EWS-Fli-1 Fusion Protein Interacts with Hyperphosphorylated RNA Polymerase II and Interferes with Serine-Arginine Protein-mediated RNA Splicing*

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Ewing’s sarcoma displays a characteristic chromosomal translocation that results in fusion of the N-terminal domain of the Ewing’s sarcoma protein (EWS) to the C-terminal DNA-binding domain of the ETS family transcription factor Fli-1 (Friend leukemia integration-1). EWS possesses structural motifs suggesting a role in transactivation as well as RNA binding. We demonstrate that wild-type EWS protein functions as an adapter molecule coupling transcription to RNA splicing by binding to hyperphosphorylated RNA polymerase II through the N-terminal domain of EWS and recruiting serine-arginine (SR) splicing factors through the C-terminal domain of EWS. The oncopgenic EWS-Fli-1 fusion protein retains the ability to bind to hyperphosphorylated RNA polymerase II but lacks the ability to recruit SR proteins because of replacement of the C-terminal domain of EWS by Fli-1. In an in vivo splicing assay, the EWS-Fli-1 fusion protein inhibits SR protein-mediated E1A pre-mRNA splicing in a dominant-negative manner. These results indicate that EWS-Fli-1 interferes with the normal function of EWS and implicate uncoupling of gene transcription from RNA splicing in the pathogenesis of Ewing’s sarcoma.

The EWS gene was originally identified in Ewing’s sarcoma with the t(11;22) chromosomal translocation, where it is fused to the DNA-binding domain of the ETS family transcription factor Fli-1 (1). Subsequent studies revealed that in Ewing’s sarcoma EWS may be fused to one of five different members of the ETS family, namely Fli-1 (1), ERG (2), ETV-1 (3), E1AF (4, 5), and FEV (6). In addition to fusions with ETS transcription factors in Ewing’s sarcoma, EWS has been shown to form fusion proteins with a number of other partners including ATF-1 in malignant melanoma of soft parts (7), WT-1 in desmoplastic small round cell tumors (8, 9), TEC in extraskeletal myxoid malignant melanoma of soft parts (7), WT-1 in desmoplastic tumors (10), and CHOP in myxoid liposarcoma (11).

In EWS fusion proteins, the N-terminal domain (NTD) of EWS is retained, whereas the C-terminal domain (CTD) of EWS is replaced by the corresponding fusion partner.

Understanding the mechanism of transformation by EWS fusion proteins will probably require knowledge regarding the functions of the wild-type proteins. In this regard, the N-terminal domain of EWS is rich in glutamine, serine, and tyrosine, residues that are commonly found in transcriptional activation domains. EWS is known to associate with a specific subpopulation of the TFIIID basal transcription factor and with certain subunits of the RNA polymerase II (Pol II) complex (12). However, the C-terminal domains of EWS contain ribonucleo-protein consensus sequence and multiple arginine-glycine-glycine (RGG) repeats, both of which are signatures of RNA-binding proteins (13).

The Ewing’s sarcoma protein EWS (1), the translocation in liposarcoma protein (TLS) (14, 15), and the TATA-binding protein associated factor (TAF1) (16) comprise a unique family of proteins with shared structural features. We previously reported that wild-type TLS not only binds to RNA Pol II, but it also interacts with two newly characterized serine-arginine (SR) splicing factors (17, 18). The structural similarities between EWS and TLS led to an assessment of the interaction of EWS with RNA Pol II and these TLS-associated SR (TASR) splicing factors. We determined that both EWS and EWS-Fli-1 interact with the hyperphosphorylated largest subunit of the RNA Pol II complex (Pol IIo) through the N-terminal domain of EWS. However, EWS interacts with TASR proteins, whereas EWS-Fli-1 is unable to interact with TASR proteins because of replacement of its C-terminal domain by the Fli-1 fusion partner. These biochemical differences between EWS and EWS-Fli-1 have functional consequences because EWS-Fli-1 interferes with TASR-mediated splicing in an in vivo E1A splicing assay. These results suggest that the EWS-Fli-1 fusion protein may contribute to cellular transformation through an effect on the coupling of transcription to RNA splicing.

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1 The abbreviations used are: Fli-1, Friend leukemia integration-1; EWS, Ewing’s sarcoma protein; NTD, N-terminal domain; CTD, C-terminal domain; pol II, polymerase II; TLS, translocation in liposarcoma protein; SR, serine-arginine; TAF1, TAF1-associated SR, PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; pol IIo, hyperphosphorylated largest subunit of RNA Pol II; pol IIa, hypophosphorylated largest subunit of RNA Pol II.

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Plasmids—The cDNAs for EWS, EWS-Fli-1, and Fli-1 were cloned into the pSG5-Flag vector with the Flag epitope at the N-terminal end. The EWS-NTD deletion mutant consists of amino acids 1–245 of the EWS protein, and the EWS-CTD deletion mutant contains amino acids 267–656 of the EWS protein. Myc-TASR-1 and -2 expression vectors were constructed by cloning full-length TASR cDNAs into pCS2-MT vector (Sigma) with the Myc epitope at the N-terminal end. For in vivo splicing assay, TASR cDNAs were inserted into pMH vector (Sigma) to generate pMH-TASR with one copy of the influenza hemagglutinin epitope at the C-terminal end of TASR proteins. Reporter plasmid pCS2-MT-E1A was a kind gift from Dr. Moreau-Gachelin (19).

Immunoprecipitation and Western Blotting—For expression of Flag- or Myc-tagged proteins, 10 μg of the pSG5-Flag expression construct and 10 μg of the pCS2-Myc expression construct were introduced into

Experimental Procedures

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RESULTS

The C-terminal Domain of EWS Interacts with SR Proteins—To investigate the in vivo interaction between EWS and the TASR proteins, plasmids expressing Flag-tagged EWS, EWS-Fli-1, and Fli-1 were constructed (Fig. 1a) and co-transfected into COS-7 cells with plasmids expressing Myc-tagged TASR-1 (lanes 1–3) and TASR-2 (lanes 4–6) were co-transfected into COS-7 cells with plasmids expressing Flag-tagged EWS, EWS-Fli-1, or Fli-1. Total cell lysates were blotted with M2 anti-Flag antibody and 9E10 anti-Myc antibody, or C-21 rabbit polyclonal anti-RNA Pol II antibody (Santa Cruz Biotechnology). For immunoprecipitation of endogenous EWS and EWS-Fli-1 fusion proteins, 30 μl of protein A/G plus agarose was pre-charged with 10 μl of 8WG16 anti-Pol II or control mouse IgG and then incubated with 0.2 ml of fresh lysate for 20 min. After four washes with radioimmune precipitation buffer, the immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis in a 6% gel, and the proteins were detected with anti-Myc antibody, Flag-EWS co-immunoprecipitated with N-18 rabbit polyclonal anti-EWS (Santa Cruz Biotechnology) and C119 rabbit polyclonal anti-Fli-1 (20). Protein bands were visualized using the ECL Western blotting analysis system (Amer sham Pharmacia Biotech).

Identification of Hypo- and Hyperphosphorylated Forms of the Pol II Largest Subunit—For immunoprecipitation of hyperphosphorylated RNA polymerase II, 15 μl of mouse monoclonal H5 or H14 antibody (Research Diagnostics, Inc.) was coupled to 40 μl of protein A/G plus agarose beads. HeLa cells from a 100-mm dish were lysed with 0.6 ml of cell lysate buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate). Insoluble material was removed via centrifugation at 15,000 × g for 5 min, and the immunoprecipitation was then carried out by incubating 0.2 ml of lysate with antibody pre-charged agarose beads at 4 °C for 4 h. After being washed three times with ice-cold cell lysis buffer, the immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis in a 6% gel. The hyperphosphorylated RNA Pol IIa was detected with the 8WG16 antibody, and the hyperphosphorylated RNA Pol IIo was detected with the H14 antibody as described previously (21).

Transfection of HeLa Cells and RT-PCR—For in vivo splicing of E1A pre-mRNA, 2 μg of pCS3-MT-E1A and 2 μg of plasmid expression plasmids expressing Flag-EWS, EWS-NTD, and EWS-CTD were co-transfected into COS-7 cells with plasmids expressing Myc-TASR-1 and Myc-TASR-2. c, plasmids expressing Flag-tagged EWS, EWS-NTD, and EWS-CTD were co-transfected into COS-7 cells with plasmids expressing Myc-tagged TASR-1 (lanes 1–3) and TASR-2 (lanes 4–6). The cell lysates were immunoprecipitated with the 9E10 anti-Myc antibody, and the immunoprecipitates were blotted with the anti-Flag antibody to detect interaction between EWS and TASR proteins (lanes 7–12). The immunoprecipitates were also blotted with the anti-Myc antibody to detect Myc-TASR-1 and Myc-TASR-2. The cell lysates were immunoprecipitated with the 9E10 anti-Myc antibody, and the immunoprecipitates were blotted with the anti-Flag antibody to detect interaction between EWS and TASR proteins (lanes 7–12). The immunoprecipitates were also blotted with the anti-Myc antibody to detect Myc-TASR-1 and Myc-TASR-2.

FIG. 1. EWS interaction with SR proteins. a, schematic of EWS, Fli-1, EWS-Fli-1, EWS-NTD, and EWS-CTD. Distinct sequence features are as follows: QST, glutamate-, serine-, and tyrosine-rich domain; RGG, region with multiple Arg-Gly-Gly repeats; RNP-CS, ribonuclease-protein consensus sequence. b, plasmids expressing Myc-tagged TASR-1 (lanes 1–3) and TASR-2 (lanes 4–6) were co-transfected into COS-7 cells with plasmids expressing Flag-tagged EWS, EWS-Fli-1, or Fli-1. Total cell lysates were blotted with M2 anti-Flag antibody and 9E10 anti-Myc antibody to detect the epitope-tagged proteins (lanes 1–6). The cell lysates were immunoprecipitated with the 9E10 anti-Myc antibody, and the immunoprecipitates were blotted with the anti-Flag antibody to detect interaction between EWS and TASR proteins (lanes 7–12). The immunoprecipitates were also blotted with the anti-Myc antibody to detect Myc-TASR-1 and Myc-TASR-2.
whereas Flag-EWS-Fli-1 and Flag-Fli-1 did not co-immunoprecipitate with either TASR protein (Fig. 1b, lanes 8, 9, 11, and 12). Because the C-terminal domain of EWS is replaced by the Fli-1 sequence in the EWS-Fli-1 fusion protein, these results suggested that wild-type EWS might mediate interaction with the TASR proteins through its C-terminal domain. To test this possibility, the N-terminal domain of EWS (EWS-NTD) and the C-terminal domain of EWS (EWS-CTD) were cloned separately into the pSG5-FL expression vector for transfection (Fig. 1a).

When expressed in COS-7 cells, Flag-EWS-CTD co-immunoprecipitated with both TASR proteins (Fig. 1c, lanes 9 and 12), whereas the Flag-EWS-NTD did not, demonstrating that EWS interacts with TASR-1 and -2 through the C-terminal domain of EWS.
phosphorylated Pol IIo form in both immunoprecipitation and Western blotting (21) (Fig. 3a, lanes 3 and 4). Repeated attempts to co-immunoprecipitate EWS with H5 and H14 were not successful (data not shown), and it is likely that binding by H5 or H14 causes Pol IIo to be released from the multi-protein complex as previously suggested (26). As an alternative approach, RNA Pol II was co-immunoprecipitated with a rabbit polyclonal antibody against the C terminus of EWS, whereas a rabbit polyclonal antibody against the N terminus of EWS failed to co-immunoprecipitate RNA Pol II (Fig. 3a, lanes 6 and 7). This different ability to co-immunoprecipitate RNA Pol II suggests that the rabbit antibody against the C terminus of EWS is less disruptive to the maintenance of the EWS-Pol II multi-protein complex than is the antibody against the N terminus of EWS. The RNA Pol II subpopulation associated with EWS appeared to be predominantly the hyperphosphorylated Pol IIo form because the co-immunoprecipitated RNA Pol II is detectable by H14 but not by 8WG16 in Western blotting (Fig. 3a, lane 6, upper and lower panels).

To co-immunoprecipitate RNA Pol II with the EWS-Fli-1 fusion protein, plasmids expressing Flag-tagged EWS, EWS-Fli-1, and Fli-1 were transfected into HeLa cells, and the lysates were used in co-immunoprecipitation with a goat polyclonal anti-Flag antibody. Under our experimental conditions, only Flag-EWS-Fli-1 co-immunoprecipitated with hyperphosphorylated Pol IIo (Fig. 3b, lane 10), whereas Flag-EWS and Flag-Fli-1 did not co-immunoprecipitate with Pol IIo (Fig. 3b, lanes 9 and 11). Because the Flag epitope is tagged at the same N-terminal ends of EWS and EWS-Fli-1 and because the immunoprecipitation was carried out with the same goat anti-Flag, these results suggested that EWS and EWS-Fli-1 might associate differently with RNA Pol IIo. The EWS-Fli-1 fusion protein may have a higher affinity than EWS toward the same site on Pol IIo, and this high affinity may preserve the interaction between Pol IIo and EWS-Fli-1 despite interference by the anti-Flag antibody binding. Another potential explanation is that EWS-Fli-1 may bind to a different site on Pol IIo that is less prone to disruption by the antibody.

**EWS-Fli-1 Inhibits RNA Splicing Mediated by SR Proteins**—To investigate whether this interaction of EWS with RNA Pol IIo and the TASR proteins played a role in RNA splicing, we tested both wild-type EWS and the EWS-Fli-1 fusion protein in an E1A splicing assay in HeLa cells. The alternative splicing of E1A pre-mRNA in HeLa cells results in the generation of five different splicing isoforms designated 13 S, 12 S, 11 S, 10 S, and 9 S (Fig. 4a) (28), and co-expression of individual SR proteins is known to alter the splicing pattern of E1A isoforms (29).

Analysis of E1A splicing products by RT-PCR indicated that expression of TASR-1 promoted splicing to the 11 S, 10 S, and 9 S isoforms (Fig. 4b; compare lanes a and e), whereas expression of TASR-2 promoted splicing to the 9 S isoform (Fig. 4b, compare lanes a and i). Endogenous EWS is constitutively expressed in HeLa cells, and co-expression of EWS did not alter the E1A splicing profile (Fig. 4b, lanes f and j). Co-expression of EWS-Fli-1 resulted in a marked inhibition of E1A splicing by both TASR-1 and TASR-2 (Fig. 4b, compare lane f with lane g, and lane j with lane k); however, co-expression of Fli-1 had little effect on E1A splicing (Fig. 4b, lanes h and l). The band designated “reporter DNA” represents the PCR-amplified E1A genomic sequence from residual plasmid DNA.

To demonstrate that the results obtained by RT-PCR were not due to selective amplification of specific sequences, we developed an RNase protection assay to directly measure the profile of E1A alternative splicing in HeLa cells. In the RNase protection assay, TASR-1 expression increased the E1A fragments corresponding to the 11 S, 10 S, and 9 S isoforms (Fig. 4c). Transfection of EWS-Fli-1 strongly inhibited E1A pre-mRNA splicing by both TASR proteins (Fig. 4c, lanes g and k). Transfection efficiency among different samples was similar in that comparable levels of EWS, EWS-Fli-1, Fli-1, and TASR proteins were expressed in the transfected HeLa cells (Fig. 4c, bottom panels). Unspliced E1A pre-mRNA was not detected by the RNase protection assay, consistent with previous reports that the unprocessed E1A pre-mRNA molecule is unstable in HeLa cells (28).

The effects of EWS deletion mutants on TASR-mediated E1A splicing were tested to determine whether the N-terminal domain or the C-terminal domain of EWS alone was responsible...
for the alteration in E1A splicing. Even though in HeLa cells both EWS deletion mutants were expressed at levels comparable with EWS-Fli-1, neither the EWS-NTD nor the EWS-CTD inhibited TASR-mediated E1A pre-mRNA splicing (Fig. 4d, lanes m–r), indicating that inclusion of Fli-1 in the fusion protein is necessary for EWS-Fli-1 inhibition of RNA splicing.
proteins expression are shown at the right on the labeled at the length antisense E1A as the probe (lanes). RNA markers (lanes a–l) are in HeLa cells were analyzed by RNase protection assay using full-length E1A reporter gene.

The steady-state levels of E1A pre-mRNA of 9S.E.1A reporter gene. The steady-state levels of E1A pre-mRNA in HeLa cells were analyzed by RNase protection assay using full-length antisense E1A as the probe (lanes a–l). RNA markers (lanes a–l) are shown at the bottom. IP: immunoprecipitation.

Fig. 5. Effects of EWS, EWS-Fli-1, and Fli-1 on the expression of 9S.E.1A reporter gene. The steady-state levels of E1A pre-mRNA in HeLa cells as analyzed by RNase protection assay using full-length antisense E1A as the probe (lanes a–l). RNA markers (lanes M) are labeled at the left, protected antisense E1A RNA fragments are shown on the right with exons designated by numerals in boxes, and levels of protein expression are shown at the bottom. IP: immunoprecipitation.

Inclusion of the Fli-1 sequence in the fusion protein may result in changes in protein folding and/or accessibility of the EWS N-terminal domain to RNA Pol II, thus endowing the EWS-Fli-1 fusion protein with a higher affinity toward certain subunits of RNA Pol II such as hSRP7 (22). This might enable the fusion protein to associate preferentially with the RNA Pol II complex, resulting in a dominant-negative effect on RNA splicing.

Although these results are consistent with the hypothesis that EWS-Fli-1 uncouples E1A reporter gene transcription from E1A pre-mRNA splicing and leads to degradation of the unprocessed E1A pre-mRNA transcripts in HeLa cells, the observed decrease in E1A splicing products could also be explained by EWS-Fli-1 inhibition of transcription from the pCS3-MT-E1A reporter or by EWS-Fli-1-induced degradation of the E1A splicing products. To test whether EWS-Fli-1 suppresses transcription from the pCS3-MT-E1A reporter or destabilizes the E1A splicing isoforms, we constructed an additional reporter plasmid, pCS3-MT-E1A-9S, which was generated from the same vector as pCS3-MT-E1A but contains a cDNA insert corresponding to the 9S.E.1A splicing isoform. The 9S.E.1A isoform is devoid of intron sequence; therefore its expression should be controlled primarily by gene transcription rather than by splicing. When analyzed in HeLa cells under the same conditions as described above, the resultant 9S.E.1A mRNA levels were not decreased by co-expression of EWS-Fli-1 or altered by TASR-1 or TASR-2 (Fig. 5). These results indicate that EWS-Fli-1 does not suppress transcription from the pCS3-MT-E1A vector or selectively destabilize the 9S.E.1A splicing isoform. Thus, the decrease in the steady-state level of E1A transcripts most likely results from EWS-Fli-1 inhibition of TASR-mediated splicing.

DISCUSSION

The EWS-Fli-1 fusion protein has been known to bind to specific DNA sequences and to transactivate target genes with Fli-1 binding sites in their promoter regions (30–33). Because deletion of either the EWS or the Fli-1 domain in the fusion protein results in loss of transforming ability (34), the EWS-Fli-1 fusion protein is believed to lead to malignant transformation via inappropriate activation of Fli-1 target genes (35). However, increasing evidence suggests that an alternative mechanism may also be involved in transformation by EWS-Fli-1. First, although EWS-Fli-1 is a more potent transactivator than Fli-1, deletional studies indicate that the domain within EWS required for transactivation differs from the domain required for transformation (36). Second, a recent mutagenesis study demonstrated that a point mutation abolishing the DNA binding activity of EWS-Fli-1 did not abolish its transforming ability (37). Third, the N-terminal domain of EWS has been reported to interact with splicing factors SF1 (38) and U1C (39) to regulate gene expression. Fourth, the RNA binding motifs in the C-terminal domain of EWS are replaced in the EWS-Fli-1 fusion protein, thus implicating a potential loss of function in RNA processing by the fusion protein.

Our findings provide evidence for a pathway whereby wild-type EWS functions as a docking molecule that binds via its N-terminal domain to hyperphosphorylated RNA Pol II and recruits specific SR splicing factors through its C-terminal domain. EWS fusion proteins, on the other hand, bind to RNA Pol II but fail to recruit TASR splicing factors. This uncoupling of RNA processing from transcription would be expected to alter gene expression in tumor cells with EWS fusions. EWS fusion proteins might block splicing by SR proteins, leading to degradation of the unprocessed target pre-mRNA and downregulation of target gene expression, or lead to alternative splicing of the target pre-mRNA. In this regard, abnormal RNA splicing in Ewing’s sarcoma cells has been reported to affect a variety of molecules such as the fragile histidine triad (FHIT) tumor suppressor (40), the p53-inducible P2X3 ion channel (41), and the PAX3 and PAX7 regulators of myogenic and neural development (42).

Our findings also demonstrate that the EWS-Fli-1 fusion protein interferes with E1A pre-mRNA splicing despite the presence of endogenous EWS protein. These results appear to be relevant to the pathogenesis of Ewing’s sarcoma because in Ewing’s sarcoma cells one EWS allele remains intact (43). In our splicing assays the EWS fusion protein functions in a dominant-negative manner. This dominant-negative effect could be partly explained by the fact that EWS-Fli-1 is present exclusively in the nucleus, whereas EWS is detectable in both the nucleus and the cytoplasm (43). The exclusive nuclear localization of the EWS-Fli-1 fusion protein in effect increases the nuclear concentration of the EWS-Fli-1 fusion protein and enhances its chance to compete with EWS for interaction with the RNA Pol II complex. Alternatively, EWS and EWS-Fli-1 may each associate with different subunits of the RNA Pol II complex, and these associations may be mutually exclusive. This notion is supported by the findings that nuclear complexes containing EWS and EWS-Fli-1 have different sedimentation profiles when fractionated through a glycerol gradient (12) and that the EWS-Fli-1 fusion protein possesses a much higher affinity toward certain subunits of the RNA Pol II complex than does wild-type EWS, as shown in this study and by others (22). It is also possible that high affinity binding to certain subunits of RNA Pol II by EWS-Fli-1 dominantly negatively affects the
recruitment of SR splicing factors by EWS to RNA Pol II, leading to disruption of normal splicing mediated by these EWS-associated SR proteins. The role of competition between EWS and EWS-Fli-1 will be investigated in future studies using inducible expression of EWS in Ewing’s sarcoma cell lines.

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