The EWS gene when fused to transcription factors such as the ETS family ATF-1, Wilms tumor-1, and nuclear orphan receptors upon chromosomal translocation is thought to contribute the development of Ewing sarcoma and several malignant tumors. Although EWS is predicted to be an RNA-binding protein, an inherent EWS nuclear function has not yet been elucidated. In this study, we found that EWS associates with a transcriptional co-activator CREB-binding protein (CBP) and the hypophosphorylated RNA polymerase II, which are included preferentially in the transcription preinitiation complex. These interactions suggest the potential involvement of EWS in gene transcription, leading to the hypothesis that EWS may function as a co-activator of CBP-dependent transcription factors. Based on this hypothesis, we investigated the effect of EWS on the activation of nuclear receptors that are activated by CBP. Of nuclear receptors examined, hepatocyte nuclear factor 4-dependent transcription was selectively enhanced by EWS but not by an EWS mutant defective for CBP binding. These results suggest that EWS as a co-activator requires CBP for hepatocyte nuclear factor 4-mediated transcriptional activation.

The EWS gene was originally identified in Ewing's sarcoma with the t(11,22) chromosomal translocation where it is fused to the ETS transcription factor Flt-1 gene (1). Subsequent studies indicated that other ETS transcription factor genes fuse to the EWS gene and produce fusion proteins in Ewing's sarcoma. In addition to the fusion with EWS transcription factors in Ewing's sarcoma, the EWS gene has been shown to form fusion proteins with ATF-1 in malignant melanoma of soft parts, WT-1 in desmoplastic small round cell tumors, and nuclear orphan receptors in myxoid chondrosarcomas (2). In EWS fusion proteins, the N-terminal domain (NTD) of EWS is retained, whereas the C-terminal domain of EWS is replaced by corresponding fusion partners. However, the roles of EWS in normal cellular functions and the mechanisms whereby EWS fusion proteins lead to these malignant tumors remain unclear.

EWS contains the transcriptional activation domain in its NTD and the RNA-recognition motif and arginine-glycine-glycine repeats (RGG), both of which are found in RNA-binding proteins (3) in its C-terminal domain. Transcriptional and post-transcriptional processing are closely coupled events in vivo, and based on its structural features, it is likely that EWS participates in RNA transcription and mRNA synthesis. Furthermore, the NTD of EWS associates with the basal transcription factor TFIIID (5) and with certain subunits of the RNA polymerase II complex (5, 6). On the other hand, EWS interacts with the splicing factors SF1 (7), U1C (8), TAF-1/TTF2-2 translocation liposarcoma protein-associated serine-arginine protein (9), and Y-box protein-1 (10). A current expectation is that EWS may act as an adaptor molecule linking between gene transcription and mRNA processing by interacting with RNA polymerase II and the splicing factors (2).

Interestingly, in this study, we indicated the interaction of EWS with the transcriptional co-activator CBP and the hypophosphorylated RNA polymerase II. CBP enhances many DNA-binding transcriptional activator proteins including nuclear receptors and other signal-regulated activators by its histone acetyltransferase activity and recruitment of RNA polymerase II-dependent basal transcription complex or other cofactors to target gene promoters (11, 12). Therefore, these identified interactions suggest the potential involvement of EWS in gene transcriptional activation. However, the fact that EWS does not have any significant DNA-binding motifs or binding activity to specific gene promoters prompted us to propose the hypothesis that EWS may function as a co-activator of CBP-dependent DNA-binding transcription factors. Based on this hypothesis, we investigated the possible effects of EWS on transcription mediated by nuclear receptors. We showed that EWS selectively enhances the nuclear receptor hepatocyte nuclear factor 4 (HNF4)-mediated transcription cooperatively with CBP. These results suggest that EWS not only...
functions as an adaptor molecule for splicing events but also as a co-activator with CBP for transcriptional initiation.

EXPERIMENTAL PROCEDURES

Plasmids—Human EWS deletion mutants were generated by PCR-based subcloning into pGEX5X-1 (Amersham Biosciences) or FLAG-tagged pcDNA3 (Invitrogen). pHNF4x8-tk-Luc and p8REx2-tk-Luc have been described previously (13, 14). pPPREx1-sv-Luc were constructed by ligating the three copies of the peroxisome proliferator-activated receptor (PPARα) binding site encompassing the nucleotide region (−104 to −92) of the 3-hydroxy-3-methyl-glutaryl-CoA synthase to the pGL3-SV40 promoter/luciferase fusion vector (Promega). Apolipoprotein CIII (ApoCIII)-Luc and glucose 6-phosphatase (G6P)–Luc were constructed by PCR-based methods. Human ApoCIII promoter (−890 to +44) or human G6P promoter (−826 to +3) fragment was inserted into pPicaGene-Basic vector II. Angiotensinogen 13-Luc was constructed by digesting a 1266-bp (−1229 to +44) fragment from human angiotensinogen promoter 13cat, which has been described previously (15), into pPicaGene-Basic vector II (Toyo-ink). pcDNA3-mCBP-HA and pcDNA3-mCBP-FLAG were generated by inserting full-length mouse CBP into HA or FLAG-tagged pcDNA3. pcDNA3-HA-HNF4 was generated by reverse transcription-PCR-based cloning into HA-tagged pcDNA3.

Antibodies—Anti-EWS rabbit polyclonal antibody was generated against a GST-EWS (246–504). Anti-HNF4α rabbit polyclonal antibody was generated against a GST-HNF4α (133–366). Anti-CBP rabbit polyclonal antisemur (5614) was described previously (16). Anti-HA monoclonal antibody (12CA5) was purchased from Roche Molecular Biochemicals; anti-FLAG monoclonal antibody (M2) was from Sigma; anti-HNF4α (C-19) and anti-RNA polymerase II (N-20) polyclonal antibodies were from Santa Cruz Biotechnology; and anti-RNA pol II monoclonal antibodies (S5W16 and H14) were from BAbCo.

Cells Culture and Transfections and Reporter Gene Assays—HEK293T cells and HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, and HEK293 cells were maintained in minimum Eagle’s medium containing 10% fetal bovine serum. For the reporter gene assay, transfections were performed by the calcium phosphate method. A Rous sarcoma virus-MLV packaging system was used to produce retroviral particles. A Rous sarcoma virus–MLV-AV7-4E2 packaging cell line was used to transduce HEK293T cells with HA-tagged CBP expression plasmids. For co-immunoprecipitation experiments, 35S-labeled extracts were prepared from HEK293T cells transfected with HA-tagged CBP expression plasmid and GST or EWS deletion mutants fused to GST. Western blot analysis was performed with anti-HA antibody (12CA5).

Immunoprecipitation—HEK293T cells were transfected using FuGENE 6 reagents (Roche Molecular Biochemicals), and nuclear extracts were prepared as described previously (13). Nuclei were extracted in buffer with anti-FLAG coupled with protein G-agarose in buffer A (20 mM HEPES, pH 7.9, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1% Nonidet P-40, 5% glycerol, 1 mM Na3VO4, 5 mM NaF, and protease inhibitors) for 4 h at 4 °C. The binding complexes were washed with the same buffer. For co-immunoprecipitation of endogenous EWS, CBP, and HNF4, nuclear extracts of HepG2 cells were subjected to immunoprecipitation with anti-EWS, anti-HNF4α, or anti-FLAG (5614) in Co-immunoprecipitation buffer (10 mM HEPES, pH 7.5, 100 mM KCl, 0.1% Nonidet P-40, and protease inhibitors).

GST Pull-down Assay—The GST fusion protein of each EWS mutant was expressed in Escherichia coli strain TopX (Invitrogen) and purified using glutathione-Sepharose beads (Amersham Biosciences). Protein extracts of HEK293T cells were incubated with each GST fusion protein bound to resin in 1 ml of buffer A for 4 h at 4 °C. After washing the beads with buffer A, bound proteins were fractionated by SDS-PAGE and analyzed by Western blotting.

Immunofluorescence—HEK293T or HEK293 cells were plated onto glass coverslips. HEK293T cells were transfected using FuGENE 6 reagents. After 24 h, cells were fixed with 8% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 1% bovine serum albumin. Cells then were incubated with the primary antibody, mouse anti-FLAG (1:500 dilution), rabbit anti-EWS (1:250 dilution), mouse anti-Pol IIa (1:200 dilution), or mouse anti-Pol IIo (1:100 dilution) and followed by staining with Alexa Fluor 488 anti-rabbit, Alexa Fluor 488 anti-mouse IgG, Alexa Fluor 488 anti-rabbit IgG, or Alexa Fluor 568 anti-mouse IgG in 0.2% glycine-free PBS. Stained cells were observed and photographed using a confocal microscope (TCS 4D, Leica).

RESULTS

Physical Association of EWS with CBP and RNA Polymerase II—To isolate CBP-interacting proteins, we used a GAL4 DNA-binding domain fused to CBP as a bait in a yeast two-hybrid screening assay (13) in which we identified EWS as a CBP-binding protein. To biochemically characterize the interaction of EWS with CBP, we constructed a series of EWS deletion mutants fused to GST and bacterially expressed products of them for the following assays (Fig. 1A). GST pull-down assays were performed on this series of EWS deletions with HEK293T lysates expressing HA-tagged CBP. Western blotting of the bound proteins using anti-HA antibody showed that CBP was specifically retained on beads coupled with GST-EWS (1–656), GST-EWS (1–333), and GST-EWS (83–227) (Fig. 1B). These results show that EWS can specifically interact with CBP through its amino acids 83–227 located on the NTD of EWS.

Two isoforms of RNA polymerase II (Pol II) exist in vivo, the hypophosphorylated (Pol IIA) and the hyperphosphorylated (Pol IIB) forms required for transcriptional initiation and elongation, respectively. Pol IIa is shown to possess the poly-A-binding domain at the C-terminal domain of the largest subunit and interacts with a range of general transcription factors at the promoter (17, 18). We investigated the relationship of EWS with different forms of Pol II using three anti-Pol II antibodies: N-20, which recognizes the common NTD of both Pol IIa and Pol IIB (17, 18); and 8G16, which only recognizes Pol IIA in immunofluorescence staining and Western blotting. To test the possible interaction of EWS with distinct forms of Pol II, we first examined their cellular localization. The staining patterns of Pol IIa and Pol IIB were compared with nucleoplasmic EWS distribution in double-labeling experiments followed by a confocal microscopy. The overlay images showed that the nucleoplasmic structures of EWS, and both Pol II forms partially overlapped (Fig. 2A). To further confirm the biochemical interaction of EWS with both Pol IIa and Pol IIB, we performed GST pull-down assays using HEK293 nuclear extracts. GST-EWS combined with both Pol IIa and Pol IIB in vitro, whereas GST alone did not (Fig. 2B). As shown in Fig. 2C, both EWS and CBP associated with the
preinitiation form of Pol II (pol IIa), whereas EWS but not CBP co-immunoprecipitated with Pol IIo in vivo. These results suggest that EWS is spatially and physically included in a part of both the preinitiation and elongation Pol II complexes.

Selective Enhancement of HNF4-mediated Transactivation by EWS—To examine the potential role of EWS in nuclear receptors-mediated transactivation, we evaluated the effects of EWS on the activation of three nuclear receptors including HNF4, retinoic acid receptor (RAR), and PPARγ/H9251 with pPPREx3-sv-Luc. As shown Fig. 3C, no effect of EWS was found on the activity of this receptor. Taken together, these results suggest that EWS is a selective co-activator for HNF4-mediated transactivation.

Formation of EWS, CBP, and HNF4 complex and Cooperative Enhancement of HNF4-dependent Transactivation—HNF4, which belongs to nuclear receptor superfamily, is a liver-enriched DNA-binding transcription factor. Binding sites for HNF4 have often been found in the regulatory regions of a large number of genes involved in fatty acid metabolism (19), gluconeogenesis (20), and blood pressure control (15) and in determining the hepatic phenotype (21). To test for transcriptional co-activations by EWS on the endogenous promoter sequences, we carried out transfection experiments using lucif...
EWS as a Co-activator for HNF4

Fig. 3. Selective enhancement of HNF4-mediated transactivation by EWS. A, HEK293 cells were transiently transfected with 50 ng of pHNF4x8-tk-Luc reporter plasmid and absence (open bars) or presence (filled bars) of 25 ng of HNF4a and 100 ng of EWS expression plasmid. B, HEK293 cells were transfected with 100 ng of pβREx2-tk-Luc for retinoic acid receptor and 100 ng of EWS expression plasmid. After transfection, cells were treated with Phenol Red-free minimum Eagle's medium with 10% fetal bovine serum either alone (open bars) or with 1 μM all-trans-retinoic acid (filled bars). C, HEK293 cells were transfected with 10 ng of pPPREx3-sv-Luc for peroxisome proliferator-activated receptor α and 100 ng of EWS expression plasmid. After transfection, cells were incubated in the absence (open bars) or presence (filled bars) of 500 μM Bezafibrate. The results are the means ± S.E. of at least three independent experiments performed in duplicate. DMSO, Me2SO.

Fig. 4. Formation of EWS, CBP, and HNF4 complex and cooperative enhancement of HNF4-dependent transactivation. Enhancement of endogenous promoter sequences by EWS. HEK293 cells were transfected with 10 ng of ApoCIII-Luc (A), G6P-Luc (B), or 50 ng of angiotensinogen 13-Luc reporter plasmid (C), 25 ng of HNF4a expression plasmid, and the indicated amounts of EWS expression plasmid. These results represent the mean ± S.E. of at least three experiments. D, co-immunoprecipitation of endogenous CBP/HNF4, CBP/EWS, and EWS/HNF4 using anti-EWS, anti-HNF4, or anti-CBP antibodies. Nuclear extracts from HepG2 cells were immunoprecipitated with anti-EWS (αEWS; left), anti-HNF4 (αHNF4; center), and anti-CBP (αCBP; right) antibodies or normal rabbit IgG as a control and then were subjected to immunoblotting with anti-EWS, anti-CBP-(5614) or anti-HNF4a (C-19) antibodies. E, EWS and CBP cooperatively enhance HNF4-mediated transactivation. Cells were transfected with 50 ng of pHNF4x8-tk-Luc reporter plasmid, 25 ng of HNF4a expression plasmid, 100 ng of EWS expression plasmid, and 250 ng of CBP expression plasmid. This result is the means ± S.E. of at least three independent experiments performed in duplicate.

erase reporter plasmids containing each HNF4-targeted gene promoter. EWS enhanced ApoCIII (Fig. 4A), G6P (Fig. 4B), or angiotensinogen (Fig. 4C) reporters, which were activated by HNF4 in a dose-dependent manner. To confirm the interaction of HNF4, CBP, and EWS under more physiological conditions, endogenous EWS, HNF4, and CBP in HepG2 nuclear extracts were co-immunoprecipitated with anti-EWS, anti-HNF4, and anti-CBP antibodies, respectively. As shown in Fig. 4D, endogenous EWS, HNF4, and CBP could be efficiently co-immunoprecipitated with endogenous CBP/HNF4, CBP/EWS, and
EWS/HNF4, respectively. To assess the regulatory effects of EWS and CBP on HNF4-mediated transactivation, we performed the reporter assay. Transfected HNF4 activity evaluated by co-transfection with an HNF4-tk-Luc reporter was further increased 3–4-fold by overexpression of EWS or CBP. When co-transfected together, both vectors induced reporter activity 13-fold in HNF4-transfected but not in non-transfected cells, suggesting that these transcription factors function cooperatively in HNF4-dependent transactivation (Fig. 4E).

Requirement of CBP for EWS Function—To determine whether the interaction with CBP is required for EWS-mediated activation, we constructed EWSΔCBP, which lacks the amino acids 83–227 in EWS. To assess the regulatory effects of EWS and CBP on HNF4-mediated transactivation, we performed the reporter assay. Transfected HNF4 activity evaluated by co-transfection with an HNF4-tk-Luc reporter was further increased 3–4-fold by overexpression of EWS or CBP. When co-transfected together, both vectors induced reporter activity 13-fold in HNF4-transfected but not in non-transfected cells, suggesting that these transcription factors function cooperatively in HNF4-dependent transactivation (Fig. 4E).

**Requirement of CBP for EWS Function**—To determine whether the interaction with CBP is required for EWS-mediated activation, we constructed EWSΔCBP, which lacks the amino acids 83–227 in EWS. We first compared expression patterns of FLAG-tagged EWS or EWSΔCBP in transfected HEK293 cells by immunofluorescence and Western blot analyses with an anti-FLAG antibody. As shown in Fig. 5A, B and C, FLAG-tagged EWSΔCBP as well as EWS was localized in the nucleus, and these expression levels were nearly equal. We next tested the binding activity of EWSΔCBP with CBP or HNF4 by GST pull-down assays. As shown in Fig. 5D, EWSΔCBP lost the interaction with transfected HA-tagged CBP (upper panel) but still bound to HA-tagged HNF4α (lower panel). We then assessed the effect of EWSΔCBP on HNF4-mediated transactivation by transient transfection assay. In HEK293 cells, an HNF4-tk-Luc reporter was inactive because of little expression of endogenous HNF4 protein (Fig. 5E, lanes 1–3). The luciferase reporter was activated up to 3-fold by co-transfection with HNF4 (Fig. 5E, lane 4). The addition of EWS resulted in additional 2.5-fold activation (Fig. 5E, lane 5). However, in the presence of HNF4, co-transfection with EWSΔCBP did not activate the HNF4-dependent transactivation (Fig. 5E, lane 6). These results suggest that interaction with CBP is required for the activity of EWS on HNF4-dependent transactivation through the CBP-binding region.

**DISCUSSION**

EWS was originally identified as a fusion protein with Fli-1 in Ewing's sarcoma and was later found in several malignancies in which the NTD of EWS was fused to the DNA-binding domain of the ETS family such as ATP-1, WT-1, CHN, and C/EBP homologous protein (2). For all of the above malignancies, the EWS fusion proteins are thought to act as potent transcriptional activators in a manner that is dependent on the transcriptional activation domain in the NTD of EWS (22). However, the transcriptional function of native EWS is not very well understood. It has been shown that transcriptional
events are closely coupled with processing events in vivo (17, 18). The C-terminal domain of the largest subunit of Pol II plays a central role in this coupling by reversible phosphorylation during the transcription cycle. Once initiation of transcription begins, the hypophosphorylated form of C-terminal domain (Pol IIa) becomes hyperphosphorylated (Pol IIo) during the transition from the preinitiation complex to the elongation-processing complex, and this form of pol II is able to recruit mRNA processing factors (23). EWS was previously reported to interact with only Pol IIo as an adaptor molecule to recruit splicing factors in elongation stages (2).

In this study, we identified the interaction of EWS with CBP and with both of the preinitiation (Pol IIa) and the elongation (Pol IIo) forms of Pol II. CBP associates only with Pol IIa (24) (Fig. 2) and activates transcriptional initiation as a bridging/co-activating/chromatin-remodeling factor. These findings suggest that EWS may activate transcriptions in collaboration with CBP and basal transcription machinery including the preinitiation form (Pol IIa) of Pol II complex.

A previous study (25) showed the interaction of EWS with CBP/p300 and the activation of c-fos, Xcnt-2, and Erb2 promoters by EWS. Because EWS does not have any obvious DNA-binding domain, it has been described that EWS may be recruited to target promoters by protein-protein interaction with DNA-binding transcription factors and act as a co-activator. However, a target DNA-binding transcription factor has not yet been identified. We demonstrated that EWS selectively potentiates the DNA-binding HNF4 transcription factor-mediated transactivation (Fig. 3). As transient transfection analyses using the ApoCIII, G6P, and angiotensinogen promoters illustrated that these were activated by HNF4 in conjunction with EWS but not in the absence of HNF4, HNF4 is a high affinity site for the entry of EWS to the target promoters. On the other hand, EWS repressed RAR-mediated transactivation (Fig. 3B) and did not associate with RAR under the conditions used in this assay (data not shown). This finding suggested that RAR is not a direct target molecule of EWS and that the repression by EWS artificially arises from squelching CBP, RNA polymerase II, TFIIID, or other factors in the transcriptional complex for RAR.

We found that EWS interacted with CBP and HNF4 and played cooperatively with CBP to further reinforce the transactivation of HNF4 (Fig. 4). In addition, the co-activational activity of EWS was abolished by the deletion of the CBP-binding region of EWS (amino acids 83–227) (Fig. 5). In previous studies, Ohno et al. (26) and Lessnick et al. (27) demonstrate that the transcriptional activity of EWS is included in amino acids 83–265 when fused to the DNA-binding domain of GAL4 or Fli-1. These findings reveal that the co-activator function of CBP mediates the transactivation ability of EWS or EWS fusion proteins through the NTD.

Although we demonstrated that EWS interacts with CBP and the preinitiation form of Pol II and enhances HNF4-mediated transactivation in a manner dependent on the co-activator function of CBP, additional EWS functions were suggested by other studies. For example, EWS contains motifs like RNA-binding proteins and actually binds to RNA and functions including fatty acid metabolism, gluconeogenesis, and blood pressure control may be exerted efficiently as results of coupling of between transcription and mRNA processing by EWS and/or PPAR/y co-activator-1.

Because EWS is a ubiquitously expressed factor (31) as compared with the limited expression profile of PPARy co-activator-1 in the brown fat, kidney, heart, and brain under the normal conditions (32), it is likely that EWS participates in various biological events through other nuclear receptors or CBP-directed DNA-binding transcription factors. In contrast, we confirmed that a EWS fusion gene, EWS-Fli-1, lost the activity as a co-activator by a minimal reporter gene system using HNF4 recognition site (data not shown). It is expected that impaired (lost or acquired) biological systems of native EWS caused by a gene fusion event may be related to the tumorigenesis. Therefore, further analysis regarding the native EWS function will be helpful to understand the physiological significance of EWS in addition to the mechanisms of transformation by EWS fusion proteins.

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