The Transcriptional Repressor ZFM1 Interacts with and Modulates the Ability of EWS to Activate Transcription*

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The ZFM1 protein is both a transcriptional repressor and identical to the splicing factor SF1. ZFM1 was shown to interact with and repress transcription from the glycine, glutamine, serine, and threonine-rich transcription activation domain of the sea urchin transcription factor, stage-specific activator protein (SSAP). EWS, a human protein involved in cellular transformation in Ewing's sarcoma tumors, contains an NH2-terminal transcriptional activation domain (NTD) which resembles that of SSAP in both amino acid composition and the ability to drive transcription to levels higher than VP16 in most cell types. Here we report that ZFM1 also interacts with EWS in both two-hybrid assays and glutathione S-transferase pull-down experiments. The region on EWS which interacts with ZFM1 maps to 37 amino acids within its NTD. Overexpression of ZFM1 in HepG2 cells represses the transactivation of reporter gene expression driven by Gal4-EWS-NTD fusion protein and this repression correlates with ZFM1 binding to EWS. Furthermore, two proteins, TLS and hTAF68, which have extensive homology to EWS, also interact with ZFM1. Recently, it was discovered that EWS/TLS/hTAF68 are each present in distinct TFIID populations and EWS and hTAF68 were also found to be associated with the RNA polymerase II holoenzyme. The association of ZFM1 with these proteins implies that one normal cellular function for ZFM1 may be to negatively modulate transcription of target genes coordinated by these cofactors.

In many human cancers, tumor-specific chromosomal rearrangements can create chimeric transcription factors with the ability to transform cells in which they are expressed (1). EWS/Fli-1 is such a fusion protein formed by (11;22) chromosomal translocation in Ewing’s sarcoma and related tumors (2). It consists of the amino-terminal 265 amino acids of EWS fused to the DNA-binding domain of Fli-1, a protein belonging to the eTS transcription factor family. The translocated portion of EWS, referred to as NTD, is rich in glycine, glutamine, tyrosine, and serine and, the fusion protein functions as a much more potent transcriptional activator than the wild type intact Fli-1 (3). Therefore, the EWS/Fli-1 chimera may function as a powerful transforming gene by deregulating Fli-1 target gene expression.

Although it was well known that the NTD of EWS is involved in the sarcoma formation, the function of native EWS protein is not understood. Recent studies on EWS, TLS, and hTAF68 demonstrated that these three proteins play roles in transcription initiation (4, 5). hTAF68 was initially isolated as a component of the TFIID complex and was also found in the preinitiation complex. TLS, which is involved in liposarcoma formation in a similar manner to EWS (6), was detected in a different and distinct subpopulation of TFIID than the one containing hTAF68. Moreover, EWS and hTAF68 can interact with several TAFs in the TFIID complex. These data suggest that EWS, TLS, and hTAF68 may function in transcription initiation.

Stage-specific activator protein (SSAP),1 an embryonic transcription factor in sea urchins, resembles EWS in structure (7). SSAP contains in its amino terminus two RNA recognition motifs as its DNA-binding domain (8). The transcriptional activation domain of SSAP consists of a central glycine and glutamine-rich domain and a COOH-terminal domain rich in serine and threonine (referred as GQC domain) (9). The GQC domain of SSAP shows a striking similarity to the NTD of EWS. Moreover, the GQC domain when fused to the Gal4-DNA-binding domain functions as a very strong transcription activation domain in mammalian cells (9). Its ability to drive high levels of transcription is comparable to the NTD of EWS.2

Recently, we isolated several human cDNAs encoding proteins that specifically interact with the GQC domain of SSAP by yeast two-hybrid screening (10). One of them encodes ZFM1, a protein initially discovered at the chromosomal locus linked to multiple endocrine neoplasia type 1 and subsequently identified as presplicing factor SF1 (11, 12). In addition, ZFM1 was independently isolated as one of eight genes activated during the process of p53-induced apoptosis (13). ZFM1 encodes a nuclear protein with a KH domain and a zinc knuckle motif which are implicated in binding nucleic acid and a long proline-rich region near the COOH terminus (12). Within this proline-rich region, ZFM1 shows significant homology to part of the Wilms’ tumor suppressor gene (WT1) (11). We have established that the interaction of ZFM1 with the transcription activation domain of SSAP results in transcriptional repression (10). Overexpression of ZFM1 protein in mammalian cells represses Gal4-GQC mediated transcription in a dose-dependent manner and this repression effect requires the GQC-interaction region on ZFM1. Moreover, when tethered to the TK promoter containing Gal4-binding sites, Gal4-ZFM1 fusion protein functions as an active repressor. Using this assay system, we were able to map the active repression domain of ZFM1 to its NH2-terminal 137 amino acids.

Considering the similarity between EWS and SSAP, we asked whether ZFM1 can interact with the NTD transcriptional activation domain of EWS and repress its transcriptional activity. In this paper, we established an interaction between EWS and ZFM1 in both two-hybrid interaction assays and GST pull-down experiments. The same region on ZFM1 which is re-

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1 The abbreviations used are: SSAP, stage-specific activator protein; GST, glutathione S-transferase; PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; PAGE, polyacrylamide gel electrophoresis.

2 D. Zhang, unpublished data.
The domain structure of EWS and the various EWS constructs used. The open reading frame of EWS constructs are represented by boxes and the numbers below the boxes refer to the amino acid sequence. The hatched region of the NH2-terminal 285 amino acids represents the glycine, glutamine, serine, and tyrosine-rich NTD of EWS. The RNA recognition motif (RRM) locates between the first two of the three shaded areas, which represent glycine-, arginine-, and proline-rich region.

**Fig. 1.** EWS interacts with ZFM1 in the yeast two-hybrid system. Various pBTM116-ZFM1 constructs and either pGAD GH-EWS-(10–656) or pGAD424 are co-transformed into L40 yeast strain in the combinations as listed in the left panel of the figure. The β-galactosidase activity of the yeast extract from the resulting transformations are shown in the right panel. The β-galactosidase value is an average plus standard error of at least three quantitation experiments for a given transformation tested.

**Fig. 2.** EWS interacts with ZFM1 in the yeast two-hybrid system. Various pBTM116-ZFM1 constructs and either pGAD GH-EWS-(10–656) or pGAD424 are co-transformed into L40 yeast strain in the combinations as listed in the left panel of the figure. The β-galactosidase activity of the yeast extract from the resulting transformations are shown in the right panel. The β-galactosidase value is an average plus standard error of at least three quantitation experiments for a given transformation tested.
EWS Interacts with Transcriptional Repressor ZFM1

**RESULTS**

ZFM1 Interacts with EWS in the Yeast Two-hybrid Assay—We have used the yeast two-hybrid system to screen cDNA libraries derived from HeLa cells and HL-60 cells for proteins that interact with the GQC transcriptional activation domain of SSAP (10).3 We isolated three cDNAs encoding different overlapping segments of the EWS protein from the HeLa cell library. The three EWS cDNAs include parts of the protein between amino acids 10 and 656, 188 and 656, and 228 and 656, respectively, fused in-frame with the Gal4 activation domain in the two-hybrid cloning vector pGAD GH (Fig. 1). The functional significance of this interaction between EWS and the GQC transcription activation domain is not clear. However, given the fact that SSAP forms homodimers and EWS is highly related to SSAP in amino acid composition, we assume that SSAP can form a heterodimer with the EWS protein.

Considering the similarity between the GQC domain and the NTD of EWS, we asked if ZFM1 was capable of interacting with EWS. To test this idea, we co-transformed pGAD GH-EWS-(10–656), which contains a nearly full-length EWS into the pCR3.1 mammalian vector (Invitrogen). Mammalian cell culture, transfection, and CAT assays were conducted according to Ref. 10.

To confirm the specificity of this interaction, we also co-transformed pBTM116-ZFM1-A with empty pGAD424 vector. Predictably, we observed a 30-fold less β-galactosidase activity in this yeast strain (Fig. 2). We also tested the ability of other ZFM1 constructs to interact with EWS using the yeast two-hybrid assay. ZFM1-A is a splicing isoform with the same NH2-terminal half as ZFM1-E but containing a longer proline-rich segment and a different COOH-terminal tail. When cloned in pBTM116 vector, ZFM1-A can also interact with EWS-(10–656) (Fig. 2). We previously showed that amino acids 321 to 484 of ZFM1 mediates its interaction with the GQC transcriptional activation domain. This same region of ZFM1 can interact with EWS-(10–656) as well. On the other hand, ZFM1-(1–320), which lacks the region responsible for interaction with the GQC domain, fails to interact with EWS-(10–656) in the two-hybrid assay (Fig. 2). These data suggest that the same region on ZFM1 which binds to the GQC transcription activation domain of SSAP is responsible for interacting with EWS.

The Region on EWS Which Interacts with ZFM1 Maps to 37 Amino Acids within Its NTD—We further refined the region on EWS that mediates its interaction with ZFM1 by making several additional EWS constructs. We co-transformed pBTM116-ZFM1-E with two-hybrid cloning vector containing different regions of EWS fused in-frame with the Gal4 activation domain. In addition to EWS-(10–656), we found that ZFM1-E interacts with EWS-(188–656) and EWS-(228–656) (Fig. 3). These EWS constructs were isolated from the HeLa cell cDNA library in our original screen using the GQC domain as bait. The common region of all these three constructs spans amino acids 228 and 656 of EWS, which encode part of the NTD and the whole COOH-terminal part of EWS. To determine whether the NH2-terminal or the COOH-terminal domain of EWS mediates this interaction, we put them separately in pGAD424 vector (Fig. 1). We observed that the NH2-terminal domain but not the COOH-terminal region of EWS mediates its interaction with ZFM1-E (Fig. 3). The region shared by all these constructs that interact with ZFM1-E is 37 amino acids between 228 and 264 of EWS. It was previously demonstrated that the NTD of

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3 D. Zhang and G. Childs, unpublished experiments.
EWS can be functionally subdivided into two subdomains (14). Domain A, which consists of amino acids 1 to 82, mediates nearly all the transformation activity of EWS but has little transactivation activity while domain B, which consists of amino acids 83 to 265, contains nearly all the transactivation activity of EWS but transforms cells less efficiently. The region on EWS which interacts with ZFM1 falls within this B domain. This implies that ZFM1 may modulate the transactivation activity of EWS-NTD.

**ZFM1 Interacts with the NTD of EWS in Vitro**—To confirm our observations in the yeast two-hybrid assays, we tested the in vitro association between ZFM1 and EWS by GST pull-down assays. We fused the EWS-NTD with glutathione S-transferase and expressed the GST-EWS-NTD chimeric protein in bacteria. The majority of GST-EWS-NTD protein is insoluble, however, the soluble fraction contains some fusion protein that can be coupled to the glutathione-Sepharose beads. ZFM1-A, ZFM1-E, and ZFM1-(1–320) were in vitro translated and incubated with glutathione-Sepharose beads coupled with either GST-EWS-NTD or GST alone. After extensive washing, we found that both ZFM1-A and ZFM1-E were retained on the beads coupled with GST-EWS-NTD but not with GST alone. As a further control for the specificity of this interaction, ZFM1-(1–320), which lacks the region responsible for binding to EWS, fails to interact (Fig. 4). These results confirmed the two-hybrid data showing that both ZFM1-A and ZFM1-E isoforms interact with EWS.

**Overexpression of ZFM1-A in HepG2 Cells Represses the Transactivation of Reporter Genes Driven by Gal4-EWS-NTD**—Previously we demonstrated that overexpression of ZFM1-A protein in HepG2 cells represses Gal4-GQC-mediated transcription in a dose-dependent manner (10). Moreover, the NH$_2$-terminal 137 amino acids of ZFM1 when fused with the Gal4 DNA-binding domain represses thymidine kinase promoter driven transcription when directly tethered to the reporter gene containing Gal4-binding sites.

To assess the significance of the interaction between ZFM1 and EWS, we asked whether overexpression of ZFM1-A modulates the transcriptional activity of the Gal4-EWS-NTD fusion protein. When pCR3.1-ZFM1-A was co-transfected into HepG2 cells, the levels of expression of tagged ZFM1-A correlate well with the amounts of DNA transfected (Fig. 5A). We tested an EWS construct in which nearly all of the EWS-NTD and a small portion of the Fli-1 COOH-terminal domain from EWS/Fli-1 chimeric protein are fused to the Gal4 DNA-binding domain in pSG424 (Gal4-EWS-NTD). This same Gal4-EWS-NTD fusion protein functions as a potent transcriptional activator in mammalian cells (3). When increasing amounts of pCR3.1-ZFM1-A were co-transfected into HepG2 cells along with pSG424-EWS-NTD and reporter G5E1BCAT, we observed that the reporter expression is repressed by ZFM1-A in a dose-dependent manner (Fig. 5A). At the highest levels of ZFM1-A transfected, transcription driven by Gal4-EWS-NTD is repressed by 4–5-fold.

**The Repression Effect of ZFM1 on EWS-NTD-mediated Transactivation Depends on Its Binding to EWS**—We have
shown that the same region of ZFM1 is capable of interacting with either the SSAP GQC domain or the EWS NTD domain. Overexpression of a ZFM1 mutant which lacks this interaction region (ZFM1-(1–320)) in HepG2 cells fails to repress Gal4-GQC mediated transactivation (10). To test whether the same interaction region on ZFM1 is also critical to mediate the repression effect of ZFM1 on transactivation of reporter gene driven by Gal4-EWS-NTD, we co-transfected HepG2 cells with pSG424-EWS-NTD, G5E1BCAT, and increasing amounts of pCR3.1-ZFM1-(1–320). Western blot showed that the expression of ZFM1-(1–320) correlates to the amount of transfected DNA (Fig. 6B). As expected, we do not observe any repression by expression of this ZFM1 mutant (Fig. 6A). This implies that ZFM1 represses EWS-NTD-mediated transactivation and Gal4-GQC mediated transcription by the same mechanism. The association between EWS and ZFM1 brings ZFM1 to the proximity of the promoter region where its repression domain may function.

ZFM1 Interacts with hTAFII68 and TLS in Vitro—hTAFII68 and TLS are two proteins which share extensive homology to EWS over its full-length (4, 6). The identification of an interaction between EWS and ZFM1 raises the possibility that ZFM1 may interact with hTAFII68 and TLS as well. To test this possibility, GST fusion proteins with hTAFII68-(1–198) and TLS-(1–286), respectively, were made and their interactions with in vitro translated various forms of ZFM1 were tested in similar experiments as that with GST-EWS-NTD. We found that both hTAFII68-(1–198) and TLS-(1–286), like EWS-NTD, interact specifically with ZFM1-A and ZFM1-E, but not with ZFM1-(1–320) (Fig. 7). While we observed that a significant fraction of input ZFM1 protein can be bound to the beads coupled with GST-EWS-NTD and GST-hTAFII68-(1–198), under the same conditions, significantly less ZFM1 is bound to the beads coupled with GST-TLS-(1–286). This implies that TLS may bind to ZFM1 with lower affinity.
DISCUSSION

Human EWS protein contains an NH₂-terminal domain rich in glycine, glutamine, tyrosine, and serine and a COOH-terminal region with a single RNA recognition motif (2). In this paper, we showed that ZFM1 physically interacts with the NH₂-terminal but not COOH-terminal region of EWS. The evidence for their interaction come from both two-hybrid interaction assays and in vitro GST pull-down experiments. Similarly, we observed specific interaction between ZFM1 and hTAF₁₀₈ and TLS in GST pull-down assays. These interactions are mediated through a region within their NTDs as well. The NTD of EWS can function as a potent transcriptional activation domain when fused to the COOH terminus of FlI-1 protein by chromosomal translocation in Ewing’s sarcoma (3). We have observed that overexpression of ZFM1 in HepG2 cells down-regulates the transactivation ability of Gal4-EWS-NTD in a dose-dependent manner. Similarly, we speculate that ZFM1 may inhibit transcription driven by the NTDs of TLS and hTAF₁₀₈ as well.

In Ewing’s sarcoma, EWS/FlI-1 fusion protein can function as a powerful transforming gene (15). Although the mechanism used to transform cells is not entirely clear, it is widely believed that the aberrant transcription activity of the NTD of EWS may deregulate a set of FlI-1 target genes. Our observations that ZFM1 represses EWS-NTD function suggests that ZFM1 may modulate EWS/FlI-1 target gene expression. However, the concentration of ZFM1 in the cell is certainly not high enough to prevent transformation and this unusual situation does not address any potential biological function for this interaction in normal cells. Using representational difference analysis, it was found that the expression of eight genes are up-regulated when NIH3T3 cells are transformed by EWS/FlI-1, including EAT-2 and the manic fringe gene (MFNG) (16). EAT-2 is a novel SH2 domain containing protein (17). MFNG is a member of the Fringe gene family instrumental in somatic development. Overexpression of MFNG in NIH3T3 cells renders them tumorigenic in mice with severe combined immunodeficiency disease (18). It will be interesting to examine whether overexpression of ZFM1 can affect the expression of these target genes.

Although the NTD of EWS acts as a transcriptional activation domain in Ewing’s sarcoma, native EWS protein does not function as a sequence-specific transcriptional activator. Recent studies indicate that EWS, along with its family members, TLS and hTAF₁₀₈, may function in transcription initiation as TAFs in different distinct subpopulations of the TFIID complex (4, 5). However, they may not function as core TAFs because hTAF₁₀₈ is present in substoichiometric amounts compared with TBP and the core TAFs. In addition, hTAF₁₀₈ and TLS were found in two chromatographically separable subpopulations of TFIID complexes. Therefore, these proteins may function as specific TAFs which could coordinate transcription from distinct promoters. In addition to EWS, TLS and hTAF₁₀₈, hTAF₂₀₃ and hTAF₁₀₅ have also been shown to be associated with distinct subpopulations of TFIID (19, 20). hTAF₁₀₅ was shown to be B-cell specific while hTAF₂₀₃ was identified within a distinct subpopulation of TFIID required for transcriptional activation by the estrogen receptor. Moreover, it was observed that additional TFIID subpopulations mediate transcriptional stimulation by distinct activators (21). Our findings of associations between ZFM1 and EWS/hTAF₁₀₈/TLS imply that ZFM1 may specifically inhibit the function of these distinct TFIID complexes. Consequently, the transcription driven by those activators whose activities are coordinated by either of these three TAFs are likely to be modulated by ZFM1 function. Currently, this model is not testable because no such activators have been firmly identified. The tight association observed between several nuclear hormone receptors and TLS suggests that receptor-activated target genes may be ZFM1 targets (22).

In addition to playing a role in transcription initiation, EWS/hTAF₁₀₈/TLS may serve additional roles in mRNA metabolism. This was initially implied by the presence of an RNA recognition motif in the COOH-terminal region of each of these three proteins and their abilities to bind RNA substrates (23, 24). Further observations that TLS is identical to hnRNP P2 and may engage in nucleo-cytoplasmic shuttling suggest a role for TLS in the packaging of newly transcribed mRNAs (24, 25). Moreover, it was observed that hTAF₁₀₈ and EWS copurify with RNA polymerase II (4, 5). These proteins directly interact with several subunits of the RNA polymerase II. These observations indicate that EWS and hTAF₁₀₈ may also function in the process of transcription elongation. In addition to its role as a repressor of transcription, ZFM1 was independently isolated as a presplicing factor SF1. SF1 is one of several proteins that is required for the formation of a presplicing complex in the early stage of the splicing of nuclear pre-mRNA (12, 26–28). The interactions between ZFM1 and EWS, TLS and hTAF₁₀₈ suggest that these proteins may also have dual functions. In addition to their roles in transcription, they could cooperate with ZFM1 in mRNA processing during transcription elongation.

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