitotic agents seems to be a general phenomenon. Efforts to generate clones that constitutively express $p42^{C/EBP\alpha}$, a differentiation protein that inhibits cell proliferation, were also reported to be unsuccessful (19).

The pivotal role of the IGF-I-R in transformation and proliferation events became evident from experiments which showed that fibroblast cell lines derived from mouse embryos in which the receptor gene has been disrupted by targeted homologous recombination cannot be transformed by simian virus 40 T antigen or by an activated Ras (36). Reintroduction of a functional IGF-I-R renders the cells susceptible to the transforming abilities of these oncogenes. Further support of the central role of the IGF-I-R in malignancy comes from studies which showed that overexpression of the receptor in NIH 3T3 fibroblasts results in a ligand-dependent, highly transformed phenotype, which includes the formation of tumors in nude mice (17). Introduction of antisense RNA to the IGF-I-R inhibited IGF-I-mediated growth in monolayers and clonogenicity in soft agar (33).

The expression of the IGF-I-R gene can be regulated by a number of growth factors and oncogenes, suggesting that activation of this receptor may be a common pathway in tumorigenesis (2). Thus, PDGF has been shown to increase the levels of IGF-I-R binding, mRNA, and promoter activity (6, 35). A short fragment of the promoter region containing a c-*myc* canonical binding site is sufficient to mediate this effect. Likewise, the proliferative effects of estrogens on breast carcinoma cell lines are probably related to their up-regulating effect on the IGF-I-R gene, which sensitizes the cells to the proliferative effects of IGFs (37).

The results of this study clearly show that in addition to being a target for growth factors and oncogenes, the IGF-I-R gene can also be regulated by negative factors, i.e., tumor

suppressor gene products. Loss of WT1 activity in Wilms' tumor may result from constitutional deletions (4, 12, 34), missense or nonsense mutations often affecting the zinc finger region (20), or alternate splicing involving exon 2 that produces a transcriptional activator WT1 variant (13). All of these mechanisms may result in transcriptional derepression of the IGF-II and IGF-I-R genes. Activation of the IGF-I-R by locally produced IGF-II may elicit a mitogenic event which may be a key mechanism for the progression of Wilms' tumor. The potential contributions of other genes known to be regulated by WT1, i.e., the PDGF A-chain, transforming growth factor β, and colony-stimulating factor 1 genes, in Wilms' tumor etiology remain to be clarified.

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