The IGF-I Receptor Gene Promoter Is a Molecular Target for the Ewing's Sarcoma-Wilms' Tumor 1 Fusion Protein*

(Received for publication, March 11, 1996, and in revised form, May 24, 1996)

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Desmoplastic small round cell tumor (DSRCT) is an abdominal malignancy in children which is characterized by a recurrent chromosomal translocation, t(11;22)(p13;q12). This rearrangement results in the fusion of the ubiquitously expressed EWS1 gene to the Wilms' tumor suppressor (WT1) gene. The chimeric protein contains the N-terminal domain of EWS1 fused to the DNA-binding domain of WT1, including zinc fingers 2–4. Because WT1 has been shown previously to bind and repress the insulin-like growth factor I (IGF-I-R) promoter, we investigated whether this promoter is, in addition, a target for the aberrant EWS/WT1 transcription factor.

EWS/WT1 activated the IGF-I-R promoter 340%, whereas a fusion protein containing a three-amino acid insert (KTS) between zinc fingers 3 and 4 had no effect. On the other hand, expression vectors encoding either WT1 or EWS1 reduced the activity of the promoter to 46 and 58% of control values, respectively. Results of gel shift assays indicate that the binding affinity of EWS/WT1 to a fragment of the 5'-flanking region of the receptor promoter was higher than the affinity of WT1 itself. Consistent with the results of functional assays, the binding of EWS/WT1(+)KTS was significantly reduced.

Due to the central role of the IGF-I-R in tumorigenesis, activation of the receptor promoter by EWS/WT1 may constitute a potential mechanism for the etiology and/or progression of DSRCT.

The insulin-like growth factor I receptor (IGF-I-R) is a tyrosine kinase membrane-bound receptor that mediates the growth and differentiation actions of the IGFs (IGF-I and IGF-II). It is constitutively expressed by most tissues, where it is basically required for progression through the cell cycle (1–4). Thus, deletion of the IGF-I-R gene in mice by homologous recombination is incompatible with postnatal life (5, 6). 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 (7) and by the inability of a number of oncogenes to transform cell lines lacking IGF-I-R (8–10).

Some human malignancies are characterized by recurrent chromosomal translocations, frequently resulting in the fusion of genes (11). One of the best characterized rearrangements is the translocation of the c-myc gene in Burkitt's lymphoma (12). In this type of cancer, the c-myc protooncogene is juxtaposed to an immunoglobulin gene by chromosomal fusion, thereby activating the oncogene. A second example of a chromosomal translocation in hematopoietic tumors is the fusion of the bcr and c-abl genes on the Philadelphia chromosome in chronic myelogenous leukemia, with generation of an oncogenic fusion protein (13). More recently, a number of pediatric malignant tumors, such as soft-tissue sarcomas, were shown to be characterized by recurrent chromosomal translocations, frequently resulting in the fusion of genes (11). These fusion gene products often comprise potential transcription factors and nucleic acid-binding proteins. An example of this growing family of disrupted transcription factors is the (11;22) chromosomal translocation found in nearly 90% of Ewing's sarcomas (EWS) and primitive neuroectodermal tumor of childhood (14–17). This translocation results in the fusion of the 5' region of the ubiquitously expressed EWS1 gene to the 3' region of the FLI-1 gene. This chimeric gene product (EWS/FLI-1) is an aberrant transcription factor which contains the transcriptional domain of EWS1, which is usually involved in protein-protein interactions, and the DNA-binding domain of FLI-1. Although capable of promoting tumorigenesis (18, 19), the target gene(s) of EWS/FLI-1 are not yet fully known.

Similarly, desmoplastic small round cell tumor (DSRCT), a distinctive primitive tumor in children, is associated with a recurrent translocation, t(11;22)(p13;q12) (20–22). Recently, a genomic DNA fragment containing an EWS1 and Wilms' tumor (WT1) fusion gene has been isolated from these tumors (23–25). Analyses of chimeric transcripts showed fusion of RNAs encoding the N-terminal domain of EWS1 to both alternatively spliced forms of the last three zinc fingers of the DNA-binding domain of WT1. As for the EWS/FLI-1 chimeric protein, the functional target gene(s) of EWS/WT1 remain unknown.

WT1 itself is a tumor suppressor gene product that functions as a transcription factor and whose deletion or mutation has been implicated in the etiology of a subset of Wilms' tumors (26–29). WT1 interacts with target promoters containing the consensus sequence GCGGGGCGC by means of four zinc finger motifs located at its C terminus, and usually suppresses their activity by means of its N-terminal activation domain (30–32). We have previously shown that the IGF-I-R gene is overex-
pressed in Wilms' tumor, consistent with the IGF-I-R gene promoter being a target for the inhibitory action of WT1 (33). Accordingly, WT1 expression in Chinese hamster ovary (CHO) cells results in a dose-dependent decrease in the activity of a co-transfected IGF-I-R promoter-luciferase reporter construct (34, 35). This effect of WT1 involves the interaction of its zinc finger domain with multiple consensus binding sites in both the 5′-flanking and 5′-untranslated regions of the IGF-I-R gene.

Since the EWS/WT1 chimeric product obtains the three C-terminal zinc fingers of the DNA binding domain of WT1 it is anticipated that this fusion protein may modulate transcription of target genes containing WT1 binding motifs, such as the IGF-I-R gene. Due to the pivotal role of IGF-I-R in transformation events we have investigated the potential molecular mechanisms for the regulation of the IGF-I-R gene promoter by the EWS/WT1 fusion protein at the transcriptional level.

EXPERIMENTAL PROCEDURES

Cell Cultures and Plasmids—Saos-2 and G401 cells were obtained from the American Type Culture Collection (Rockville, MD). Saos-2 is a human osteosarcoma-derived cell line and G401, initially considered a Wilms' tumor-derived cell line, is now thought to be a sarcoma-derived cell line (36). Saos-2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 5% calf serum. G401 were grown in McCoy's 5A medium, containing 10% fetal bovine serum. Media were supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin, 2.5 μg/ml fungizone, and 2 mM l-glutamine.

For transient co-transfection experiments, a genomic IGF-I-R fragment containing 476 base pairs of 5′-flanking region and 640 base pairs of 5′-untranslated region was subcloned upstream of a promoter-less firefly luciferase reporter gene, pO TLC. The promoter activity of this construct, p(-476)/640LUC, which includes most of the proximal promoter region, has been previously described (34). In some transient transfection experiments, the following fragments of the IGF-I-R gene promoter were used: −455/+30, −115/+60, and −40/+60 (nucleotide no. 1 corresponds to the transcription start site of the IGF-I-R gene).

Two EWS/WT1 expression vectors were employed, pCMV-EWS/WT1 and pCMV-EWS/WT1(+KTS) (see Fig. 1A). Both constructions were constructed via polymerase chain reaction-mediated overlapping gene fusion techniques (30), and the resulting chimeric genes were subcloned into the cytomegalovirus (CMV) promoter-containing expression vector, pCB6 (+) (23). Both plasmids encode the N-terminal domain of EWS1 fused to the three C-terminal zinc fingers of WT1. Expression vector pCMV-EWS/WT1(+KTS) contains a 9-base pair insert encoding a 3-amino acid fragment (Lys-Thr-Ser) following residue 390 of the WT1 sequence. pCMV-EWS/WT1 expression vector encodes an EWS/WT1 protein lacking this insert.

The WT1 expression vector (pCMV-hWT1) was constructed by inserting a 2.1-kilobase pair human WT1 DNA downstream of the CMV promoter in the pCB6 (+) vector, as described previously (32). The EWS expression vector (pCMV-EWS1) was constructed by subcloning a 2.2-kilobase pair NotI-ClaI human EWS1 DNA (a gift from Dr. Christopher Denny, Department of Pediatrics, University of California, Los Angeles) into the NotI and ClaI sites of pCB6 (+). A β-galactosidase expression vector (pCMV β-galactosidase, Clontech) was used as a control for transfection efficiency.

DNA Transfections—Saos-2 cells were transfected using a calcium phosphate transfection kit (5 Prime—3 Prime Inc., Boulder, CO). Each 100-mm dish received 10 μg of reporter plasmid, 2.5 μg of pCMV-β-gal, and variable amounts of pCMV-EWSWT1 (without or with KTS), or of pCMV-hWT1 or pCMV-EWS1. In each case the total amount of DNA transfected (15 μg) was kept constant using pCB6 (+) DNA. Four hours after transfection, DNA-containing medium was changed to complete medium, and the plates were incubated for an additional 40 h at 37 °C. G401 cells were transfected using 50 μg of Lipofectin® reagent (Life Technologies, Inc.) in Opti-minimal Eagle's medium. Each dish received 1 μg of reporter plasmid, 20 μg of expression vector, and 4 μg of pCMV-β-galactosidase. After 16 h the Lipofectin reagent-containing medium was changed to McCoy's 5A medium, and the plates were incubated for an additional 48 h at 37 °C. At the indicated times, cells were harvested, lysed, and luciferase and β-galactosidase activities were measured as described previously (37).

Gel Retardation Assays—A fragment of the IGF-I-R 5′-flanking region extending from −331 to −40 was isolated by digestion of a genomic IGF-I-R clone with XmnI and PmlI. Following purification from agarose gels, the fragment was end-labeled with [γ-32P]ATP, using T4 polynucleotide kinase, and separated from unincorporated nucleotide using 5′-32P-labeled columns (Schleicher & Schuell).

The following recombinant proteins were produced as histidine-fusion proteins in Escherichia coli and employed in gel retardation assays: EWS/WT1, EWS/WT1(+KTS), and the zinc finger domain of WT1, or WTZ2F (23). The EWS/WT1 fusion proteins contain 21 EWS1-encoded amino acids N-terminal of the fusion point and zinc fingers 2, 3, and 4 of WT1. They either lack or contain the three-amino acid insert between zinc fingers 3 and 4. Recombinant protein WTZF contains all four zinc fingers of WT1; the binding activity of this protein was described previously (30).

DNA gel retardation assays were performed by preincubating 0.5, 20, and 50 ng of the purified proteins in 5 μl of 20 mM Hepes, pH 7.5, 70 μM KCl, 12% glycerol, 0.05% Nonidet P-40, 100 μM ZnSO4, 0.05 mM dithiothreitol, 1 mg/ml bovine serum albumin, and 0.1 mg/ml poly(dI-dC), with or without the indicated unlabeled competitor DNA, on ice. After 15 min, 75,000 dpm (0.2–1 ng) of the labeled fragment was added, and the reaction was incubated for an additional 10 min. Shifts in mobility were assessed by scanning through a 5% polyacrylamide gel that was run at 250 V for 2 h at 4 °C.

DNase I Footprinting—DNase I footprinting reactions were performed as described previously (34) using a labeled fragment of the IGF-I-R promoter extending from −331 to +115. Maxam-Gilbert A and G sequencing reactions were run as markers.

RESULTS

Regulation of IGF-I-R Promoter Activity by the EWS/WT1 Fusion Protein—In previous studies, we have identified the promoter region of the IGF-I-R gene as a molecular target for tumor suppressor WT1 (33-35). We showed that WT1 binds both upstream and downstream of the IGF-I-R gene transcription start site and suppresses transcription of transfected IGF-I-R promoter constructs, as well as of the endogenous gene. Since the chimeric EWS/WT1 protein retains most of the DNA binding domain of WT1, we began to analyze the transcriptional regulation of the IGF-I-R promoter by EWS/WT1. For this purpose, we cotransfected the human osteosarcoma cell line Saos-2 with a reporter construct containing most of the proximal region of the IGF-I-R promoter upstream of a luciferase gene. This fragment, extending from nucleotide −476 in the 5′-flanking region to +640 in the 5′-untranslated region, contains 12 WT1 binding sites as shown in Fig. 1B, cotransfection of an EWS/WT1 expression vector lacking the 3-amino acid insert (pCMV-EWS/WT1) increased the activity of the IGF-I-R promoter −340%. The effect of EWS/WT1 was dose-dependent, with maximal activity seen already at 2.5 μg of expression vector (Fig. 1C). On the other hand, an EWS/WT1 expression vector including the 3-amino acid insert (pCMV-EWS/WT1(+KTS)) was unable to stimulate promoter activity (Fig. 1B). For control purposes we cotransfected cells with a full-length WT1 expression vector. As shown previously in other cell lines (34, 35), WT1 suppressed promoter activity (46% of control levels) in Saos-2 cells. Interestingly, full-length EWS1 similarly reduced the activity of the IGF-I-R promoter to 58% of control values (Fig. 1B).

Cotransfections were also performed in G401, a Wilms' tumor-derived cell line in which we previously studied the tumor-suppressing effect of WT1. Similarly to Saos-2 cells, EWS/WT1 stimulated the activity of the IGF-I-R promoter, although the effect was much less pronounced. EWS/WT1(+KTS) had no effect, whereas both native WT1 and EWS reduced promoter activity (Fig. 2).

To determine the region of the IGF-I-R promoter responsible for the response to EWS/WT1, cotransfection experiments were performed in Saos-2 cells using a number of IGF-I-R promoter/reporter plasmids containing different portions of 5′-flanking and 5′-untranslated regions (Fig. 3). Construct p(−455/+30)LUC, in which all six potential WT1 sites in the 5′-un-
A. Expression vectors

B. 10 micrograms of the p(−2476/+640)LUC reporter plasmid (shown in Fig. 3) were cotransfected into Saos-2 cells with 2.5 µg of the indicated expression vector using the calcium phosphate method. After 40 h, the cells were harvested, and the levels of luciferase and β-galactosidase activities were measured. The luciferase values, normalized for β-galactosidase, are expressed as percentage over the activity of the empty pCB6+ expression vector. Experiments were performed between three and seven times, each time in duplicate. Bars are mean ± S.E.

C. Dose-dependence of the effect of EWS/WT1. Ten micrograms of the p(−476/+640)LUC were cotransfected in Saos-2 cells with increasing amounts of EWS/WT1 expression vector. Cells were processed as indicated above.

**Fig. 1.** Activation of IGF-I-R promoter activity by the EWS/WT1 fusion protein in Saos-2 cells. A, schematic representation of the expression vectors used in transient transfection experiments. Full-length cDNAs encoding EWS1, WT1, and EWS/WT1 (with and without KTS) were subcloned downstream of the CMV promoter in the pCB6+ vector. B, 10 micrograms of the p(−2476/+640)LUC reporter plasmid (shown in Fig. 3) were cotransfected into Saos-2 cells with 2.5 µg of the indicated expression vector using the calcium phosphate method. After 40 h, the cells were harvested, and the levels of luciferase and β-galactosidase activities were measured. The luciferase values, normalized for β-galactosidase, are expressed as percentage over the activity of the empty pCB6+ expression vector. Experiments were performed between three and seven times, each time in duplicate. Bars are mean ± S.E. C, dose-dependence of the effect of EWS/WT1. Ten micrograms of the p(−476/+640)LUC were cotransfected in Saos-2 cells with increasing amounts of EWS/WT1 expression vector. Cells were processed as indicated above.
translated region were removed, was stimulated by EWS/WT1 to an extent that was not significantly different from the effect seen with p(−476/+640)LUC. Likewise, reporter construct p(−188/+640)LUC, in which WT1 sites at positions −262/−254, −250/−242, −220/−212, and −196/−188 were removed, was stimulated by the chimeric protein. Removal of the WT1 site at position −163/−155 in the p(−40/+640)LUC construct resulted in a drastic reduction in basal promoter activity to levels resembling those of the promoterless luciferase reporter pGLUC (data not shown). Reporter plasmid p(−400/+640)LUC was not responsive to EWS/WT1 (Fig. 3).

Interaction of EWS/WT1 Fusion Protein with the IGF-I-R Promoter.—To analyze the interactions between EWS/WT1 and the promoter region of the IGF-I-R gene, gel retardation assays were performed using a fragment of the 5′-flanking region extending from −331 to −40, together with the following recombining proteins: EWS/WT1, EWS/WT1(+KTS), and WTZF. EWS/WT1 proteins contained the 21-amino acid fragment of EWS1 located N-terminal to the fusion point and most of the C-terminal domain of WT1, including zinc fingers 2–4 (Fig. 3A). Incubation of the labeled −331/−40 fragment with increasing amounts of the fusion protein lacking the KTS insert resulted in the appearance of five retarded bands (at 50 ng of protein), consistent with the number of WT1 sites footprinted in this region (Fig. 4B). Incubation with WTZF generated four bands, which suggests that the chimeric protein had an increased affinity for WT1 binding sites in comparison to the native WT1 protein. EWS/WT1(+KTS) had a largely reduced affinity for WT1 sites, a fact that is consistent with: (a) the inability of an expression vector encoding EWS/WT1(+KTS) to stimulate promoter activity (Fig. 1B) and (b) the results of early studies which suggested that the KTS insert interferes with the binding of native WT1 protein to the IGF-I-R promoter region (34).

The formation of the DNA-protein complexes was greatly diminished when the binding reactions were performed in the presence of a 30–300-fold molar excess of the −331/−40 unlabeled fragment, but not when an upstream DNA fragment (−455/−331) lacking WT1 sites was used as competitor (Fig. 5A). Conversely, incubation of the labeled −455/−331 fragment with EWS/WT1 or WTZF proteins generated a single retarded band which appears to be the result of a nonspecific interaction, since its formation was not abolished by excess of cold competitor. When combined, these results indicate that interaction between EWS/WT1 and the IGF-I-R promoter occurs only at specific WT1 sites (Fig. 5B).

Finally, to determine whether the fusion protein and WT1 bind to the same or to different sites on the IGF-I-R promoter, DNase I footprinting was employed using a 32P-labeled fragment extending from −331 to −115, together with purified EWS/WT1 and WTZF proteins. As shown in Fig. 6, both proteins generated the same footprints in this region of the receptor gene, indicating that the fusion protein binds to the same sites as the native protein.

**DISCUSSION**

Tumor-specific chromosomal translocations resulting in chimeric transcription factors emerged as a general theme in oncogenesis (11). The modular organization of transcription factors is disrupted by the chromosomal event, and gain-of-function mutant genes harboring motifs derived from unrelated genes are usually generated. Many of these fusion proteins have been shown to be highly oncogenic.

DSRCT is a very aggressive clinicopathological subtype of small cell round tumors (SCRT) which occurs most frequently in adolescent males and is mainly circumscribed to the abdomen (38). Histopathological studies showed the presence of epithelial, mesenchymal, and neuronal elements, similarly to triphasic Wilms’ tumors which also contain epithelial, mesenchymal, and stromal cells (39). Cytogenetic analyses revealed the presence of a recurrent chromosomal translocation,
The IGF-I-R has a central role in transformation and proliferation events, and its presence is a fundamental prerequisite for the transforming ability of simian virus 40 large T antigen, Ras, and other oncoproteins (1, 8–10). Under most physiological conditions the expression of the potent IGF-I-R promoter is negatively controlled by a number of tumor suppressors, including p53 and WT1 (45). Inhibitory control of IGF-I-R gene expression by WT1 can be overcome by the pathologic fusion of EWS1 to WT1, an event which abrogates the tumor suppressor
Fig. 6. DNase I footprinting analysis of the 5′-flanking region of the IGF-I-R gene promoter with EWS/WT1 and WTZF. A 32P-labeled probe extending from −333 to +115 was incubated with 15 or 60 ng of purified EWS/WT1 or WTZF (or without protein) and subsequently digested with DNase I, as described previously (34). Thick bars denote the location of consensus WT1-like sequences. A+G Maxam-Gilbert sequencing reaction; M, molecular weight marker.

effect of WT1 and which generates an oncogenic chimera. By means of the WT1-derived DNA binding domain, EWS/WT1 is able to recognize and activate the same set of target genes which were previously negatively regulated by WT1.

In conclusion, we have presented evidence that the IGF-I-R promoter is a molecular target for the EWS/WT1 fusion protein. Relief from negative regulation by WT1 and activation by EWS/WT1 constitute a novel paradigm in tumorigenesis.

Acknowledgment—We thank Dr. Guck Ooi for critical review of the manuscript.

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TABLE 1

| Protein (ng) | A+G M |
|-------------|-------|
| 15          | 60    |
| 60          | 60    |