Transcriptional Activation by the Ewing’s Sarcoma (EWS) Oncogene Can Be Cis-repressed by the EWS RNA-binding Domain*

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The Ewing’s sarcoma (EWS) oncogene contains an N-terminal transcriptional activation domain (EWS activation domain, EAD) and a C-terminal RNA-binding domain (RBD) (Fig. 1). Knowledge of EWS is mostly derived from studies of a group of dominant oncogenes (EWS fusion proteins, EFPs) that arise due to chromosomal translocations in which EWS (or the related TLS/FUS gene) is fused to a variety of cellular transcription factors (reviewed in Refs. 1–3). EFPs are very potent transcriptional activators (4–9) dependent on the EAD and a C-terminal RNA-binding domain contributed by the fusion partner. The spectrum of malignancies associated with EFPs are thought to arise via EFP-induced transcriptional deregulation, with the tumor phenotype specified by the EWS fusion partner and cell type.

Studies of EFPs have provided insights into the mechanism of trans-activation by the EAD (4, 5, 9–12). EWS/ATF1 (the EFP that causes malignant melanoma of soft parts (13), see Fig. 1) is a potent constitutive activator of ATF-dependent promoters (4, 5, 12). Trans-activation requires both the EAD (4, 12) and the DNA-binding domain (bZIP domain) of ATF1 (5, 12). The EAD acts directly in the transcription complex (10, 12) and contains multiple dispersed elements that cooperate synergistically (10, 12). In the case of EWS/ATF1, the N-terminal region (residues 1–86) of the EAD plays a relatively important role in trans-activation (4, 12). Significantly, this region of the EAD binds directly to the RNA polymerase II subunit hsRPB7 (Fig. 1), and this interaction has been proposed to be important for trans-activation (14).

In contrast to understanding of EFPs, the normal function of EWS remains poorly characterized. EWS together with the related genes TLS/FUS and hTAFII68 encode a sub-group (the TET family (15)) within the RNP family of RNA-binding proteins that are probably involved in several aspects of RNA biogenesis and function (16). TET proteins (TETs) bind to RNA (17, 18) but sequence-specific RNA binding by TETs has yet to be demonstrated, and the relatively high abundance of EWS and TLS suggests that TETs might interact with many RNA targets (3). TLS rapidly shuttles from the cytoplasm to the nucleus suggesting a role in RNA transport (18, 19). Other evidence for a cytoplasmic role for TETs is that EWS interacts with a protein tyrosine kinase (Pyk2) and relocates from the cytosol to ribosomes upon Pyk2 activation (20).

The evidence that TETs are involved in transcription is, for the most part, indirect or circumstantial. First, as mentioned above, the EAD functions as a potent activation domain in EFPs (4–9). Second, a TBP-associated factor (hTAFII68) (15) is a member of the TET family. Third, TETs directly interact with several transcription factors including components of the general transcriptional machinery (14, 15, 21), TAFs (21) and activator proteins (22, 23). TETs are present in sub-stoichiometric amounts in TFIID complexes (15), and different TETs are present in distinct TFIID subpopulations (15) indicating that TETs are not general transcription factors. A major question concerns the potential role of the RBD in transcription. EWS binds to the polymerase II subunit hsRPB3 but not to hsRPB5 or hsRPB7, whereas the isolated EAD binds hsRPB5 and hsRPB7 but not hsRPB3 (21). Thus the RBD might play a pivotal role in differentiating the transcriptional properties of EFPs and EWS. It is also significant that EFPs, without exception, lack the RBD, strongly suggesting that loss of the RBD is necessary for EFP-induced oncogenesis and that, in turn, the RBD may block trans-activation by the EAD.

Here we describe a functional approach for elucidating the role of the RBD in transcription. We show that a cis-linked RBD can strongly and specifically repress trans-activation by the EAD. Further studies of the repression phenomenon should help to illuminate the key molecular interactions that distinguish EFPs and EWS/TETs and provide insights into the normal cellular function of EWS/TETs.

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The abbreviations used are: EWS, Ewing’s sarcoma oncogene; EAD, EWS activation domain; RBD, RNA-binding domain; EFPs, EWS fusion proteins; ATF, activating transcription factor; RRM, RNA recognition motif; CAT, chloramphenicol acetyltransferase; hnRNP, heterogeneous nuclear ribonucleoprotein; PKA, protein kinase A.

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**Fig. 1. Functional regions of EWS, ATF1, and EWS/ATF1.** EWS contains an N-terminal transcriptional activation domain (EAD) and a C-terminal RNA-binding domain (RBD). Residues 1–82 of the EAD (R78B) bind to the RNA polymerase II subunit hRPB7 (14), residues 228–264 (ZFM1) bind to the transcriptional repressor/splicing protein ZFMSF1 (32), and residues 258–280 (IQ) contain an IQ domain involved in calmodulin binding and protein kinase C phosphorylation (33). The RBD contains two elements (RRM and RGG boxes) commonly found in RNA-binding proteins (31) and a C2-C2 putative zinc finger (54). The RRM motif consists of ~100 residues with a conserved three-dimensional structure (33) and three RGG-rich boxes (RGG1, RGG2, and RGG3) containing 5, 4, and 12 tri-peptide RGG motifs, respectively. ATF1 is a PKA-inducible activator (27, 55). The bZIP domain (amino acids 214–271) mediates dimerization and DNA binding and consists of the basic region (55) that directly contacts DNA and the leucine zipper (ZIP) that allows dimerization. Q2 represents a glutamine-rich constitutive activation domain (56), and PKA represents the kinase-inducible domain (55) including a single PKA-phosphoacceptor site. The NTR is the N-terminal 30 residues of ATF1 that diverge from CREB (55, 57). EWS/ATF1 is an oncoprotein that is associated with malignant melanoma 453 cell parts (13). The chromosomal cross-over point that produces EWS/ATF1 is shown with a X resulting in the EAD fused to the C-terminal region of ATF1 (residues 66–271). EWS/ATF1 lacks the PKA-phosphoacceptor site of ATF1 and functions as a potent constitutive activator of ATF1-dependent promoters (4, 5, 12) dependent on the EAD and the bZIP domain of ATF1.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Constructions—** pΔ1–71/SomCAT contains the somatostatin promoter to position –71, fused to the chloramphenicol acetyltransferase (CAT)-coding sequences (24). pVIP25CAT, pVIP4CAT (4), pCAT-BstN1 (25), and pG1E4TCAT (26) are as described previously.

pRM1 and pRM2 were obtained by digestion of pRM6 (EWS residues 326–345) with XhoI and in-frame religation. pRMO contains RGG1 and (EWS residues 346–656) and insertion of an oligonucleotide to recreate the reading frame and delete EWS residues 558–623. pRM5 lacks RGG3 and was obtained by digestion of pNC with XbaI and insertion of an oligonucleotide to recreate the reading frame. pSVERZA was obtained by insertion of an HindIII sticky/BglII-blunted fragment from pNC (EWS residues 1–656) into pSVZATF1 (27) digested with HindIII (sticky) and SacI blunt. pSVERZA expresses a protein containing intact EWS sequence, all of ATF1 except residues 21–27 and the ZbZIP domain, and the bZIP domain (ZbZIP) from the Zta bZIP protein. pSVEZA was containing from pSVERZA by ApaI digestion. BamHI partial digestion, blunting, and religation. pSVEZA expresses a protein corresponding to SVERZA but lacking the RBD (EWS residues 246–623). pSVEZA was obtained by inserting an XcmI/XbaI fragment from pERZA into XcmI/XbaI-digested pΔ287. pSVEZA expresses a protein similar to pΔ287 with the ATF1 bZIP domain replaced by the Zta bZIP domain. PMc is as described previously (28). pSVG4vec was previously obtained from the Gal4 DNA-binding domain (residues 1–147) fused to the EAD (residues 1–245). pSVG4vec was obtained by inserting a SalI/BamHI-ended oligonucleotide containing a multiple cloning site into pSVG4/245 digested with SalI/BamHI. pG4NC was obtained by cloning a SalI/BglII fragment from pNC into pSVG4vec digested with SalI and BglII. pG4NC expresses a protein containing the Gal4 DNA-binding domain at the N terminus and the entire EWS sequence at the C terminus.

**Transfections, CAT Assays, and Western Blotting—** JEG-3 cells were grown in Dulbecco’s modification of Eagle’s medium containing 10% fetal calf serum. Transfections (12), CAT assays (29), and Western blotting (4) were carried out as described previously. For quantitation, percent conversion of unacetylated to acetylated [14C]chloramphenicol was determined by excision of spots from the TLC plate and quantitation of radioactivity using a liquid scintillation counter.

**Nuclear Extracts and Affinity Purification—** Preparation of nuclear extracts and sequence-specific DNA-affinity purification were carried out as described previously (30).

**RESULTS**

**Effect of the RBD on Trans-activation—** To test the effect of a cis-linked RBD on trans-activation (Fig. 2) by EWS/ATF1, we used a previously described transient assay (4, 12). An expression vector for the test protein and an ATF-dependent reporter (pΔ1–71/SomCAT) were introduced into JEG-3 cells, and trans-activation was monitored by CAT assay (Fig. 2B). A protein called ∆287C (Fig. 2A), which closely resembles EWS/ATF1, strongly activates transcription as previously shown (4, 12), and a protein called NC containing the RBD (Fig. 2A) has much reduced activity (1.4% of ∆287C or 70-fold repression). ∆287C and NC are expressed at similar levels as shown by Western blot analysis of epitope-tagged proteins (Fig. 2B). To verify that repression is due to a cis-effect of the RBD, we co-expressed ∆287C and a protein (G4NC, Fig. 2A) in which the ATF1 portion of NC is replaced by the Gal4 DNA-binding domain (Fig. 2B). G4NC is unable to bind to the ATF reporter and has no effect on trans-activation by ∆287C. We conclude that the RBD can strongly repress the EAD but only when linked in cis.

We tested several ATF-dependent promoters and all were sensitive to repression (Fig. 2C), although repression varied from 7-fold for ESICAT to 70-fold for ∆1–71/SomCAT. To ask whether the ATF1 portion of EWS/ATF1 plays a direct role in repression, we replaced ATF1 with the DNA-binding domain of Gal4 (Fig. 2A) and tested for repression using a Gal4 reporter (Fig. 2D). As previously shown (10, 11) G4/245 gives high levels of trans-activation, and inclusion of the RBD (G4NC) effectively represses trans-activation (41-fold repression). We conclude that the ATF1 portion of NC has no direct role in repression and that a specific DNA-binding domain is not required.

**Elements within EWS Required for Repression—** To determine the requirements for repression, we performed deletion analysis of the RBD (Fig. 3A). The RBD contains a number of structural features (notably the RRM and RGG boxes) that are characteristic of other RNA-binding proteins (Ref. 31, see Fig. 1). The RRM together with the RGG boxes cover the entire RBD except for a single C2/C2 zinc finger (Fig. 1). Deletion of the zinc finger (RM6) has no effect on repression indicating that the known RBD elements are sufficient for repression. In
addition, deletion of the RRM (RM4) also has no effect, and addition of the RRM in the presence of RGG1 (compare RM1 with RMD) has only a minimal repressive effect (~2-fold repression). The above results demonstrate that the zinc finger and the RRM play no obvious role in repression. In contrast, deletion of RGG1 alone (RM3) reduces repression slightly (~5-fold more active than NC) and deletion of RGG3 alone (RM5) results in substantial loss of repression (17-fold more active than NC). Deletion of residues 345–545 (RM2) also results in significant loss of repression (~20-fold more active than NC). Since this latter region contains RGG2 and only two other elements (RRM and the zinc finger) that are not required for repression, the RGG2 region may also contribute to repression. However, RM2 suffers a large deletion (~200 amino acids), and loss of repression might be explained by dislocation of RGG3 relative to the EAD. In summary, the above results indicate that regions RGG1–3 are necessary and sufficient for repression.

Three elements within the EAD (binding sites for hsRPB7 (14), ZFM1/SF1 (32), and the IQ domain (33), see Fig. 1) might be required for repression, and we tested previously characterized mutants lacking these elements (4, 12) for sensitivity to repression (Fig. 3B). Results are presented as fold repression (~RBD/+RBD). For the CAT assay shown, different amounts of extract were used for each pair of proteins, and levels of trans-activation in the absence of the RBD are not the same (4, 12). C-terminal deletion of the EAD leaving residues 1–167 (compare Δ167C with 167R or residues 1–87 (compare Δ87C with 87R) has a small effect (5-fold less repression) or no obvious effect on repression, respectively. The reason that 167R is somewhat less susceptible to repression than 87R is not clear. However, the strong repression observed for 87R indicates that both the IQ domain and the ZFM1-binding site are not critical for repression but that the hsRPB7 binding region alone is sensitive to repression. In contrast, removal of the hsRPB7 binding region almost completely abolishes repression (compare Δ78 with 78R). Although the activity of both Δ78 and Δ87C is ~20-fold lower than that of EWS/ATF1 (4, 12), this residual activity is, nonetheless, highly significant (~100-fold activation). A protein containing a duplicated hsRPB7-binding site (Δ87CD) is a very potent activator (the activity of Δ87CD is only ~3-fold lower than EWS/ATF1 (12)) and is still efficiently repressed by the RBD (compare Δ87CD with 87RΔ). Analysis of the above deletion mutants demonstrates that the region of the EAD coincident with the hsRPB7-binding site is highly sensitive to repression.

Mechanism of Repression—To gain insight into the mechanism of repression, we examined the effect of the RBD on nuclear accumulation (Fig. 4A). Cells were transfected with pΔ87C (~RBD, see Fig. 3B) and p87R (+RBD) followed by Western blot analysis of whole cell (T) and nuclear extracts (N). The proportion of Δ87C and 87R proteins present in the nuclear fraction is comparable. We next tested in vitro DNA binding by Δ87C and 87R present in nuclear extracts from transfected cells. Δ87C and 87R proteins were purified using an ATF-DNA affinity resin as described previously (4) and were detected by Western blotting (Fig. 4A). Δ87C and 87R bind specifically to the ATF-DNA affinity resin as indicated by competition by an oligonucleotide containing a consensus ATF1-binding site (4), and the amount of binding is comparable for Δ87C and 87R. Together, the above results show that the RBD has no effect on nuclear accumulation or DNA binding in vitro.

The above findings suggested that RBD-mediated repression might occur directly at the promoter. To test this possibility and, at the same time, to ask whether the RBD can repress another activator, we examined the properties of a novel hybrid protein (ERZA). ERZA essentially contains intact EWS and intact ATF1 and therefore has the potential to act as a constitutive activator (via the EAD) or as a PKA-inducible activator (via ATF1). We could not use native ATF1 for this experiment because endogenous ATF1/CREB activate ATF-dependent reporters (24), and to circumvent this, we used a previously described bZIP swap approach (27) employing the heterologous bZIP domain of the Zta protein (ZbZIP) together with a reporter containing Zta-binding sites. ZATF1 corresponds to ATF1 except with the bZIP domain of Zta and activates the Zta reporter in a PKA-inducible manner (Fig. 4B) as previously shown (27). Significantly, ERZA behaves like ZATF1, giving a low basal...
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**DISCUSSION**

Mechanism of Repression—We have established several characteristics of repression as described under “Results.” A major issue concerns whether binding of the RBD to nascent mRNA transcripts might be involved in repression. Although the RRM is dispensable for repression, the RGG3 region is important, and this region is sufficient for binding to poly(G) and poly(U) substrates in vitro (17). Similarly, the RGG boxes of TLS (8) and the Fmrp protein (35) may be sufficient for ribopolymer binding and poly(G) binding, respectively. Thus, we cannot completely exclude a role for RNA binding in repression. However, we feel that such a role is unlikely for two reasons. Lack of involvement of the RRM indicates that high affinity RNA binding is not involved in repression (31), and selective repression of the EAD demonstrates that the RBD does not act via a transcript-dependent shut down of the promoter.

Considering the above, we favor a model whereby repression results from the RBD directly interfering with trans-activation by the EAD. This view is also prompted by several other indications that the EAD and RBD functionally interact. First, for both TLS (8) and EWS (17) the EAD alters the RNA binding specificity of the RBD in vitro. Second, phosphorylation of the IQ domain of the EAD inhibits RNA binding by EWS (33). Third, the RBD prevents interaction of the EAD with at least two transcriptional components (hsRPB7 and hsRPB8) (21), and it has been suggested that the ability to bind hsRPB7 is required for EAD-mediated trans-activation (14). In light of the above and our finding that the N-terminal region of the EAD (residues 1–86) is highly sensitive to repression and binds to hsRPB7 (14), we propose that repression results from steric hindrance of EAD binding to RPB7. This might be achieved by direct contacts between the RBD and the EAD or via additional factors that participate in a ternary complex. Further analysis using the repression assay to correlate more precisely hsRPB7/residues remaining. The N-terminal hsRPB7-binding site (R7BS), ZFM1/IQ domains, and the RBD are shown. Plasmids and proteins containing the RBD are denoted R in the nomenclature. For each pair of proteins (containing the same region of the EAD in the presence or absence of the RBD) the fold repression is shown to the right. For the representative CAT assay different amounts of extract were used for each pair of proteins, and levels of trans-activation in the absence of the RBD are not the same (4, 12).
EAD binding with trans-activation will enable a rigorous test of the role of hsRPB7 in trans-activation by EFPs.

Normal EWS/TET Function—Although it is clear that EFPs are transcription factors, it has not been definitively established that EWS/TETs normally function in transcription. Since transcriptional activation domains can be generated at a surprisingly high frequency by fusion of random sequences to a DNA-binding domain (36), it remains possible that the normal function of EWS has nothing to do with transcription. In this event, the effect of the RBD that we score as cis-repression of EFS might normally be to prevent aberrant association of EWS (via the EAD) with transcriptional components. The presence of TETs in polymerase II complexes is not inconsistent with this suggestion (15, 21) because this characteristic might reflect a role for TETs in coupling transcription with RNA processing or transport rather than transcription, as previously suggested (3).

If EWS does function in transcription then how does it work? The finding that the RBD can antagonize the EAD on a broad range of promoters or (viewed another way) that “promoter-bound” EWS does not activate transcription suggests that EWS might normally function as a repressor. In this regard, it is pertinent that several proteins that share characteristics with EWS act as transcriptional repressors. Examples include the yeast protein Nrd1 (37), hnRNP-U (38), and NELF (39) all of which have an RNA-binding component and repress transcriptional elongation. We cannot exclude that lack of activation by promoter-bound EWS might reflect “imprisoning” of EWS in the promoter region thereby preventing a positive role in elongation, but previous findings for other activators suggest that this is unlikely. Specifically, tethering a normally RNA-dependent activator (TAT) (40) or components of polymerase II (41) to the promoter via a DNA-binding domain or, alternatively, recruiting a normally promoter-bound activator via an RNA ligand (42) in both cases preserves the capacity to activate transcription. The flexibility observed for other activators suggests that the inability of promoter-bound EWS to activate transcription means that EWS is normally not an activator. In this case EWS would differ from some other hnRNPs proteins (including hnRNP D (43), hnRNP K (44), and hnRNP DOB (45)) that play positive roles in transcription.

Our findings are of significance for evaluating the possible effects of recruitment of TETs to the promoter via interactions with other transcription factors. Such interactions have recently been identified, including high affinity binding of TLS to nuclear hormone receptors (23). In this latter case the functional consequences have not been determined, but our experiments with EWS suggest that recruitment of TETs to the promoter is unlikely to play a role in transcriptional activation.

Repression and Oncogenesis—Our finding that a cis-linked RBD can repress trans-activation by the EAD and the fact that oncogenic EFSs never contain the RBD strongly supports the role of trans-activation in oncogenesis. Furthermore, our results provide a rationale, in molecular terms, for obligatory loss of the RBD during oncogenesis. Although the fusion proteins that we have studied are contrived, it is possible (and even likely) that such proteins naturally occur due to somatic mutation but escape discovery because they are not oncogenic. For example, a fusion protein containing EWS exons 1–16 (including the entire RBD) fused to ATF1 (residues 66–271) would be in frame (34) and almost identical to the NC protein. Thus the proteins that we have characterized correspond to alternative EWS/ATF1 hybrids that can contribute to understanding of the molecular mechanism of oncogenesis.

We have shown that repression can occur on a variety of promoters and in the context of a heterologous DNA-binding domain (Gal4) suggesting that repression is likely to be operative for all EFSs. The recent finding that promoter-bound hTAF68 does not activate transcription (46) supports this suggestion. To date there is no transformation assay for EWS/ATF1, but for hTAF68 inclusion of C-terminal sequences (including a major part of the RBD) blocks both trans-activation and transformation (46). It will be of interest to test the effect of the RBD using the established transformation assays for other EFSs (47–49). The work of several groups has revealed that EFSs are involved in tumor maintenance (50–53), raising the possibility that EFS inhibitors will have therapeutic potential. The absolute tumor specificity of EFSs together with the clear functional distinctions between EFSs and TETs further suggests that EFSs may be attractive therapeutic targets. Our results suggest that the RGG boxes (and hence possibly the tri-peptide RGG motif) might represent potent and selective EAD inhibitors. By using the repression assay, it will be of interest to test this possibility further with a view to creating RGG-related compounds that might serve as useful leads for drug development.

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