Modulation of EWS/WT1 activity by the v-Src protein tyrosine kinase

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Abstract Desmoplastic small round cell tumor (DSRCT) is a malignant human cancer that is associated with a specific t(11;22) chromosome translocation, where 265 amino acids from the EWS amino-terminus are fused to the DNA binding domain of the WT1 tumor suppressor gene. We have noticed the presence of several SH3 interacting domains within the amino-terminus of EWS and have assessed the potential of EWS/WT1 to interact with such motifs. We find that EWS/WT1 can associate with the SH3 domain of several proteins, including v-Src. Ectopic expression of v-Src phosphorylates EWS/WT1 in vivo, as well as enhances the transactivation ability of the EWS amino-terminal domain. Structural alteration of the v-Src SH2 or SH3 domains produced mutants that could not interact with EWS/WT1 nor augment the transcriptional properties of EWS. Taken together, our results suggest the possibility that some transcriptional properties of EWS/WT1 may be regulated by a cytoplasmic signaling pathway.

Key words: EWS/WT1; v-Src; Tyrosine kinase; Transcription activation

1. Introduction

Desmoplastic small round cell tumor (DSRCT) is an aggressive neoplasm with a poor prognosis that affects children and young adults. This tumor is characterized by: (i) nests and clusters of undifferentiated small round cells separated by a prominent desmoplastic stroma, (ii) polyphenotypic differentiation, and (iii) a reciprocal chromosomal translocation t(11;22)(p13;q12), which fuses the amino-terminal domain (NTD) of the *EWS* gene in-frame to three of the four carboxy-terminal zinc fingers of the *WT1* tumor suppressor gene [1–3]. The *EWS* gene is involved in several tumor-related chromosome translocations that produce fusions between the EWS amino-terminal domain (NTD) and a carboxy-terminal domain often consisting of a DNA binding domain derived from one of a number of transcription factors [4,5]. The Wilms' tumor suppressor gene, *WT1*, encodes a zinc finger transcription factor belonging to the EGR (early growth response) family of Cys_2–His_2 zinc finger proteins and is mutated in 5–15% of sporadic WTs [6]. The pre-mRNA is alternatively spliced at two coding exons to produce four WT1 polypeptide isoforms containing four zinc fingers [7]. The first alternative splice event produces WT1 isoforms containing or lacking 17 amino acids approximately midway within the coding region, whereas the second alternative splice site inserts (+) or removes (−) three amino acids (±KTS), between zinc fingers III and IV and alters the DNA binding specificity of the protein [8].

EWS/WT1 is a chimeric protein that contains the NTD of EWS and three of the four C-terminal zinc fingers from WT1, as well as retaining the second alternative splicing event between zinc fingers III and IV [1,3]. The portion of EWS that displaces the NTD of the WT1 tumor suppressor gene encodes a potent transcriptional activator [9]. This chimeric protein has been shown to up-regulate putative WT1 downstream targets, such as PDGF-A [10], EGR-1 [11], and IGF-1R [12], which are normally down-regulated by WT1 (reviewed in [6]). In addition, the EWS/WT1 fusion protein plays an important role in the development of DSRCT. Overexpression of EWS/WT1(−KTS), but not EWS/WT1(+KTS), is oncogenic when introduced into NIH3T3 cells [13].

Transformation of cells by non-nuclear oncoproteins leads to deregulated transcription in the nucleus – a decisive event in cell transformation resulting in altered transcription of certain genes (reviewed in [14]). One of the possible themes is that different proto-oncogene products function as components of a network that transduces signals from the exterior of the cell to transcription factors in the nucleus [14]. For example, many of the biological activities of v-Src (an oncogene of the Rous sarcoma virus and a member of non-receptor tyrosine kinases) involve changes in gene transcription and this protein is capable of activating a variety of transcription factors (reviewed in [15,16]). It has been reported that v-Src mediated transcriptional activation involves multiple transcription control elements, including NF-κB, ATF/CRE, AP-1, CArG, and SIE sites [17–21].

For example, Bruton's tyrosine kinase (Btk), a member of the Tec family of non-nuclear receptor tyrosine kinases containing SH1, SH2, and SH3 domains but lacking the typical myristylation site and negative regulatory tyrosine present in Src family kinases [22], has been demonstrated to enhance TFII-I mediated transcriptional activation [23]. Interestingly, Btk associates with TFII-I and phosphorylates TFII-I on tyrosine residues in vivo. Transcription activation of the *c-fos* promoter by TFII-I in serum stimulated NIH3T3 cells requires an intact Ras signalling pathway [24]. Taken together, these data suggest that certain transcription factors, including TFII-I, link signal transduction to transcription events in the cell.

In this study, we report that v-Src can associate with, and phosphorylate, EWS/WT1. We also find that transcriptional

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activation by the EWS-NTD is stimulated by v-Src. These data suggest that the transcriptional activation properties of EWS(NTD) can be influenced by a cytoplasmic signaling pathway.

2. Materials and methods

2.1. Materials and general methods

Restriction endonucleases and DNA modifying enzymes were purchased from New England Biolabs (Beverly, MA, USA). [^{35}S]deoxyadenosine 5′ (α-thio) triphosphate (1000–1500 Ci/mmol), [^{35}S]methionine (>1000 Ci/mmol), and D-threo-[dichloroacetyl-1-^{14}C]chloramphenicol (54.0 Ci/mmol) were obtained from New England Nuclear. Preparation of plasmid DNA, restriction enzyme digestion, agarose gel electrophoresis of DNA, DNA ligation, and bacterial transformations were carried out using standard methods ([25] and references therein). Subclones of DNA PCR amplifications were always sequenced to ensure the absence of unwanted secondary mutations.

2.2. Plasmid constructions

Descriptions of EWS/WT1(−KTS), 5×Gal4·E1B·CAT, GAL4, GAL4-EWS(NTD), pcDNA3/GST, and pcDNA3/GST-EWS/WT1(−KTS) have been previously presented [9,26]. To generate pcDNA3/v-Src, the *Bam*HI fragment of MMTV/v-Src (obtained from Dr. John Bell, Ottawa, Canada) was subcloned into the *Bam*HI site of pcDNA3. For generating GST/Unique v-Src, the unique domain of v-Src was amplified by the PCR using primer 5′-Src/Uni (5′-GATCGAATTCATGGGGAGTAGCAAGAGC-3′) and primer 3′-Src/Uni (5′-GATCCTCGAGGGCACGCTGCGGCGACGT-3′), digested with *Eco*RI and *Xho*I, and cloned into the same sites of pGEX(4T-1). For GST/SH3 v-Src, the SH3 domain of v-Src was generated by the PCR using primer 5′-Src/SH3 (5′-GATCGAATTCGGGGCACTGGCTGGCGGC-3′) and primer 3′-Src/SH3 (5′-GATCCTCGAGGGGGTTTTCGGGGTTGAG-3′), digested with *Eco*RI and *Xho*I, and cloned into the same sites of pGEX(4T-1). To generate GST/SH2 v-Src, the SH2 domain of v-Src was amplified by the PCR using primer 5′-Src/SH2 (5′-GATCGAATTCTGGTACTTTGGGAAGATC-3′) and primer 3′-Src/SH2 (5′-GATCCTCGAGGGTCTGGGGCTTGGACGT-3′), digested with *Eco*RI and *Xho*I, and cloned into the same sites of pGEX(4T-1). To generate GST/Kinase v-Src, the kinase domain of v-Src was amplified by the PCR using primer 5′-Src/Kinase (5′-GATCGAATTCCAGGGACTCGCCAAGGAC-3′) and primer 3′-Src/Kinase (5′-GATCCTCGAGCTACTCAGCGACCTCCAA-3′), digested with *Eco*RI and *Xho*I, and cloned into the same sites of pGEX(4T-1). The restriction enzyme sites for *Eco*RI (5′-GAATTC-3′) and *Xho*I (5′-CTCGAG-3′) in the PCR primers are indicated by underlines.

Mammalian expression plasmids pcDNA3/v-Src ΔSH3·SH2, v-Src ΔKinase, and v-Src R295 were constructed by the PCR. (A) pcDNA3/v-Src ΔSH3·SH2. The unique domain of v-Src was generated by the PCR using primer 5′-Src-1 (5′-GATCAAGCTTATGGGGAGTAGCAAGAGC-3′) and 3′-Src-76 (5′-GGCACGCTGCGGCGACGT-3′) and the kinase domain of v-Src was generated by PCR using the primer 5′-Src-253 (5′-CAGGGACTCGCCAAGGAC-3′) and 3′-Src-4 (5′-GATCTCTAGACTACTCAGCGACCTCCAA-3′). These PCR products were kinased and ligated together, digested with *Hin*dIII and *Xba*I, and cloned into the same sites of pcDNA3. (B) pcDNA3/v-Src ΔKinase. The v-Src ΔKinase domain (aa 1–258) was generated by the PCR using primers 5′-Src-1 and 3′-Src/ter-258 (5′-CTAGTCCTTGGCGAGTCCCTG-3′), digested with *Hin*dIII after kinasing with T4 polynucleotide kinase, and subcloned into the *Hin*dIII and *Eco*RV sites of pcDNA3. (C) pcDNA3/v-Src R293. PCRs were performed with primers 5′-Src-1 and 3′-Src-2 (5′-GGTGCCGGGCTTCAGAGTCCTTATGGCCACTCTGGTGGT-3′) for the N-terminal part and 5′-Src-3 (5′-ATGTCCCCGGAGGCCTTC-3′) and 3′-Src-4 for the C-terminal part of v-Src, respectively. These PCR products were ligated, digested with *Hin*dIII and *Xba*I, and cloned into the same sites of pcDNA3. The *Hin*dIII (AAGCTT) and *Xba*I (TCTAGA) sites are indicated by underlines.

Description of plasmids GST-EWS/WT1 and GST-EWS/WT1(NTD), used for GST pull-down assays, have been described previously [26]. (A) GST-EWS/WT1(1–245). The *Bam*HI fragment of pGEX(RC)-EWS/WT1 was subcloned into the *Bam*HI site of pGEX(RC). (B) GST-EWS/WT1(1–163). Plasmid pGEX(RC)-EWS/WT1 was digested with *Avr*II and *Hin*dIII, blunt end repaired with klenow, and re-ligated. (C) GST-EWS/WT1(1–109). Plasmid pGAL4/EWS(1–109) [9] was cleaved with *Eco*RI and *Not*I and cloned into the same sites of pGEX(4T-1). (D) GST-EWS/WT1(1–35). Plasmid pGEX(RC)-EWS/WT1 was cleaved with *Nde*I and *Hin*dIII, blunt end repaired with klenow and re-ligated. (E) GST-EWS/WT1(70–200). The EWS/WT1(70–200) fragment was generated by the PCR using primers 5′-aa70 (5′-GATCGAATTCTATGGACAGCCTCCCACT-3′) and primer 3′-aa200 (5′-GATCCTCGAGCGGCTGTGTAGAGGAATA-3′), digested with *Eco*RI and *Xho*I, and cloned into the same sites of pGEX(4T-1). (F) GST-EWS/WT1(192–265). The EWS/WT1(192–265) PCR product was produced by the PCR using primers 5′-aa192 (5′-GATCGAATTCCCTACCAGCTATTCCTCT-3′) and 3′-aa265 (5′-GATCCTCGAGACTCTGCTGCCCGTAGCT-3′), cleaved with *Eco*RI and *Xho*I and ligated into the same sites of pGEX(4T-1). (G) GST-EWS/WT1(245–362). Plasmid pcDNA3-EWS/WT1(−KTS) was digested with *Bam*HI and *Xho*I and the excised fragment was cloned into the same sites of pGEX(RC). (H) GST-EWS/WT1(70–163). The PCR product from amino acids 70–163 of EWS/WT1 was produced by the PCR using primers 5′-aa70 and 3′-aa163 (5′-GATCCTCGAGTAGGCTGGGCTGGTTGTA-3′), cleaved with *Eco*RI and *Xho*I, and cloned into the same sites of pGEX(4T-1). (I) GST-EWS/WT1(140–200). The *Eco*RI and *Xho*I cleaved EWS/WT1(140–200) was produced by the PCR using primer 5′-aa140 (5′-GATCGAATTCCAGGATGGAAACAAGCCC-3′) and primer 3′-aa200, digested with *Eco*RI and *Xho*I and ligated into the same sites of pGEX(4T-1).

2.3. In vitro transcription and translation

In vitro transcriptions and translations were performed as previously described [9]. Briefly, pcDNA3:EWS/WT1 or pcDNA3/v-Src were linearized with *Sma*I or *Dra*I, respectively, and in vitro transcriptions performed with T7 RNA polymerase. In vitro translations were performed in the presence of [^{35}S]methionine in rabbit reticulocyte lysates, essentially as described by the manufacturer's instructions (Promega). For analysis of integrity, all in vitro translation products were electrophoresed on 10% SDS–PAGE.

2.4. GST pull-down assays

All GST fusion proteins were purified according to the manufacturer's recommendations with slight modifications [9]. Briefly, bacterial extracts containing GST or GST fusion proteins were incubated with a 50% slurry of glutathione-agarose beads (Pharmacia) in coupling buffer [50 mM Tris–HCl (pH 8.0), 1% NP-40, 2 mM EDTA, 150 mM NaCl, 2 mM of PMSF (phenylmethylsulfonyl fluoride), 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml antipain] with rocking. GST or GST fusion proteins bound to beads were then collected by brief centrifugation and washed four times in wash buffer (coupling buffer plus 10% glycerol). In vitro synthesized [^{35}S]methionine labeled v-Src was incubated with immobilized GST or GST-EWS/WT1 fusion proteins in binding buffer (wash buffer plus 1 mM DTT) for 1 h at 4°C. The beads were collected by centrifugation and washed four times with binding buffer. Bound proteins were eluted from the beads by boiling in 1×SDS loading buffer (62.5 mM Tris–HCl (pH 6.9), 10% glycerol, 2% SDS, 5% β-mercaptoethanol) and were separated by electrophoresis. Protein gels were treated with EN^{3}HANCE (NEN Research Products) before drying and exposed to Kodak X-Omat film.

2.5. Cell culture, transfection, affinity precipitation, immunoblotting, and CAT assays

C33A or 293T cell lines were maintained in minimum essential medium (α-MEM) or Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% heat inactivated fetal calf serum (Gibco-BRL), penicillin, and streptomycin. Using calcium phosphate precipitation, cells were co-transfected with 10 μg of pcDNA3/v-Src together with either 10 μg of pcDNA3:GST or pcDNA3:GST-EWS/WT1. After 48 h, cells were harvested and lysed in a buffer containing 50 mM Tris–HCl (pH 8.0), 1% NP-40, 2 mM EDTA, 150 mM NaCl, 1 mM DTT, 2 mM of PMSF (phenylmethylsulfonyl fluoride), 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml antipain for 30 min on ice. Lysates were clarified by centrifugation at 16 000×*g* for 15 min at 4°C and incubated with glutathione beads for 1 h. Beads were collected by brief centrifugation and washed with the same buffer four

times. The affinity-precipitated proteins were eluted in the same manner as described above, separated by electrophoresis through a 10% polyacrylamide gel, and transferred onto an Immobilon-P polyvinylidone difluoride membrane (Millipore). This filter was blocked with 5% non-fat in TBS-T (20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.1% Tween 20) for 1 h at room temperature, then affinity-purified monoclonal anti-Src antibody (LA074, Quality Biotech., National Cancer Institute) and HRP (horseradish peroxidase)-conjugated goat anti-mouse antibody (Santa Cruz Biotechnology, Inc.) were used as primary and secondary antibody, respectively. The blot was incubated and visualized by ECL (DuPont NEN) according to manufacturer's instructions.

For CAT assays, C33A cell lines were transfected with the indicated amounts of DNAs. Individual DNA precipitates were adjusted to contain equal amounts of total DNA by the addition of the empty expression vector, pcDNA3. To normalize for transfection efficiency, cells were co-transfected with 2 μg of pRSV/β-gal. At 48 h after transfection, cells were harvested and assayed for β-galactosidase and CAT activity [27]. Following thin layer chromatography analysis, regions containing acetylated [^{14}C]chloramphenicol, as well as unacetylated [^{14}C]chloramphenicol, were quantitated by direct analysis on a PhosphoImager (Fujix BAS 2000).

For anti-phosphotyrosine blots, $(His)_6$-tagged EWS/WT1(−KTS) proteins were purified according to the manufacturer's recommendations (QIAGEN) with slight modifications. Briefly, total cell extracts containing $(His)_6$-tagged proteins were prepared by sonication, centrifuged, and the supernatant collected by centrifugation. The supernatant was incubated with an Ni-NTA agarose resin (QIAGEN) in binding buffer (8 M urea, 0.1 M sodium phosphate, 10 mM Tris–HCl (pH 8.0)) and washed with wash buffer (8 M urea, 0.1 M sodium phosphate, 10 mM Tris–HCl (pH 6.3)) four times. The bound protein was eluted with elution buffer (wash buffer supplemented with 0.1 M EDTA). The affinity-purified EWS/WT1(−KTS) proteins were separated by electrophoresis through a 10% polyacrylamide gel, and transferred onto an Immobilon-P polyvinylidone difluoride membrane (Millipore). To quantitate levels of tyrosine phosphorylation, 5% bovine serum albumin (Amresco) in TBS-T was used for pre-blocking and the anti-phosphotyrosine antibody (4G10, UBI) was used for probing. To quantitate the amounts of EWS/WT1 protein, filters were stripped with stripping buffer (62.5 mM Tris–HCl (pH 6.8), 2% SDS, 97.9 mM β-mercaptoethanol) for 30 min at 50°C and reprobed with an antibody that recognizes the C-terminus of the WT1 zinc fingers (C-19 (Santa Cruz Biotechnology)). The blot was incubated and visualized by ECL according to manufacturer's instructions (DuPont NEN).

3. Results

3.1. EWS/WT1 interacts with SH2 and SH3 domain containing proteins

We have previously documented that EWS/WT1 is a substrate for phosphorylation by c-Abl, and that this event inhibits DNA binding and self-association by EWS/WT1 [26]. Since proline-rich domains, of the type found in the EWS-NTD (see below) are known to interact with SH3 domains, we assessed whether EWS/WT1 could interact with other SH3 domain containing proteins.

In this experiment, [^{35}S]Met labeled EWS/WT1 protein was prepared by in vitro translation and GST pull-down assays were performed with GST fusion proteins containing the SH3 regions of various protein tyrosine kinases and adapter proteins. The EWS/WT1 protein was retained by fusion proteins containing the c-Abl and v-Src SH3 domains (Fig. 1A). A small amount of binding was also noted to the SH3 domain of Crk(N) (Fig. 1A). However in this assay, the EWS/WT1 protein did not bind to the SH3 domains of Spectrin, RasGAP, PLC-γ, Grb2(N), Grb2(C), and p85α (the regulatory subunit of P13 kinase) (Fig. 1A). Thus, EWS/WT1 binds differentially to the SH3 domains tested and provides a molecular selectivity that can distinguish among SH3 domains. The lack of reactivity of several different SH3 domains with EWS/WT1 suggests that the EWS/WT1 interaction is specific and that different types of SH3 domains are not equivalent.

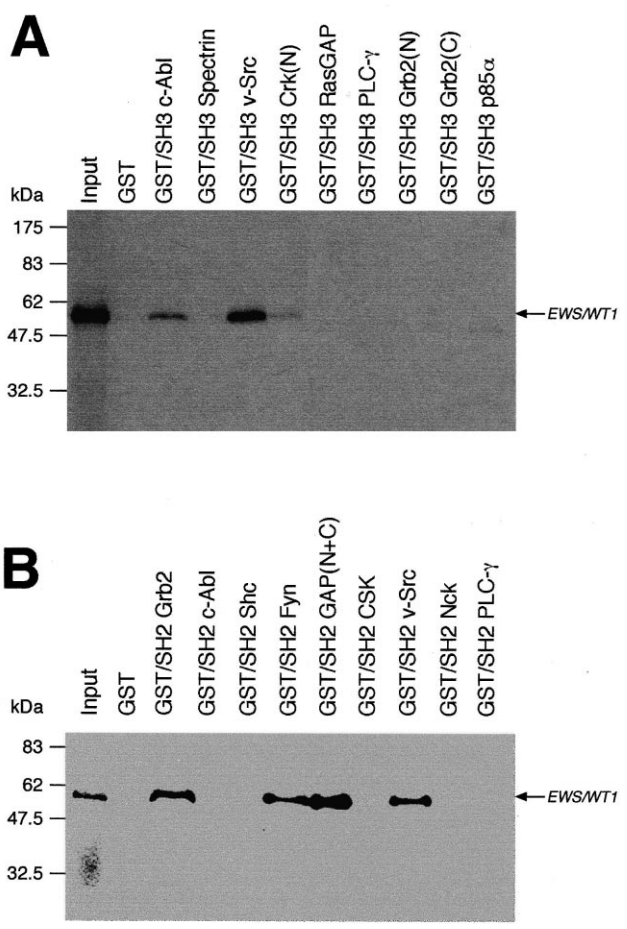


Fig. 1. EWS/WT1 can bind to SH3 and SH2 domains. A: Differential binding of EWS/WT1 to SH3 domains. [^{35}S]Met labeled EWS/WT1 was incubated with GST fusion proteins containing the SH3 regions of c-Abl, Spectrin, v-Src, RasGAP, PLC-γ, Grb2(N), Grb2(C), or p85α. An aliquot of the input (20%) and the pellet from the various GST-SH3 pull-downs were analyzed on a 10% SDS–PAGE and the bound EWS/WT1 protein was visualized by fluorography. The GST-SH3 domains from various proteins are indicated at the top of each lane. The positions of migration of the molecular weight markers are indicated to the left, and EWS/WT1 is indicated by an arrow to the right. B: Binding analysis of EWS/WT1 to SH2 domains. The SH2 domains indicated above each lane were expressed as GST fusion proteins and utilized in GST pull-down assays with [^{35}S]Met labeled EWS/WT1 protein. An aliquot of input (10%), as well as pellets from the various GST-SH2 pull-downs were analyzed on a 10% SDS–PAGE and visualized by fluorography. The positions of migration of molecular weight markers and of EWS/WT1 are indicated.

EWS/WT1 is also expected to be an SH2 domain interacting protein, since it is tyrosine phosphorylated [26] and contains potential interacting motifs for SH2 domains. We thus performed a series of GST pull-down assays to assess whether EWS/WT1 was capable of interacting with these. As shown in Fig. 1B, EWS/WT1 was able to bind to the SH2 domains of Grb2, Fyn, GAP(N+C), and v-Src. However, EWS/WT1 did not show any binding to the c-Abl, Shc, CSK, Nck, and PLC-γ SH2 domains. These results indicate that EWS/WT1 can bind to SH2 domains, in addition to SH3 domains. For the

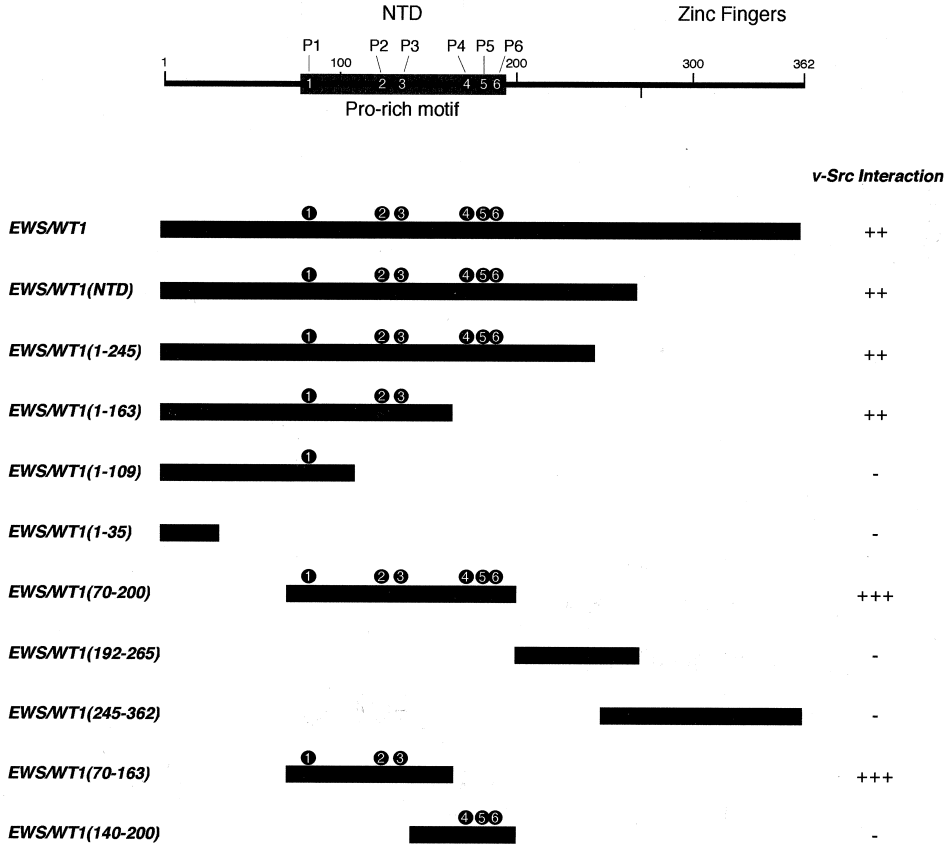


Fig. 2. Mapping of the v-Src interacting domain of EWS/WT1. The EWS/WT1 cDNA fragments fused in-frame to the GST gene in pGEX vector are schematically shown. Proteins from *E. coli* that expressed recombinant pGEX vectors encoding the various GST-EWS/WT1 fragment fusion proteins were incubated with [^{35}S]Met labeled in vitro translated v-Src protein. Following washing, the bound proteins were eluted with SDS loading buffer and proteins were analyzed by 10% SDS–PAGE. The + or − symbols to the left refer to the ability (or inability) to bind to v-Src. The six putative EWS/WT1 SH3 binding motifs (P-X-X-P) are indicated as P1–P6.

purpose of the current study, we focused on better defining and characterizing the interaction between EWS/WT1 and v-Src.

3.2. The proline-rich motif of EWS/WT1 is involved in v-Src interaction

To define the minimal region within EWS/WT1 required to bind to v-Src, a series of deletion mutants were generated. The proline-rich domain of EWS/WT1 contains six P-X-X-P motifs (P1 (^{80}PTAP^{83}), P2 (^{122}PAYP^{125}), P3 (^{136}PTRP^{139}), P4 (^{173}PQVP^{176}), P5 (^{180}PMQP^{183}), and P6 (^{188}PSYP^{191})) which could act as SH3 domain interacting sites. To determine which domain(s) within EWS/WT1 might mediate v-Src interaction, in vitro binding experiments were performed with a series of EWS/WT1 deletion mutants (Fig. 2). In this experiment, GST-EWS/WT1 fusion proteins were used in affinity precipitation experiments utilizing radiolabeled v-Src produced from in vitro translations. v-Src bound to GST-EWS/WT1, GST-EWS/WT1(NTD), GST-EWS/WT1(1–245), and GST-EWS/WT1(1–163), but failed to bind to: (i) C-terminal deletion mutants GST-EWS/WT1(1–109) and GST-EWS/WT1(1–35), (ii) the internal domain mutant (GST-EWS/WT1(192–265)), and (iii) the DNA binding domain mutant (GST-EWS/WT1(245–265)) (Fig. 2). Fusion protein GST-EWS/WT1(70–200), which contains all six P-X-X-P motifs (amino acids 80–191) bound to in vitro translated v-Src, as did GST-EWS/WT1(70–163), which contains the first three P-X-X-P motifs (P1–P3) (Fig. 2). GST-EWS/WT1(140–200), containing P-X-X-P motifs P4–P6, lost the ability to interact with v-Src, indicating that amino acids 70–163 contain the responsible domain(s) for v-Src interaction (Fig. 2).

3.3. Only the SH3 and SH2 domains of v-Src interact with EWS/WT1

Although we have shown that EWS/WT1 can interact with both SH2 and SH3 domains of v-Src (Fig. 1), these results do not preclude additional sites within v-Src which could interact with EWS/WT1. To address this issue, a series of in vitro GST pull-down assays were performed with GST fusions of v-Src (Fig. 3A). The v-Src functional regions, such as the unique domain (aa 1–76), the SH3 domain (aa 77–168), the SH2 domain (aa 148–252), and the kinase domain (aa 253–526), were generated by the PCR, individually expressed as GST fusion proteins in *Escherichia coli*, and coupled to glutathione-Sepharose beads. Following incubation with [^{35}S]Met labeled EWS/WT1 protein and extensive washing, only GST fusions containing the SH3 or SH2 domains were able to specifically retain EWS/WT1 (Fig. 3B). EWS/WT1 did not interact with the amino-terminal unique domain or the carboxy-terminal kinase domain (Fig. 3B). These results suggest that the association of EWS/WT1 with v-Src can be mediated independently by the SH2 or SH3 domains.

3.4. Association between EWS/WT1 and v-Src in vitro and in vivo

To determine whether the interaction between EWS/WT1 and v-Src occurred in vivo, we performed coaffinity precipitations following transfection of 293T cells with expression vectors driving the synthesis of both proteins. Plasmid pcDNA3:GST-EWS/WT1 (a mammalian expression vector containing EWS/WT1 fused to GST) or pcDNA3:GST (a mammalian expression vector containing the GST domain) were co-transfected with pcDNA3/v-Src into 293T cells. Cells were lysed 48 h after transfection, GST or GST-EWS/WT1 fusions were affinity-precipitated with glutathione beads, and subsequently immunoblottings performed on the eluents using an anti-Src antibody (LA074, Quality Biotech., National Cancer Institute) to detect the presence of v-Src (Fig. 4A). Consistent with in vitro binding assays, v-Src is specifically coprecipitated with GST-EWS/WT1, but not with GST alone (Fig. 4A). These results suggest that EWS/WT1 and v-Src can associate in vivo.

To eliminate the possibility that an uncharacterized eukaryotic adapter molecule was mediating the interaction between EWS/WT1 and v-Src, we performed the following experiment. Recombinant $(His)_6$-tagged EWS/WT1 protein was purified from *E. coli* and used in pull-down assays with GST/SH3 v-Src which had also been produced in *E. coli*. As shown in Fig. 4B, both bacterially produced proteins could interact, suggesting that the v-Src-EWS/WT1 interaction does not require an adapter protein.

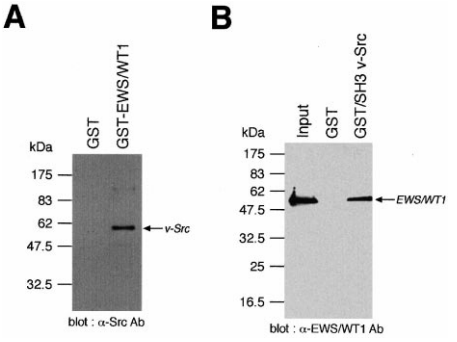


Fig. 4. Interaction between EWS/WT1 and v-Src in vivo. A: Co-affinity precipitation of v-Src with GST-EWS/WT1. Plasmid pcDNA3:v-Src was co-transfected with pcDNA3:GST (lane 1) or pcDNA3:GST-EWS/WT1 constructs (lane 2). Following lysis of the transfected cells 48 h after transfection, affinity precipitations were performed with glutathione-Sepharose 4B beads. The affinity precipitates were analyzed for the presence or absence of v-Src protein by Western blotting utilizing an anti-Src antibody (LA074). The positions of the molecular weight markers are indicated to the left and the position of migration of v-Src is indicated by an arrow to the right. B: Direct interaction between EWS/WT1 and the SH3 domain of v-Src. Bacterially produced $(His)_6$-tagged EWS/WT1 protein was incubated with GST alone or GST-SH3, and GST pull-downs performed as described in Section 2. An aliquot of the input (20%) and of the pellets from the pull-down assays was fractionated on a 10% SDS–PAGE, blotted onto PVDF membrane, and the bound EWS/WT1 protein was detected with an α-WT1 zinc finger antibody (C19).

3.5. EWS/WT1 is a substrate of v-Src in vivo

To determine whether v-Src could phosphorylate EWS/WT1, we performed in vitro kinase assays (Fig. 5). Myelin basic protein (MBP) was used as an internal control to monitor the v-Src kinase activity in each incubation. In vitro incubation of recombinant GST-EWS/WT1 with commercially available v-Src protein kinase, resulted in the phosphorylation of GST-EWS/WT1 (Fig. 5A). No corresponding radiolabeled product could be detected in v-Src or GST control lanes, respectively. These results demonstrate that v-Src tyrosine kinase can directly phosphorylate GST-EWS/WT1. The observation that GST alone is not a substrate establishes that phosphorylation occurs within the EWS/WT1 part of the fusion protein. The amino acid acceptor of EWS/WT1 was established by phospho-amino acid analysis. Only phosphotyrosine (pY) was detected in EWS/WT1 (Fig. 5B). To determine whether EWS/WT1 is a substrate for v-Src in vivo, a plasmid encoding $(His)_6$-tagged EWS/WT1 was transfected into C33A cells with v-Src cDNA or control vector, respectively. Forty-eight hours after transfection, cells were lysed under denaturing conditions and $(His)_6$-EWS/WT1 proteins were affinity-precipitated with Ni^{2+}-NTA resin. Affinity-purified EWS/WT1 proteins were fractionated by SDS–PAGE, transferred to PVDF membrane, and immunoblotted with an anti-EWS/WT1 antibody (C-19) to quantitate the amount of proteins in each sample (Fig. 5C, top panel). To detect tyrosine phosphorylation, the anti-phosphotyrosine antibody, 4G10, was used as a probe (Fig. 5C, bottom panel). Significant amounts of tyrosine phosphorylation were detected on EWS/WT1 protein in cells co-transfected with v-Src (Fig. 5C, bottom panel), but not in cells co-transfected with vector alone. These data suggest that EWS/WT1 is a substrate for v-Src in vivo although further experiments would be required to demonstrate whether the EWS/WT1 phosphorylated sites obtained in vivo are the same as those obtained in vitro.

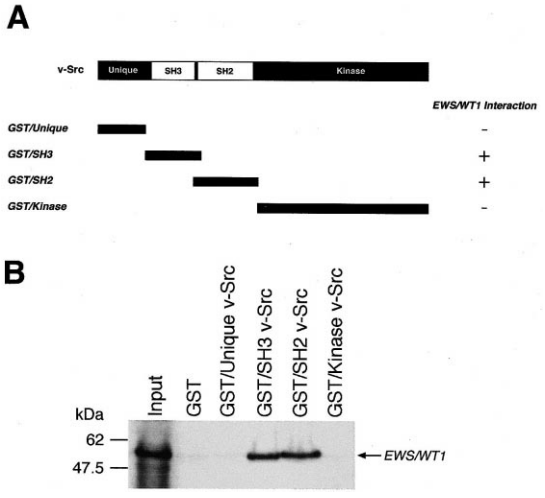


Fig. 3. The SH3 and SH2 domains of v-Src mediate EWS/WT1 binding. A: Schematic representation of the GST fusion v-Src derivatives used in this study. Constructs GST/Unique, GST/SH3, GST/SH2, and GST/Kinase represent the GST domain fused to the v-Src unique, SH3, SH2, and kinase domains, respectively. GST fusion proteins from induced lysates of *E. coli* expressing recombinant pGEX vectors encoding the various functional domains of v-Src protein were incubated with [^{35}S]Met labeled EWS/WT1 protein. Following washing, the bound proteins were eluted and analyzed by SDS–PAGE. The + or − symbols to the left refer to the ability (or inability) of the GST fusions to retain EWS/WT1. B: Binding of EWS/WT1 to the v-Src SH2 and SH3 domains. Following GST pull-down assays, eluted fractions were analyzed by SDS–PAGE. The positions of molecular weight markers are indicated to the left and the position of EWS/WT1 is indicated on the right.

3.6. v-Src stimulates transactivation by the EWS(NTD)

A number of studies have shown that v-Src can indirectly activate [28,29] or repress [30,31] the expression of genes encoding a wide variety of cellular proteins. To test whether co-expression of v-Src could influence EWS(NTD) mediated transcriptional activation, cells were co-transfected with (i) a v-Src expression plasmid, (ii) an expression vector containing a GAL4 DNA binding domain fused to the EWS-NTD and, (iii) a reporter plasmid containing the E1B minimal promoter harboring five GAL4 DNA binding sites. Consistent with previously reported data [9], introduction of GAL4-EWS(NTD) in C33A cells significantly activated transcription (~33-fold) from the reporter 5×Gal4·E1B·CAT (Fig. 6A, compare lane 4 to lane 1). Co-transfection of the expression vector driving synthesis of only the GAL4 DNA binding domain did not show activation (Fig. 6A, lane 1). Co-transfection of v-Src, but not c-Src, with GAL4-EWS(NTD) enhanced the response

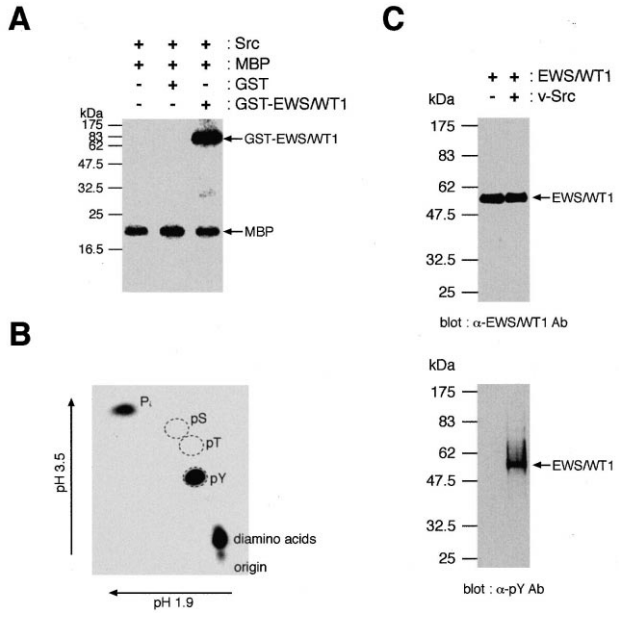
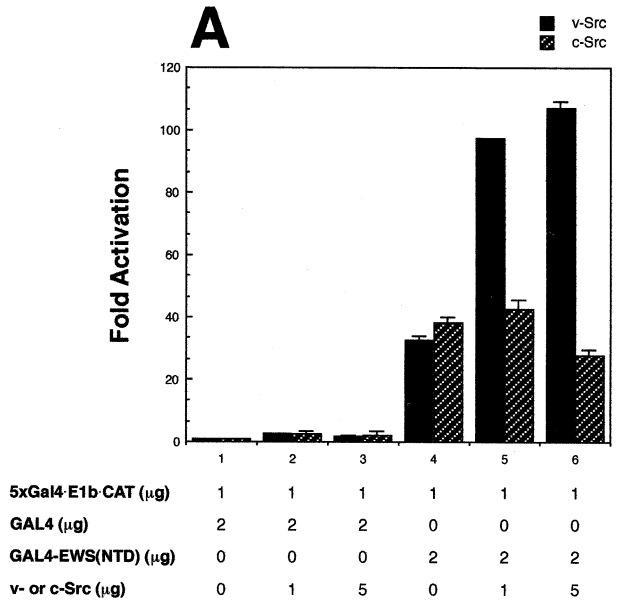
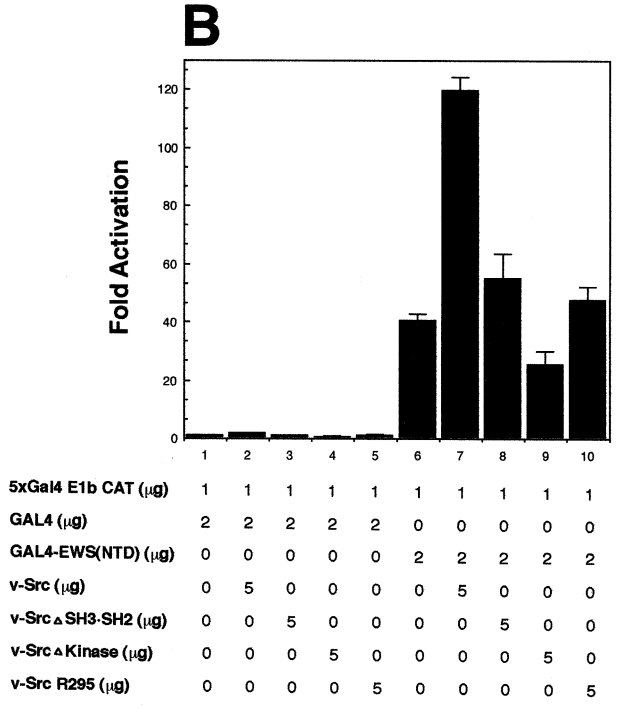


Fig. 5. Tyrosine phosphorylation of EWS/WT1 by Src. A: In vitro phosphorylation of EWS/WT1 by Src tyrosine kinase. Two micrograms of GST or GST-EWS/WT1 were incubated with commercially available Src (Oncogene Research Products, MA, USA) with 50 μM ATP and 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) in a reaction mixture for 20 min at 30°C. For each reaction, 1 μg of MBP was used as an internal control to monitor Src kinase activity. Reaction mixtures were resolved on a 15% SDS–PAGE and labeled proteins were visualized by exposing the dried gel to X-ray film (Kodak). The presence of Src, myelin basic protein (MBP), GST, or GST-EWS/WT1 in each incubation is indicated above the lanes. B: Phosphoamino acid analysis of phosphorylated EWS/WT1 protein by Src in vitro. Acid hydrolysates of phosphorylated GST-EWS/WT1 were prepared and fractionated by electrophoresis on thin layer cellulose at pH 1.9 (first dimension) and pH 3.5 (second dimension). The positions of unlabeled standards, detected with ninhydrin, are shown as pS (phosphoserine), pT (phosphothreonine), and pY (phosphotyrosine). The autoradiograph shows ^{32}P migrating with phosphotyrosine (pY), orthophosphate (Pi), and di-amino acids. C: Tyrosine phosphorylation of EWS/WT1 by ectopically expressed v-Src in vivo. Plasmid encoding $(His)_6$-EWS/WT1, and either v-Src or the empty vector (pcDNA3), were co-transfected into C33A cells. Recombinant $(His)_6$-EWS/WT1 protein was pulled down by Ni-NTA agarose. The presence of EWS/WT1 protein was confirmed with an anti-WT1 zinc finger antibody (C-19, Santa Cruz Biotechnology Inc.) (top panel) and tyrosine phosphorylation was detected with an anti-phosphotyrosine antibody (4G10) (bottom panel).





of the 5×GAL4·E1b·CAT reporter up to 107-fold in a dose-dependent fashion (Fig. 6A, compare lanes 5 and 6 to lane 4). This enhancement by v-Src was EWS(NTD)-dependent since v-Src had no effect on the transcription response mediated by GAL4 (Fig. 6A, compare lanes 2 and 3 to lane 1).

To further explore the effect of v-Src on EWS-NTD mediated transcription, three v-Src mutants (v-Src ΔSH3·SH2, lacking SH3 and SH2 domains; v-Src ΔKinase, a kinase domain deletion mutant; and v-Src R295K, a kinase negative mutant harboring a lysine to arginine missense mutation in the kinase domain) were introduced in the transfection assay described above (Fig. 6B). All three mutants failed to stimulate transcription by EWS/WT1, suggesting a requirement for

Fig. 6. Functional influence of v-Src on the transcription activity of EWS(NTD). A: Stimulation of EWS mediated transactivation by v-Src. Two micrograms of GAL4 or GAL4-EWS(NTD) plasmids were co-transfected with 0, 1, or 5 μg of v-Src (black bar) or c-Src (hatched bar) expressing plasmid into C33A cells. CAT activity was measured after normalization to β-galactosidase activity and expressed as fold activation relative to the basal level observed with the 5×GAL4·E1b·CAT reporter plasmid and the GAL4 DNA binding domain alone (lane 1). The average of two independent experiments is presented. B: v-Src SH3 and SH2 domains as well as kinase domain are required for the enhancement of EWS(NTD) mediated transactivation. Two micrograms of GAL4 or GAL4-EWS(NTD) expression plasmids were co-transfected with 5 μg of v-Src or v-Src mutants (v-Src ΔSH3·SH2 (SH3 and SH2 domain deletion mutant), v-Src ΔKinase (kinase domain deletion), and v-Src R295 (kinase inactivation mutant with lysine to arginine mutation in kinase domain)) into C33A cell line, respectively. CAT activity was measured after normalization to β-galactosidase activity and expressed as fold activation relative to the basal level observed with the 5×GAL4·E1b·CAT reporter plasmid and the GAL4 DNA binding domain alone (lane 1). The average of two independent experiments is presented.
←

a region encompassed by the SH3 and SH2 domains, and for the kinase domain of v-Src, for this effect (compare lanes 8–10 to lane 7).

4. Discussion

The results reported herein begin to explore the functional regulation of the EWS/WT1 oncoprotein. The EWS/WT1 fusion protein has been proposed to play an important role in the development of DSRCT [3]. In support of this notion, expression of EWS/WT1(−KTS) is oncogenic when introduced into NIH3T3 cells [13]. One model to explain how EWS/WT1 mediates transformation is that it binds to DNA recognition elements normally regulated by WT1, or other related zinc finger proteins, and alters the transcriptional status of these genes. One potential downstream candidate is *PDGF-A*, since this gene: (i) is overexpressed in a stable cell line expressing EWS/WT1(−KTS), (ii) is expressed in specimens of DSRCT, and (iii) responds to EWS/WT1 in vitro [10].

The modulation of EWS/WT1 activity by regulatory proteins has not been extensively studied. We have previously documented that EWS/WT1 is phosphorylated in vitro and in vivo by the nuclear tyrosine kinase, c-Abl, and that this inhibits EWS/WT1 DNA binding and self-association [26]. The results presented herein indicate that EWS/WT1 activity can also be regulated by a signaling pathway under control of (a) cytoplasmic kinase(s). A number of studies have shown that the v-Src protein tyrosine kinase can deregulate gene expression, such as *fos* and *jun* [32–36]. In addition, many signalling molecules, such as v-Src, ras, and Btk, enhance the transcriptional activity of several transcription factors, such as AP-1 [37,38] or TFII-I [23]. Also, cells expressing the v-Src oncogene have increased expression of HIF-1, VEGF, and ENO1 under both hypoxic and non-hypoxic conditions and this is associated with increased transcription [39]. Thus, in the current study, it is likely that v-Src activates a signal transduction pathway that leads to the potentiation of transcription activation by EWS/WT1 (Fig. 6).

SH2 and SH3 domains mediate a variety of physiological responses and are found together on many proteins [40], suggesting that their activities may be coordinated. Interestingly, the NTD of EWS/WT1 (aa 70–163) contains three proline-rich motifs which could provide binding sites for the v-Src SH3 (Src homology 3) domain (Fig. 2). Our results indicate that EWS/WT1 can interact with the SH2 domain of v-Src, but we have not attempted to define the responsible EWS/WT1 domain. The binding of EWS/WT1 to SH3 domains does not appear to be promiscuous since, when presented with nine possible SH3 domains, EWS/WT1 only bound to three (Fig. 1).

The EWS protein can be phosphorylated by a number of kinases. For example, protein kinase C phosphorylates EWS through an IQ domain and inhibits its RNA binding activity [41]. Since the EWS/WT1 chimeric protein does not retain the IQ domain, it is not expected to be a substrate for protein kinase C. In addition, EWS has been reported to associate with, and be a substrate for, Bruton's tyrosine kinase (BTK) and the Pyk2 tyrosine kinase [42,43]. Both of these kinases are cytoplasmic, and it is thought that extranuclear EWS is the target of these enzymes. Analysis of the EWS/WT1 protein sequence (performed at http://www.cbs.dtu.dk/services/NetPhos/) revealed the presence of 13 potential tyrosine phosphorylation sites (indicated by underscores within the sequence: ^{2}ASTD<u>Y</u>STYS^{10}, ^{25}PTQG<u>Y</u>AQTT^{33}, ^{40}SYGT<u>Y</u>GQPT^{48}, ^{48}TDVS<u>Y</u>TQAQ^{56}, ^{62}GQTA<u>Y</u>ATSY^{70}, ^{66}YATS<u>Y</u>GQPP^{74}, ^{73}PPTG<u>Y</u>TTPT^{81}, ^{154}STGG<u>Y</u>NQPS^{162}, ^{161}PSLG<u>Y</u>GQSN^{169}, ^{168}SNYS<u>Y</u>PQVP^{176}, ^{191}PPTS<u>Y</u>SSTQ^{199}, ^{199}QPTS<u>Y</u>DQSS^{207}, and ^{204}DQSS<u>Y</u>SQQN^{212}). Although our data strongly suggest that EWS/WT1 is a substrate for v-Src in vivo, it is not clear how the cytoplasmic membrane-integrated v-Src modifies the nuclear localized EWS/WT1. It is possible that extranuclear EWS/WT1 is a substrate for modification by v-Src. Alternatively, and not mutually exclusive, is the possibility that v-Src activates a pathway that leads to nuclear modification of EWS/WT1.

Our results also demonstrate that v-Src has a striking effect on the activation properties of the EWS-NTD (Fig. 6). Also, deletions of v-Src affecting a domain encompassed by the SH3 and SH2 regions, or by the kinase domain, abolished stimulation of EWS/WT1 activation (Fig. 6B) suggesting the requirement of these regions for v-Src activity in this assay. It is currently unclear to us why c-Src did not potentiate the EWS-NTD transcriptional properties (Fig. 6A). A comparative study between c-Src and v-Src has revealed that v-Src has three point mutations in its SH3 domain and one upstream of SH3, and that engineering these mutations into c-Src converted c-Src to be oncogenic and active in kinase activity [44]. In addition, three-dimensional structural analysis of c-Src has shown that interaction among domains, stabilized by binding of the phosphorylated tail to the SH2 domain, locks the molecule in a conformation that simultaneously disrupts the kinase active site and sequesters the binding surfaces of the SH2 and SH3 domains. Mutations in v-Src are predicted to break these interactions, producing an open active kinase [45]. Thus it is clear that these two proteins differ in enzymatic and functional properties.

It will be important to assess the phosphorylation status of EWS/WT1 in DSCRTs since this event could provide insight into the activity of EWS/WT1 in this malignancy. If signaling pathways involved in modifying EWS/WT1 function are usurped in this malignancy, then defining these events becomes important for understanding progression in this malignancy.

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