The Wilms’ Tumor Gene Product WT1 Mediates the Down-regulation of the Rat Epidermal Growth Factor Receptor by Nerve Growth Factor in PC12 Cells*

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Recently, we characterized the rat epidermal growth factor receptor (EGFR) promoter and demonstrated that TCC repeat sequences are required for the down-regulation of EGFR by nerve growth factor (NGF) in PC12 cells. In this study, we report that the Wilms’ tumor gene product WT1, a zinc finger transcription factor, is able to enhance the activity of the rat EGFR promoter in cotransfection assays. Gel mobility shift assays demonstrate that WT1 binds to the TCC repeat sequences of the rat EGFR promoter. Overexpression of WT1 resulted in up-regulation of the expression levels of endogenous EGFR in PC12 cells. Interestingly, NGF down-regulated the expression levels of WT1 and EGFR in PC12 cells, but not in the p140trk-deficient variant PC12nnr5 cells or in cells expressing either dominant-negative Ras or dominant-negative Src. Most importantly, we evaluated the inhibitory effect of antisense WT1 RNA on EGFR expression, and we found that antisense WT1 RNA could substantially reduce EGFR expression in either histochemical staining study or immunoblot analysis. These results indicate that NGF-induced down-regulation of the EGFR in PC12 cells is mediated through WT1 and that WT1 may play an important role in the differentiation of nerve cells.

The epidermal growth factor receptor (EGFR) is a member of the ErbB family of ligand-activated tyrosine kinase receptors, which play a central role in the proliferation, differentiation, and/or oncogenesis of epithelial cells, neural cells, and fibroblasts (1). Some years ago, we reported that PC12 cells respond to both epidermal growth factor (EGF) and nerve growth factor (NGF), but that the response to epidermal growth factor is mitogenic, whereas the response to nerve growth factor leads to differentiation (2). It was of interest to determine what would happen if the cells were exposed to both stimuli at the same time. The answer was that the cells simply did not respond to EGF after they had been treated with NGF, and the reason that they did not respond is that the EGFR is markedly down-regulated by treatment of the cells with NGF (3).

Our studies have focused on the molecular mechanism by which NGF down-regulates the EGFR. Previous studies have demonstrated that NGF-induced down-regulation of the EGFR was at the transcriptional level (4) and that the down-regulation is p140trk, Ras, and Src-dependent (5). Recently, we isolated and characterized the rat EGFR promoter region and found that TCC repeat sequences in the promoter region of the rat EGFR are required for the down-regulation of rat EGFR by NGF during the differentiation of PC12 cells (6).

WT1, the Wilms’ tumor suppressor gene, is located at chromosome locus 11p13 and encodes a zinc finger protein that is one of several transcription factors that have been found to interact with TCC repeat sequences (7–9). Experimental evidence has indicated that WT1 not only plays a role during kidney development but is also involved in the development and homeostasis of other tissues. The products of WT1 have been implicated in various cellular processes such as proliferation, differentiation, and apoptosis (10). To better understand the molecular mechanisms of down-regulation of EGFR by NGF in PC12 cells, it is important to study the possible interaction between WT1 and EGFR during the differentiation of PC12 cells.

Here, we report that the Wilms’ tumor gene product WT1 activates the activity of the rat EGFR promoter through binding to the TCC repeat sequences. Treatment of PC12 cells with NGF decreases the expression levels of WT1 and EGFR. However, the down-regulation of WT1 by NGF, like the EGFR, is not seen in PC12nnr5 cells or in cells expressing either dominant-negative Ras or dominant-negative Src. As another approach, we have studied the inhibitory effect of antisense WT1...
RNA on EGFR expression and found that antisense WT1 RNA could substantially reduce EGFR expression. These data strongly suggest that WT1 is involved in the NGF-induced down-regulation of the expression levels of EGFR receptor in PC12 cells.

**EXPERIMENTAL PROCEDURES**

**Materials—**NGF and rat type I collagen were purchased from Becton Dickinson (Bedford, MA). Monoclonal antibody against the EGFR (6F1) was obtained from Medical and Biological Laboratories, Co., Ltd. (Nagoya, Japan). Anti-WT1 antibody (C-19) and pCMV-Sp1 antisense and sense constructs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). LipofectAMINE and LipofectAMINE 2000 were purchased from Life Technologies, Inc. (Gaithersburg, MD). Cy3-linked donkey anti-mouse antibody was purchased from Jackson Immunoresearch (West Grove, PA). The Mammalian Transfection Kit was purchased from Stratagene (La Jolla, CA). Stable dominant-negative Src PC12 variant cells (SrcDN2) were generously provided by Dr. Simon Halegoua (State University of La Jolla, CA). Stable dominant-negative Ras PC12 variant cells (M-M17-26) were generously provided by Dr. Geoffrey Cooper (Dana Farber Cancer Center, Boston, MA), and PC12nr5 (a pheochromocytoma clone unresponsive to nerve growth factor) cells were graciously contributed by Dr. Lloyd Greene (Columbia University, New York, NY).

**Cell Culture—**PC12 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum, 10% horse serum, 100 μg/ml streptomycin, and 100 units/ml penicillin. For NGF treatment, 100 ng/ml NGF was added to the culture medium. In all experiments involving extended treatment with NGF, the medium was changed, and fresh NGF was added every other day. PC12nr5 cells (11) were grown in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 5% fetal bovine serum and 10% horse serum. The PC12 cell variants M-M17-26 expressing the Ha-ras Asn-17 gene under the control of the CMV immediate-early promoter (12) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cells were lysed in a buffer containing 20 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% deoxycholic acid, 10% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM sodium vanadate, and 50 mM sodium fluoride at 4°C for 30 min. Insoluble material was removed by centrifugation, and equal amounts of lysates (25 μg) were used for immunoblotting. Protein concentration was estimated using the Bio-Rad protein assay system. Samples were resolved on a 7.5% SDS-polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking with nonfat dry milk, the blot was incubated with either 6F1 anti-EGFR antibody (0.6 μg/ml) or anti-WT1 antibody (C-19; 1:10,000 dilution). Bound antibodies were detected by sheep anti-mouse immunoglobulin or donkey anti-rabbit immunoglobulin conjugated with horseradish peroxidase (Amersham Pharmacia Biotech, Piscataway, NJ) and analyzed with the Supersignal Chemiluminescent Substrate (Pierce, Rockford, IL).

**Plasmid Constructs—**The rat EGFR promoter-luciferase pRE constructs and the chimeric EGFR reporter pRE-TK-Luc plasmids have been described previously (6). The original WT1 expression vector pCMV-WT1 consisted of the full-length protein coding regions under the control of the CMV immediate-early promoter (14). pCMV-WT1 was cut with EcoRI and subcloned into pTracer-CMV2 (Invitrogen, Carlsbad, CA), which contains a SV40 early promoter that drives expression of the green fluorescent protein (GFP) gene fused to a Zeocin resistance gene and a CMV immediate-early promoter driving expression of the second cDNA. WT1 expression plasmid pCMV2-WT1-GFP and antisense WT1 expression plasmid pCMV2-antiWT1-GFP were confirmed by DNA sequencing. Plasmid DNAs were prepared from these constructs using Maxi-prep kits (Qiagen, Valencia, CA) and quantitated by UV absorbance at 260 nm. Protein concentrations were determined using a BCA protein assay system (Pierce). Samples were resolved on a 4% polyacrylamide gel, followed by autoradiography. To determine specific binding, unlabeled specific competitor oligonucleotides were added in 50-fold molar excess over labeled probe. To identify proteins in the DNA-protein complex, supershift experiments were performed with either an anti-WT1 C-19 antibody or an anti-Sp1 antibody.

**Evaluation of Endogenous WT1 Effects on EGFR Expression in PC12 Cells—**For histochemical staining, PC12 cells grown on poly-L-lysine (Sigma, St. Louis, MO)-coated LAB-TEK chamber slides (Nalge Nunc, Naperville, IL) were transiently transfected with 1 μg of pCMV2-antiWT1-GFP or GFP alone for 24 h using LipofectAMINE 2000 according to the manufacturer’s protocol. Cells were fixed in PBS containing 4% paraformaldehyde for 10 min and then washed three times in PBS. EGFR was detected with anti-EGFR antibody 6F1 at a dilution of 1:50 and with Cy3-linked donkey anti-mouse antibody at a dilution of 1:100. Cells were imaged on a Nikon ECLIPSE E800 microscope with a ×40 immersion lens. Fifteen fields were examined, and representative fields were photographed.

**RESULTS**

**WT1 Activates the Promoter Activity of the Rat EGFR Promoter—**Recently, we isolated and characterized the promoter region of the rat EGFR promoter, and we found that TCC repeat sequences in the promoter region are required for NGF-induced transcriptional down-regulation of EGFR (6). The TCC repeat sequences in the human EGFR promoter have been reported to be a DNA-binding sequence for the Wilms’ tumor repressor, WT1 (16). Thus, it is possible that WT1 may bind to this TCC-rich sequence and modulate the transcription of the rat EGFR gene. To test this hypothesis, we cotransfected different reporter plasmids expressing activator and internal deletion constructs of rat EGFR promoter into PC12 cells with pCMV-WT1 expression vectors or empty control vectors. Interestingly, expression of WT1 activated the promoter activity of the reporter plasmid about 2-fold. These results suggest that the rat EGFR gene is one of the genes transcriptionally activated by WT1 (17).
Sequence analysis of the 5'-flanking region of the rat EGFR gene shows that the TCC repeat sequences were found at −2343, −2317, and −2298 relative to the translation starting site (6). Significant transcriptional activation of the rat EGFR promoter by WT1 was observed in the constructs pRE1102, pRE1102D260, pRE318, and pRE318D260, all of which contain two or three TCC repeat sequences, whereas the construct pRE1102D318–260, which contains only one TCC repeat sequence, was activated slightly by WT1 (Fig. 1A). Also, the promoter activities of the constructs pRE260 and pRE181, both of which lack TCC repeat sequences, were not activated by WT1. Thus, we concluded that WT1 activated the promoter activity of the EGFR gene through the TCC repeat sequences in the promoter region.

To further determine whether the TCC repeat sequences in the EGFR promoter are functionally responsible for the activation of the EGFR by WT1, we cloned oligonucleotides containing the 59-base pair DNA fragments (−318 to −260 of the EGFR promoter) upstream of the herpes simplex virus thymidine kinase luciferase reporter plasmid. This reporter plasmid was cotransfected with pCMV-WT1 expression vector into PC12 cells (Fig. 1B). WT1 produced a 2- to 4-fold activation of the reporter constructs containing TCC repeat sequences and single site mutation of TCC repeat sequences. However, the activation was abolished in the reporter constructs containing no TCC repeat sequences (p260Tk-Luc) or multiple mutation of TCC repeat sequences (pM1TK-Luc). These data support the observation that the TCC repeat sequences are responsible for the transcriptional activation of the rat EGFR mediated by WT1.

**WT1 Up-regulates the Expression Levels of Endogenous EGFR in PC12 Cells**—To determine whether WT1 up-regulates EGFR expression, we transiently transfected PC12 cells with the pCMV2-WT1-GFP expression vector or with the GFP empty vector as a control and prepared GFP fluorescence-sorted cell lysates that were then tested for the levels of EGFR. Western analysis indicated that WT1 increased the protein level of EGFR in PC12 cells (Fig. 2A). Additionally, WT1 was found to lead to a 6-fold activation of the rat EGFR promoter construct pRE318 (Fig. 2B). These results indicate that expres-
NGF-induced Down-regulation of WT1 Is p140<sup>trk</sup>-dependent—Signal transduction pathways in PC12 cells have been studied thoroughly, providing the opportunity to define the pathway mediating WT1 down-regulation. We have previously shown that down-regulation of EGFR in PC12 cells caused by NGF treatment is dependent on p140<sup>trk</sup>, Ras, and Src pathways (5). It is quite reasonable to ask whether these pathways are involved in the down-regulation of WT1. To test this possibility, three cell lines were used: PC12nnr5, a cell line that expresses very little or no p140<sup>trk</sup>, the high affinity receptor for NGF (11); and two PC12 cell variants that express dominant-negative forms of Ras (M-M17-26) and Src (SrcDN2), respectively (12, 13). When cells were treated with NGF for 5 days, expression levels of both WT1 and EGFR were decreased dramatically in wild-type PC12 cells. However, no reduction of WT1 and EGFR proteins in PC12 cells is detectable 1 day after the addition of NGF, and this reduction is gradual and progressive. The correlation coefficient (r) between the relative protein level of EGFR and WT1 is 0.9758 (p < 0.001).

Endogenous WT1 Effects on EGFR Expression in PC12 Cells—To further determine the effects of endogenous WT1 on EGFR expression, we evaluated the inhibitory effect of an antisense WT1-GFP construct in PC12 cells. Histochemical staining revealed that expression of both WT1 and EGFR was observed upon NGF treatment in M-M17-26, SrcDN2, and PC12nnr5 cells (Fig. 4). These data strongly suggest that NGF-induced down-regulation of EGFR involves WT1 in a p140<sup>trk</sup>-, Ras-, and Src-dependent manner.

**Fig. 3.** The TCC repeat sequences appeared to be responsible for the WT1-mediated down-regulation of the EGFR by NGF in PC12 cells. The 59-base pair radiolabeled duplex probe (1 ng) extending from −318 to −260 was incubated with 10 μg of untreated PC12 cell nuclear extracts in the absence (lane 1) or presence of 50-fold molar excesses of unlabeled cold probe (lane 2), depicted at the top of each lane. Supershift experiments were performed with either anti-WT1 C-19 antibody or anti-Sp1 antibody (lanes 3 and 4), and electrophoresis was performed on nondenaturing 4% polyacrylamide gels. Two major protein-DNA complexes (A and B) were observed with nuclear extracts from PC12 cells.

**Fig. 2.** Exogenous expression of WT1 increases the level of endogenous EGFR in PC12 cells. PC12 cells were cotransfected with either pCMV2-WT1-GFP expression vector or empty GFP vector, together with pEG318 reporter plasmid and pRL-TK, by the calcium phosphate method. Forty-eight h after transfection, cells were sorted by GFP fluorescence using a FACScan flow cytometer. Cells with GFP expression and untransfected cells were harvested, and expression levels of EGFR and WT1 (A) and luciferase activity (B) were measured. A, immunoblot analysis of EGFR and WT1 detected by anti-EGFR antibody 6F1 and anti-WT1 antibody, respectively. B, luciferase activity induced by WT1 expression. Error bars, ± S.D. Each experimental point was performed in triplicate.

**WT1 Binds Directly to the TCC Repeat Sequences of the EGFR Promoter—**To determine whether WT1 binds directly to the TCC repeat sequences within the rat EGFR promoter (−318 to −260), electrophoretic mobility shift analysis was performed (Fig. 3). Two major protein-DNA complexes (Fig. 3, complexes A and B) were observed with nuclear extracts from PC12 cells. To determine whether WT1 was present in the protein-DNA complexes, polyclonal antibody against WT1 was used. The addition of anti-WT1 antibody decreased the intensity of complex A dramatically, whereas the addition of anti-Sp1 antibody did not, indicating that WT1 binds to the region of EGFR promoter (Fig. 3, lanes 3 and 4). In support of this result, mutations in TCC repeat sequences abolished the binding of WT1 in electrophoretic mobility shift analysis as described previously (6). Thus, some of complex A contains WT1 that reacts with anti-WT1. These results suggest that WT1 transcriptionally triggers EGFR promoter activity through direct binding to the TCC repeat sequences within the EGFR promoter.

**WT1 Expression Level Is Down-regulated by NGF in PC12 Cells—**To further determine the effects of endogenous WT1 on EGFR expression, we evaluated the inhibitory effect of an antisense WT1-GFP construct in PC12 cells. Histochemical staining revealed that expression of both WT1 and EGFR in pCMV2-antiWT1-GFP transfected cells was decreased compared with that in GFP control cells and wild-type PC12 cells (Fig. 5A). The decrease in EGFR expression was also detected by immunoblot analysis comparing extracts from pCMV2-
antiWT1-GFP-transfected cells with GFP control cells and wild-type PC12 cells (Fig. 5B). This result indicates that endogenous WT1 contributes to the regulation of EGFR expression.

**DISCUSSION**

EGFR, the prototypical member of the superfamily of receptors with intrinsic tyrosine kinase activity, is widely expressed on many cell types. Upon activation by its ligand, the intrinsic kinase phosphorylates its own tyrosines and numerous intermediary effector molecules, including closely related c-erbB receptor family members. This initiates numerous signaling pathways. The integrated biological responses to EGFR signaling are pleiotropic, including mitogenesis, apoptosis, enhanced cell motility, and differentiation. In addition to being implicated in organ morphogenesis, unregulated EGFR signaling has been correlated in a wide variety of tumors with invasion and metastasis. Thus, regulation of EGFR signaling including its own expression has potential impact on the integrity of cells.

The Wilms’ tumor gene (WT1) is one of at least three genes that are involved in the development of Wilms’ tumor, a pediatric kidney cancer. Wilms’ tumor has been the subject of intense clinical and basic scientific research because it presents a model for cancer treatment and for the relationship between development and cancer, and it illustrates the impact of genetic alterations on development and tumorigenesis. The products of WT1 have been implicated in various cellular processes such as proliferation, differentiation, and apoptosis. In agreement with the diverse functions of WT1, the number of candidate target genes is rapidly mounting (10). EGFR has been shown to be one of potential targets of WT1 (17).

The PC12 cell line is a good model for the study of NGF action and responds to NGF by differentiating into a postmitotic cell type with neuronal characteristics (18, 19). Although both EGF and NGF stimulate a common set of signaling pathways, NGF induces differentiation in PC12 cells, whereas EGF induces proliferation in PC12 cells. We have previously shown that EGFR is down-regulated by NGF treatment and controlled transcriptionally (4, 6). Here we provide evidence that the WT1 protein is directly involved in the down-regulation of EGFR in PC12 cells. First, rat EGFR promoter activity is enhanced by
expression of WT1. Second, deletion and mutation of TCC repeat sequences within the EGFR promoter abolish the enhancing effect of WT1. Third, WT1 binds to TCC repeat sequences in electrophoretic mobility shift analysis, and mutations in TCC repeat sequences diminish the binding, consistent with the result of the reporter gene assays. Fourth, overexpression of the WT1 protein increases the expression level of endogenous EGFR. Fifth, the expression level of endogenous WT1 is concomitantly decreased along with NGF treatment in PC12 cells, suggesting that WT1 regulates the transcription of EGFR. Supportive evidence is that neither the EGFR nor WT1 expression is a consequence of down-regulation of NGF in PC12 cells is mediated by WT1. It is likely that the strong demonstration that down-regulation of rat EGFR by antisense WT1 RNA substantially reduces WT1 and EGFR expression, indicating that endogenous WT1 is contributing to regulation of EGFR expression. These data strongly demonstrate that down-regulation of rat EGFR by NGF in PC12 cells is mediated by WT1. It is likely that the down-regulation of EGFR is a consequence of down-regulation of WT1 by NGF. Both events are dependent on p140trk, Ras, and Src. Finally, antisense WT1 RNA substantially reduces WT1 and EGFR expression, indicating that endogenous WT1 is contributing to regulation of EGFR expression. These data strongly demonstrate that down-regulation of rat EGFR by NGF in PC12 cells is mediated by WT1. It is likely that the down-regulation of EGFR is a consequence of down-regulation of WT1 by NGF. Both events are dependent on p140trk, Ras, and Src, which is consistent with this possibility.

Although it had been shown that WT1 protein could activate or repress the expression of a number of promoters in cotransfection experiments (17, 20–23), endogenous forms of many of these putative target genes were later shown not to be regulated by WT1 in biological systems (24). However, our data include not only cotransfection assays of the reporter plasmids and overexpression of endogenous EGFR in PC12 cells but also the endogenous WT1 effect on EGFR repression. Englert et al. (21) have reported that WT1 suppresses synthesis of the human EGFR and induces apoptosis by binding to two TC-rich repeat sequences in certain cell lines (Saos-2 and U2OS), but not in all types of cells. Our result supports the notion that cellular environment is a key aspect for the function of WT1 in vivo.

Because WT1 protein is expressed mainly in developing kidney, testis, and ovary, indicating that it is involved in the differentiation of genital tissues, it is of interest that WT1 protein is decreased along with neuronal differentiation of cells. Once neurotrophin is provided and cells are committed to being differentiated into neurons, there is suppression of a specific set of genes. Given that EGFR is a crucial factor for proliferation of cells, WT1 could have the potential to regulate differentiation. Studies using a variety of leukemic cell lines suggest that wild-type WT1 is acting to promote cellular proliferation rather than differentiation (24). Recently, amphi-regulin, a member of the epidermal growth factor family, was found to be a major target of WT1 and activated by WT1 (25). This also indicates that genes whose transcription is activated by WT1 may be critical for biological activity.

In summary, down-regulation of WT1 by NGF appears to mediate down-regulation of EGFR during the differentiation of PC12 cells induced by NGF. The molecular mechanism for the down-regulation of WT1 by NGF in PC12 cells will help us to understand details about the down-regulation of EGFR and cell differentiation.

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