Modification of EWS/WT1 functional properties by phosphorylation

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In many human cancers, tumor-specific chromosomal rearrangements are known to create chimeric products with the ability to transform cells. The EWS/WT1 protein is such a fusion product, resulting from a t(11;22) chromosomal translocation in desmoplastic small round cell tumors, where 265 aa from the EWS amino terminus are fused to the DNA binding domain of the WT1 tumor suppressor gene. Herein, we find that EWS/WT1 is phosphorylated in vivo on serine and tyrosine residues and that this affects DNA binding and homodimerization. We also show that EWS/WT1 can interact with, and is a substrate for, modification on tyrosine residues by c-Abl. Tyrosine phosphorylation of EWS/WT1 by c-Abl negatively regulates its DNA binding properties. These results indicate that the biological activity of EWS/WT1 is closely linked to its phosphorylation status.

desmoplastic small round cell tumor | DNA binding | homodimerization | c-Abl

Desmoplastic small round cell tumor is an aggressive rare tumor that generally occurs in adolescence and is located to the peritoneal surfaces of the abdomen. This malignancy is associated with a recurrent translocation t(11;22)(p13;q22), which fuses the amino-terminal domain (NTD) of the EWS gene in-frame to three of the four carboxyl-terminal zinc fingers of the WT1 tumor suppressor gene (1, 2). The EWS gene is involved in several tumor-related chromosomal translocations that produce fusions with genes postulated to function as transcription factors (for a review, see ref. 3). In each case, the translocation produces chimeric molecules containing the EWS NTD fused to the DNA domain of the partner.

There are two isoforms of EWS/WT1 generated as a result of an alternative splicing event between the third and last WT1 zinc finger and resulting in the insertion or removal of three amino acids (±KTS). This event produces EWS/WT1 isoforms with distinct DNA binding properties (4). The EWS/WT1(−KTS) isoform recognizes GC-rich WT1 binding sites [5'-GCGGGGCGG-3'] with ~10-fold higher affinity than WT1 and can transform NIH 3T3 cells (4, 5). On the other hand, EWS/WT1 (+KTS) does not bind GC-rich WT1 sites and has no transforming activity. The NTD (amino acids 1–265) of EWS/WT1 is composed almost exclusively of tyrosine, glutamine, alanine, serine, threonine, glycine, and proline residues, of which some are organized in a repeated and degenerate polypeptide motif having the consensus NSYGQQS. This domain shares distant homology with the carboxyl-terminal domain (CTD) of eukaryotic RNA polymerase II (6) and is a potent transcriptional activator (4, 7, 8). One model to account for the transforming properties of EWS/WT1 (−KTS) is that some of the genes normally under WT1 control are deregulated by EWS/WT1.

During our characterization of the EWS/WT1 oncogene we discovered that this product can self-associate and that the responsive region maps to the chimeric portion of the molecule, requiring both EWS and WT1 domains (see below). Herein, we report that phosphorylation of the EWS/WT1 chimeric product dramatically alters its biological properties, inhibiting both DNA recognition and homodimerization. In addition, we show that c-Abl is a candidate upstream modifier of EWS/WT1 biological activity.

Materials and Methods

Plasmid Constructions. The construction of EWS/WT1(−KTS) has been described (4). To generate glutathione S-transferase (GST)-EWS/WT1(−KTS), which was used to produce bacterial recombinant protein, the EcoRI (Klenow blunted)–XhoI fragment from pcDNA3-EWS/WT1(−KTS) was transferred into the blunted SacI and intact XhoI sites of pGEX-RC. For expression in mammalian cells, the HincII fragment (Klenow repaired) containing the GST domain was isolated from pGEX-3T (Amersham Pharmacia) and subcloned into the EcoRV V site of pcDNA3 (Invitrogen) to generate pcDNA3:GST. pcDNA3:GST-EWS/WT1(−KTS) was made by isolating an EcoNI–XhoI fragment from pGEX-RC:EWS/WT1(−KTS) and cloned into the same sites of pcDNA3:GST. To generate the amino terminally hemagglutinin (HA)-tagged EWS/WT1 [pcDNA3:HA-EWS/WT1(−KTS)], the EcoRI–XbaI fragment of pcDNA3-EWS/WT1(−KTS) was cloned into a pcDNA3-HA tag vector containing three copies of the HA peptide epitope (NH2–YPYDVPDYAG–COOH) (kindly provided by H. Imataka and N. Sonenberg, McGill University). For histidine-tagged recombinant EWS/WT1(−KTS) proteins, the EcoRI–HindIII fragment from pcDNA3-EWS/WT1(−KTS) was subcloned into the same sites of the pTcHisB XPRESS SYSTEM vector (Invitrogen) to generate pTcHisB-EWS/WT1(−KTS). Detailed protocols outlining plasmid constructions can be obtained from the authors on written request. All constructs involving manipulations by PCR were sequenced by using double-stranded DNA templates (9) to ensure the absence of mutations.

Cell Cultures, Transfections, Protein Purifications, and Western Blot Analyses. COS-7 or 293T cell lines were maintained in DMEM supplemented with 10% heat-inactivated FCS (GIBCO/BRL), penicillin, and streptomycin. For phosphorylation studies, 20 μg of pcDNA3/(His)6-EWS/WT1(−KTS) was used in transient transfection assays, and cells were pretreated with sodium pervanadate, prepared fresh by mixing 6% hydrogen peroxide (Pierce) and sodium orthovanadate (Fisher Scientific) to yield a final concentration of 10 mM sodium ortho-vanadate. This solution was diluted to 290 μM pervanadate with DMEM, added to the cells, and maintained at 37°C for 15 min. For in vivo self-association experiment, 10 μg of HA-tagged EWS/WT1(−KTS) plasmid, pcDNA3:HA-EWS/WT1(−KTS), was transfected with either 10 μg of pcDNA3:GST or pcDNA3:GST-EWS/WT1(−KTS) DNA. After 48 hr, cells were harvested in resuspension buffer (50 mM Tris-HCl, pH 8.0/1% NP-40/2 mM EDTA/150 mM NaCl/1 mM DTT/2 mM PMSF/1 μg/ml aprotinin/1 μg/ml leupeptine/1 μg/ml antipain) and lysed by sonication. Supernatants were collected by centrifugation at 14300–14305 | PNAS | December 7, 1999 | vol. 96 | no. 25

Abbreviations: NTD, amino-terminal domain; GST, glutathione S-transferase; HA, hemagglutinin; CTD, carboxyl-terminal domain; SH3, src homology 3; PVDF, poly(vinylidene difluoride).

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16,000 × g for 15 min at 4°C and incubated with glutathione beads (Amersham Pharmacia) for 1 hr. Beads were collected by brief centrifugation and washed with resuspension buffer four times. The affinity-selected proteins were eluted in 1× SDS loading buffer (62.5 mM Tris-HCl, pH 6.9/10% glycerol/2% SDS/5% β-mercaptoethanol).

Affinity-purified proteins were separated by electrophoresis through a 10% polyacrylamide gel and transferred onto an Immobilon poly(vinylidene difluoride) (PVDF) membrane (Millipore). This filter was blocked with 5% nonfat in TBS-T (20 mM Tris-HCl, pH 7.5/150 mM NaCl/0.1% Tween 20) for 1 hr at room temperature, and 12CA5 (anti-HA antibody) and horseradish peroxidase-conjugated donkey anti-rabbit (Amersham Life Sciences) antibodies were used as primary and secondary antibodies, respectively. The blot was incubated and visualized with ECL solution according to the manufacturer’s instructions (DuPont NEN). For antiphosphotyrosine blots, 5% BSA (Amresco, Euclid, OH) in TBS-T was used for preblocking, and the 4G10 antiphosphotyrosine antibody was used for probing.

In Vitro Transcription and Translation. In vitro transcription and translation were performed as described (4). Briefly, pcDNA3:EWS/WT1(−KTS) was linearized with SmaI and in vitro transcriptions were performed with T7 RNA polymerase. In vitro translations were performed in the presence of [35S]methionine translation mix (DuPont/NEN). Translations were performed in the presence of [35S]methionine (4), and 106 cpm of probe was prepared as described (4), and 106 cpm/ml of probe and 0.5 mM of unlabeled ATP were used in each hybridization. The blot was washed with renaturation buffer, dried, and exposed at −80°C with intensifying screen. Oligonucleotides containing the WT1 recognition site (in capital letters) [WTE: 5′-gagtcGCGTGGAG-Tagaa-3′] or, a mutant version not capable of binding WT1 [WTE(m): 5′-gagtcGCGTAGAGTagaa-3′], were used as probes.

Results

Forskolin treatment of fibroblast cells expressing WT1 leads to phosphorylation of Ser-365 and Ser-393 of zinc fingers 2 and 3, respectively, and abolishes the DNA binding activity of WT1 in vitro (12). The RNA binding activity of WT1 is not altered by phosphorylation (13). Because the EWS/WT1 contains three of the four WT1 zinc fingers, as well as several potential tyrosine phosphorylation sites, we wanted to determine whether EWS/WT1 was phosphorylated in vivo and determine whether any functional characteristics of EWS/WT1 were altered by such modification.

In Vivo Phosphorylation of EWS/WT1 Regulates Its DNA Recognition Ability. An expression vector driving the synthesis of EWS/WT1 containing six histidine residues at the amino terminus was transiently transfected into COS-7 cells. Forty-eight hours after transfection, cells were exposed for 15 min to pervanadate, a known specific inhibitor of phosphotyrosine phosphatases (14). Cell extracts probed with an antibody specific to WT1 zinc finger IV (α-C-19) revealed that the pervanadate-treated EWS/WT1 isolated from cells pretreated with corticosteroid contained phosphorylated protein (Fig. 1A Lower, lane 2). In contrast, radiolabeled WTE(m) (Fig. 1A Upper, lane 1). Because Ni2+-affinity protein preparations were used in the above experiments, we interpret these results to indicate that EWS/WT1 is a substrate for (an) endogenous protein tyrosine kinase(s).

Phosphoamino acid analysis of His-tagged EWS/WT1 used in these experiments revealed the presence of phosphotyrosine and phosphoserine residues (Fig. 1B). To assess whether in vivo phosphorylation of EWS/WT1 had a functional consequence on the DNA binding properties of EWS/WT1(−KTS), we performed Southwestern blot analysis using a 32P-labeled WT1 recognition site (called WTE) as probe (Fig. 1C). In this assay, EWS/WT1(−KTS) isolated from untreated lysates recognized the WTE probe (Fig. 1C Upper, lane 1). This binding is specific, because a mutant probe, WTE(m), containing a point mutation in the WT1 recognition site, failed to bind (Fig. 1C Upper, compare lane 3 to lane 1). Pervanadate treatment of EWS/WT1(−KTS) transfected COS-7 cells produced an isofom that no longer efficiently bound DNA (Fig. 1C Upper, compare lane 2 to 1). The differences in DNA binding are not a result of differences in protein levels, because both extracts contain equivalent amounts of EWS/WT1(−KTS) (Fig. 1C Lower, compare lane 3 to lane 1). As expected, radiolabeled WTE(m) did not bind to EWS/WT1(−KTS) isolated from pervanadate-treated cells (Fig. 1C Upper, lane 4).

EWS/WT1 Is a Substrate for the c-Abl Protooncogene. Because the CTD of RNA polymerase II is the site of multiple phosphorylation events (11), some of which are thought to be mediated by the c-Abl product (14), we decided to assess whether c-Abl resided upstream of EWS/WT1. In addition, we noted the presence of two Src homology 3 (SH3) domain interacting motifs within the EWS/WT1 NTD that corresponded to the consensus motif (PxxxDxxP) identified in a number of c-Abl targets (Fig. 2A). Indeed, GST pull-downs of recombinant His-tagged EWS/WT1 with GST or c-Abl GST/SH3 revealed that EWS/WT1 was specifically bound to the c-Abl SH3 domain (Fig. 2B). To extend these results and demonstrate an in vivo association, EWS/WT1 and c-Abl mammalian expression vectors driving synthesis of c-Abl and either GST or GST-EWS/WT1 were introduced into COS-7 cells. Forty-eight hours after transfections, cell extracts were prepared and affinity-precipitated with glutathione affinity resin (Fig. 2C). Eluted proteins were probed by Western blotting for the presence of copurifying c-Abl protein. Clearly, c-Abl can copurify with GST-EWS/WT1, but not GST, demonstrating that EWS/WT1 and c-Abl are capable of associating in vivo (Fig. 1C), although we have yet to map the interacting domain on EWS/WT1.

To determine whether EWS/WT1 is a substrate for phosphorylation by c-Abl, we isolated c-Abl protein from Jurkat cells by immunoprecipitation (Fig. 3A). Tyrosine phosphorylation of c-Abl is known to be caused by c-Abl kinase activity. Incubation of c-Abl with His-tagged EWS/WT1 followed by Southwestern blot analysis with WTE as a probe, abolished DNA binding (Fig. 3C). Immunohistochemical probing for EWS/WT1 on this same blot demonstrated that equivalent amounts of protein were present in both reactions and...
that the presence of immunoprecipitated c-Abl did not cause degradation of the substrate (Fig. 3C). Phosphoamino acid analysis of His-tagged EWS/WT1 used in these experiments revealed only the presence of phosphotyrosine residues (Fig. 3D). These results clearly demonstrate that c-Abl is capable of modifying EWS/WT1 DNA binding activity by tyrosine phosphorylation.

**Inhibition of EWS/WT1 Self-Association by Tyrosine Phosphorylation.**

During the course of these studies, we noticed that EWS/WT1 could self-associate and that phosphorylation of EWS/WT1 also affected this property (described below). Initial experiments aimed to map the EWS/WT1 homodimerization potential using GST pull-down assays performed with in vitro-translated EWS/WT1(200–310) and GST-EWS/WT1 recombinant protein purified from bacteria. As shown in Fig. 4A, 35S-methionine-labeled EWS/WT1(200–310) specifically bound to GST-EWS/WT1 (lane 3) but not to GST (lane 2).

To demonstrate that self-association was not restricted to our in vitro binding conditions, affinity coprecipitation experiments were performed directly from transfected cells (Fig. 4B). 293T cells were cotransfected with pcDNA3:HA-EWS/WT1 and pcDNA3:GST-EWS/WT1 or pcDNA3:GST, and lysates were prepared 48 hr after transfection. GST or GST-EWS/WT1 were affinity-purificated with glutathione-Sepharose beads, and subsequent immunoblotting performed by using an anti-HA antibody (12CA5) revealed that HA-EWS/WT1 is present only in extracts containing GST-EWS/WT1 (Fig. 4B).

To identify the domain(s) within EWS/WT1 that mediate self-association, in vitro GST pull-down assays were performed with a series of EWS/WT1 deletion mutants (Fig. 4C). Amino-terminal and carboxyl-terminal deletions of the EWS(NTD) were generated and fused in-frame to the GST domain. These experiments map the interaction domain to a 126-aa region of the WT1 zinc fingers and the EWS sequences immediately upstream of the fusion junction (Fig. 4C). We were unable to refine the definition of this region because a series of finer deletions (e.g., GST-EWS/WT1(200–310) or GST-EWS/WT1(185–296)) failed to self-associate.

To determine the effect of in vivo phosphorylation on dimerization of EWS/WT1, we transfected COS-7 cells with pcDNA3:HA-EWS/WT1 and 48 hr later prepared cell extracts. These were used in affinity-selection assays with immobilized GST or GST-EWS/WT1 and revealed that HA-EWS/WT1 isolated from per-
vanadate-treated cells could not self-associate, whereas EWS/WT1 from untreated cells could (Fig. 5A, compare lane 4 to 2). The differences in self-association behavior between phosphorylated or underphosphorylated EWS/WT1 species (Fig. 5A, compares lanes 4 and 2) cannot be attributed to differences in protein levels (Fig. 5A Lower). A possible alternative interpretation to our data is that phosphorylation of HA-EWS/WT1 increases self-association, such that no monomers remain when extracts are challenged with immobilized GST-EWS/WT1. To test this hypothesis, COS-7 cells were transfected with HA- and GST-tagged EWS/WT1. After treatment with vehicle or pervanadate, cell extracts were prepared and purified over a glutathione resin (Fig. 5B). If phosphorylation increases self-association, then one would expect to find HA-EWS/WT1 copurifying with GST-EWS/WT1 from pervanadate-treated cells. This was not the case because HA-EWS/WT1 copurified with GST-EWS/WT1 from untreated COS-7 cells, but not from pervanadate-treated cells (Fig. 5B), indicating that phosphorylation prevents self-association of EWS/WT1.

Self-Association of EWS/WT1 Is Not Required for DNA Binding. Because the DNA binding and self-association domains of EWS/WT1 overlap (Fig. 4C) and both are influenced by phosphorylation (Figs. 1C, 3C, and 5A), we addressed whether loss of DNA binding in response to this modification was an indirect consequence of loss of self-association (Fig. 5C). To this end, we generated a deletion mutant of EWS/WT1 containing six histidine residues, (His)6-EWS/WT1(245–362). This truncated protein contains the DNA binding domain of EWS/WT1, but cannot self-associate (Fig. 4C). Bacterially produced recombinant (His)6-EWS/WT1(245–362) was intact (Fig. 5C, Left) and was capable of
These data indicate that although the EWS position of HA-EWS ular mass markers are indicated on the left (NEB broad range markers) and the indicated expression vectors in the transfection mixture. The positions of molec-
vivo approximately 20% of the 35S-methionine-labeled protein used in the experiment was
14304 a and 3, retinoic acid receptor y
Fig. 4. Self-association of EWS WT1. (A) Retention of EWS WT1 by GST-EWS WT1 recombinant protein. In vitro-transcribed and -translated 35S-
methionine-labeled EWS WT1 was incubated with either -2 µg of GST (lane 2) or GST-EWS WT1 (lane 3) bound to glutathione-Sepharose beads. Approxi-
mately 20% of the 35S-methionine-labeled protein used in the experiment was
loaded in lane 1 as a reference. The EWS WT1 proteins were eluted with SDS
loading buffer and analyzed by 10% SDS PAGE. The position of the molecular
mass markers are indicated on the left (NEB broad range markers) and the position of EWS WT1 is indicated to the right. (B) EWS WT1 self-association in vivo. 293T cells were transfected with pcDNA3:HA-EWS WT1 and either pcDNA3:GST (lane 1) or pcDNA3:GST-EWS WT1 (lane 2), or 293T cells were transfected with only pcDNA3:GST-EWS WT1 (lane 3). Affinity-precipitations were performed with glutathione-Sepharose 4B beads and analyzed for the presence or absence of HA-EWS WT1 by Western blotting with an anti-HA antibody (12CAS). + or — indicate the presence or absence, respectively, of the indicated expression vectors in the transfection mixture. The positions of molecular mass markers are indicated on the left (NEB broad range markers) and the position of HA-EWS WT1 is indicated by an arrow. (C) The EWS WT1 self-association domain resides between amino acids 185 and 310. A schematic diagram illustrating the EWS WT1 deletion mutants used in this study is presented. The results from in vitro affinity selection experiments are summarized to the right. + or — indicates whether or not 35S-methionine-labeled full-length EWS WT1 could be retained on an affinity matrix containing the corresponding GST-EWS WT1 derivative. The amino acid numbering refers to the position of the amino acids in the fusion protein, not the numbering normally found in EWS or WT1.

binding WTE as assessed by Southwestern analysis (Fig. 5C, Right). These data indicate that although the EWS WT1 DNA binding and self-association domains overlap, self-association is not required for DNA interaction.

Discussion
The results reported herein begin to explore the functional regulation of the EWS WT1 oncoprotein. Whereas WT1 is generally a repressor of transcription, EWS WT1 is a potent activator. Many potential WT1 target genes have been identified on the basis of the presence of potential binding sites within their promoters. These include those encoding for early growth response 1 (EGR-1), epidermal growth factor receptor, insulin-like growth factor 2, insulin-like growth factor 1 receptor (IGF1R), platelet-derived growth factor A (PDGFA), PAX2, c-MYC, BCL2, the transforming growth factor-β molecules 1, 2, and 3, retinoic acid receptor α, colony-stimulating factor 1, c-MYB, p21, and WT1 itself, among many others (16, 17). EWS WT1 has been demonstrated to activate transcription of the IGF1R gene (7) and EGR-1 (8) in transient transfections and of the endogenous PDGFA promoter (15). Our results demonstrate that EWS WT1 is a tyrosine- and serine-phosphorylated protein and that specific interaction with DNA is abolished when EWS WT1 is phosphorylated (Figs. 1 and 3). There are reported differences regarding the ability of EWS WT1 to activate specific downstream targets of WT1 (e.g., see ref. 15) and a possible explanation for this could be that cell-type specific differences in the phosphorylation status of EWS WT1 may be
responsible. Regulation of DNA binding by phosphorylation of sequence-specific transcription factors is well documented and can be positively (18, 19) or negatively (20, 21) influenced. In addition, the EWS product can be phosphorylated by protein kinase C through an IQ domain and this phosphorylation inhibits DNA binding (22). In those cases in which the phosphorylation sites are separate from the DNA binding domain, it is most likely that phosphorylation alters the conformation of the protein gene leads to homotypic associations that translate into transcriptional effects, as has been proposed for the WT1 tumor suppressor gene (30). The self-association of EWS/WT1 represents an excellent target for drug design given that it resides within the junction portion of EWS/WT1 and therefore is unique to this chimera.

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