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# Gadd45 Family Proteins Are Coactivators of Nuclear Hormone Receptors

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**Gadd45 family genes encode nuclear acidic proteins composed of Gadd45, MyD118, and CR6. Sequence analysis showed that Gadd45 family proteins (Gadd45, MyD118, and CR6) contain LXXLL signature motifs considered necessary and sufficient for the binding of several coactivators to nuclear receptors. Interaction between Gadd45 or CR6 and RXR$\alpha$ was confirmed by a two-hybrid test in yeast. Results from a series of GST pulldown assays showed that these Gadd45 family proteins interact with several nuclear hormone receptors including RXR$\alpha$, RAR$\alpha$, ER$\alpha$, PPAR$\alpha$, PPAR$\beta$, and PPAR$\gamma$2 *in vitro*. Interaction between Gadd45 family proteins and nuclear hormone receptors resulted in modest activation of transactivating function of nuclear hormone receptors in reporter systems. When fused to DNA binding domain of GAL4, Gadd45 and CR6 activated the UAS-mediated transcription in mammalian cells. These results suggest that Gadd45 family proteins bind to nuclear hormone receptors and act as nuclear coactivators.** 

***Key Words:*** **Gadd45; LXXLL; nuclear receptor; binding; coactivator.**

Gadd45 gene was initially identified as a gene whose mRNA is rapidly induced by agents that cause DNA damage such as UV radiation, MMS and X-ray irradiation (1, 2). Gadd45 was widely studied as a marker for p53 activation, for appreciable induction of Gadd45 by X-ray irradiation occurred only in cells having a wild type p53 phenotype (3–6). Recent evidence showed that the Gadd45-null mice exhibited several of the phenotypes characteristic of p53-deficient mice (7). MyD118, exhibiting a significant homology with Gadd45, was identified as an immediate early responsive gene induced by IL-6 in the murine myeloid cell line M1 (8). MyD118 was rapidly induced by TGF-$\beta$1 that induced M1 cells for growth arrest and apoptosis uncoupled from differentiation (9). Another Gadd45-like gene, CR6, was originally identified as an immediate early response gene in T cells stimulated by interleukin-2 (10). Recent study showed that CR6 also was immediately induced by oncostatin M that is a member of the IL-6 family cytokines exhibited both growth stimulatory and growth inhibitory activity depending on the cells (11). Using short term transient transfection assays, it has been demonstrated that all three related genes (hereafter called Gadd45 family genes) suppressed colony formation of human lung carcinoma H1299 cells, but their mechanism of action is uncertain (12).

In an effort to understand the mechanisms of Gadd45 family genes, several cellular proteins that physically interacted with them were identified. All three Gadd45 family proteins interacted with the DNA replication and repair protein PCNA (11, 13–15) and the cyclin dependent kinase inhibitor $p21^{WAF1/CIP1}$ (11, 15). Recently Gadd45 were found directly associate with Cdc2, which played a central role with cyclin B1 as a complex in progression from G2 to M phase (16). The interaction with these cell cycle regulatory proteins implicated that Gadd45 family proteins might play important role(s) in nucleotide excision repair, G1 arrest and G2 arrest in response to DNA damage (17). Takekawa and Saito (18) recently discovered that Gadd45 family proteins also associated with MTK1, which in turn activated both p38 and JNK pathway leading to apoptosis in response to environmental stresses. Although potential roles of Gadd45 family proteins have been illustrated based on the function of their associated proteins, none of their biological significance is so clear yet. At the present time the involvement of Gadd45 in nucleotide excision repair and P38 and JNK mediated-apoptosis pathway is still in controversy among research groups (13, 18–22).

The nuclear receptor (NR) superfamily is a group of ligand dependent transcription factors characterized

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by an impressive functional diversity by controlling gene networks (23). The ligands for NRs contribute to vertebrate development and homeostasis by serving as biological signals to control cell growth and differentiation. Upon binding ligand, NRs act as dimeric transcription factors to activate or repress expression of nuclear target genes by binding to specific DNA sequences termed hormone response elements (24). Results from studying to define the mechanism by specific modulation of target gene expression achieved by NRs indicate that there might be factors common to different NRs required for efficient transcriptional activation. Subsequent works have let to the identification of several coactivators such as steroid receptor coactivator-1 (SRC-1) (25), p300/CBP/co-integrator-associated protein (p/CIP) (26), and transcription intermediate factor-2 (TIF2) (27). These coactivators enhance levels of NR-mediated transactivation several fold (28) and display a broad specificity across the NR superfamily.

In this study we demonstrated that all Gadd45 family proteins bound to several nuclear hormone receptors including RXR$\alpha$, RAR$\alpha$, ER$\alpha$, PPAR$\alpha$, PPAR$\beta$ and PPAR$\gamma$2 using yeast two hybrid system and GST pulldown assay. In addition to that, each of Gadd45 family genes was found to contain LXXLL-like motifs and a transcription modulation activity for nuclear hormone receptors that are common features of many known coactivators.

## MATERIALS AND METHODS

*Construction of plasmid DNAs.* All plasmid DNAs were generated using standard cloning procedures and verified by restriction enzyme analysis and DNA sequencing (Bionex, Seoul, Korea). For an expression in yeast, pDBD-Gadd45 and pDBD-CR6 was constructed by inserting PCR-amplified human CR6 cDNA or human Gadd45 cDNA, digested with *Eco*RI and *Xho*I from pHul45B2 (obtained from Fornace, A. J., Jr.), into *Eco*RI/*Xho*I sites of pAS2-1 (Clontech), respectively. Human RXR$\alpha$ cDNA (aa 44-462), digested with *Xma*I and *Eco*RI from pBS-RXR$\alpha$, were also cloned into *Xma*I/*Eco*RI sites of pACT2 (Clontech) and named pAD-RXR$\alpha$.

For expression of GST-fusion proteins, human Gadd45 cDNA (aa 5-159) was cloned into *Eco*RI/*Xho*I sites of pGEX-4T1 (Amersham Pharmacia Biotech). PCR amplified mouse MyD118 and human CR6 cDNAs cloned into *Eco*RI/*Xho*I sites of pGEX-4T3 and pGEX-4T1, respectively.

For mammalian expression, pFlag-Gadd45 and -CR6 plasmid DNAs were cloned by insertion of each cDNA into *Eco*RI/*Xho*I sites of pCMV-Tag2 (Stratagene). The pCMV-RXR$\alpha$ was constructed by insertion of PCR-amplified human RXR$\alpha$ cDNA in the pcDNA3 (Invitrogen). The PPAR expression vectors, pmyc-PPAR$\alpha$ and pmyc-PPAR$\gamma$2, were constructed by inserting each cDNA (from B. M. Spiegelman) into pCMV-Tag3 vectors. pPPRE-tk-Luc was constructed as described previously (29). Plasmids pFA2-Gadd45 and pFA2-CR6 were constructed by inserting these cDNAs into pFA2 vector (Stratagene) which containing the GAL4 DNA binding domain fusion. pFR-Luc, containing 5 copies of upstream activating sequence (UAS), was purchased from Stratagene.

For *in vitro* translation, PCR-amplified human RXR$\alpha$, RAR$\alpha$ and ER$\alpha$ cDNA, were cloned into pBluescript vector (Stratagene) and named pBS-RXR$\alpha$, pBS-RAR$\alpha$ and pBS-ER$\alpha$, respectively. The plasmids containing mouse PPAR$\alpha$, PPAR$\beta$, and PPAR$\gamma$2 cDNAs were obtained from B. M. Spiegelman.

*Yeast two-hybrid assay.* To access protein-protein interaction, plasmid constructs containing DBD-fusion and AD-fusion were co-transformed in yeast Y190 strain. Yeast transformants were plated on selection medium lacking leucine and tryptophan and replicated on an appropriate selection medium.

*GST pulldown assay.* Bacterially expressed GST-fusion proteins were purified as described previously (30). Coupled *in vitro* transcription/translation was done by TnT rabbit reticulocyte system (Promega) to produce [$^{35}$S]-labeled nuclear hormone receptors. GST pulldown assay was done as follow. Two micrograms of GST-fusion proteins were incubated with 10 $\mu$l of glutathione-agarose resin (Sigma) at room temperature for 30 min. After incubation, 5 $\mu$l of *in vitro* translated [$^{35}$S]-labeled nuclear hormone receptors, diluted in 300 $\mu$l of Buffer B (20 mM HEPES (pH 7.9), 50 mM NaCl, 1 mM $MgCl_2$, 17% glycerol, and 2 mM DTT) containing 1% NP-40, was added to the reactions. The reaction mixtures were incubated for additional 2 h at room temperature with rotation in the presence or absence of appropriate ligand. After brief centrifugation, beads were washed by three times with buffer B containing 1% NP-40 and the samples were subjected to SDS–PAGE, dried and scanned by Molecular Imager FX system (Bio-Rad) or exposed to X-ray film (Eastman Kodak).

*Cell culture and transfection.* NIH/3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS), 100 unit/ml penicillin, and 100 $\mu$g/ml streptomycin (Life Technologies, Inc.). For transient transfection assays, NIH/3T3 cells (~60,000 cells/well) grown in 24-well plates were transfected with the appropriate expression plasmids with reporter plasmid (100 ng) using Lipofectamine Plus (Gibco-BRL). Total amount of plasmid DNAs was adjusted to 0.4 $\mu$g per well with carrier DNA (pCMV-Tag2A). Luciferase assay was determined as manufacturer's instruction (Promega) with a Lumat LB9507 luminometer (EG&G Berthold). The luciferase activities were normalized to the protein concentration measured by Bio-Rad protein assay kit (Bio-Rad).

## RESULTS AND DISCUSSION

### *Gadd45 Family Proteins Contain LXXLL Motifs*

Ever since the nucleotide and deduced amino acid sequence of Gadd45 were first published (3) a few motifs biologically significant were reported. A recent cloning of the third Gadd45 family gene, CR6, and its sequence comparisons with Gadd45 and MyD118 enabled us to explore potential motifs in their amino acid sequences (11, 16, 18). An interesting feature contained within the sequences of Gadd45 family proteins is the presence of the three repeated LXXLL (L, leucine; X, any amino acid)-like motifs. Gadd45 and CR6 have one and MyD118 has two complete LXXLL motifs located in the specific regions of the aligned sequences as shown in Fig. 1A. The coactivators such as SRC-1, p/CIP, CBP, and TIF2 and many other factors capable of interacting with nuclear hormone receptors, share a common motif containing a core consensus sequence LXXLL (31). Because the short sequence motif LXXLL, present in these coactivators, is necessary and sufficient to mediate the binding of these proteins to nuclear hormone receptors, it is very

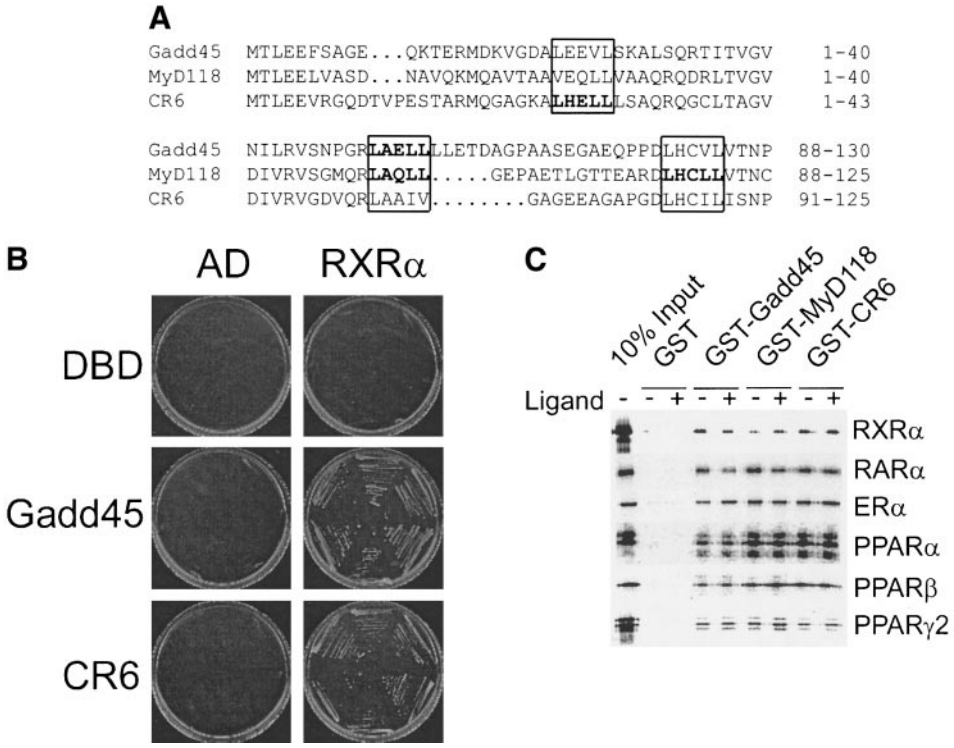


**FIG. 1.** Interaction between Gadd45 family protein and nuclear hormone receptors. (A) LXXLL motifs in the Gadd45-like proteins. Amino acid sequence alignment of Gadd45-like proteins shows that there are three repeats of LXXLL-like motifs. Boxed regions indicate the LXXLL-like motifs. Bold sequences indicate complete LXXLL motifs. Sequence alignment was done by DNAsis Ver 2.1. (B) Interaction between Gadd45 or CR6 proteins and RXR$\alpha$ in yeast. Yeast transformants containing AD-RXR$\alpha$ and DBD-Gadd45 or DBD-CR6 only can survive in the selection medium containing 35 mM of 3-AT and 1 $\mu$M of 9-*cis*-RA. To reduce the individual variation, independent six transformants were streaked on selection medium without leucine, tryptophan, and histidine. (C) Control GST protein alone or Gadd45 family proteins fused to GST were immobilized on glutathione agarose beads and incubated with different *in vitro* translated [$^{35}$S]methionine-labeled nuclear receptors and appropriate ligands or vehicle (DMSO). For RXR$\alpha$, 9-*cis*-RA (Sigma) was used at 1 $\mu$M; for RAR$\alpha$, all-*trans*-RA (Sigma) was used at 1 $\mu$M; for ER$\alpha$, 17-$\beta$-estradiol (Sigma) was used at 1 $\mu$M; for PPAR$\alpha$ and PPAR$\beta$ WY14643 (Biomol) was used at 1 $\mu$M; and for PPAR$\gamma$2 BRL49653 (GlaxoWellcome) was used at 1 $\mu$M.

plausible that Gadd45-like proteins bind to and act as coactivators of nuclear hormone receptors.

### *Gadd45 Family Proteins Interact with Nuclear Receptors*

In order to verify Gadd45 family genes also associate with nuclear receptors *in vivo*, yeast two-hybrid tests were carried out. Either human pDBD-Gadd45 or pDBD-CR6 was co-transformed with pAD-RXR$\alpha$ into yeast Y190 strain. As expected, yeast transformants containing both GAL4 DBD-fusion and GAL4 AD-fusion vectors could grow on selection medium lacking with leucine and tryptophan (data not shown). To reduce the individual variation, independent six transformants were stricked on selection medium lacking with leucine, tryptophan, and histidine. Selection medium also contained 35 mM of 3-amino-1,2,4-triazole (3-AT) to eliminate the basal transcriptional activities of DBD-Gadd45 and DBD-CR6, and 1 $\mu$M of 9-*cis*-retinoic acid (9-*cis*-RA) as ligand for RXR$\alpha$. Growth of yeast on this selection medium indicated that Gadd45 and CR6 specifically interact with RXR$\alpha$ in yeast (Fig. 1B).

A series of GST pulldown assays was carried out to confirm the interaction between Gadd45 family proteins and many other nuclear hormone receptors. Bacterially expressed GST-Gadd45, -MyD118 and -CR6 proteins were immobilized on glutathione-agarose beads and incubated with [$^{35}$S]methionine-labeled, *in vitro* translated nuclear hormone receptors. As shown in Fig. 1C, RXR$\alpha$ associates with each GST-Gadd45 family proteins but fails to bind to the control GST protein. This interaction is a ligand-independent manner, in that addition of 9-*cis*-retinoic acid (RA) at 1 $\mu$M does not significantly alter this binding. RAR$\alpha$, ER$\alpha$, PPAR$\alpha$, PPAR$\beta$, and PPAR$\gamma$2 also bind specifically to Gadd45 family proteins in the similar manner. To access the functionality of GST-fusion proteins, GST pulldown assays were also carried out with [$^{35}$S]methionine-labeled, *in vitro* translated PCNA and p21. As previously described (11, 13–15), *in vitro* translated PCNA and p21 can bind to bacterially expressed GST-

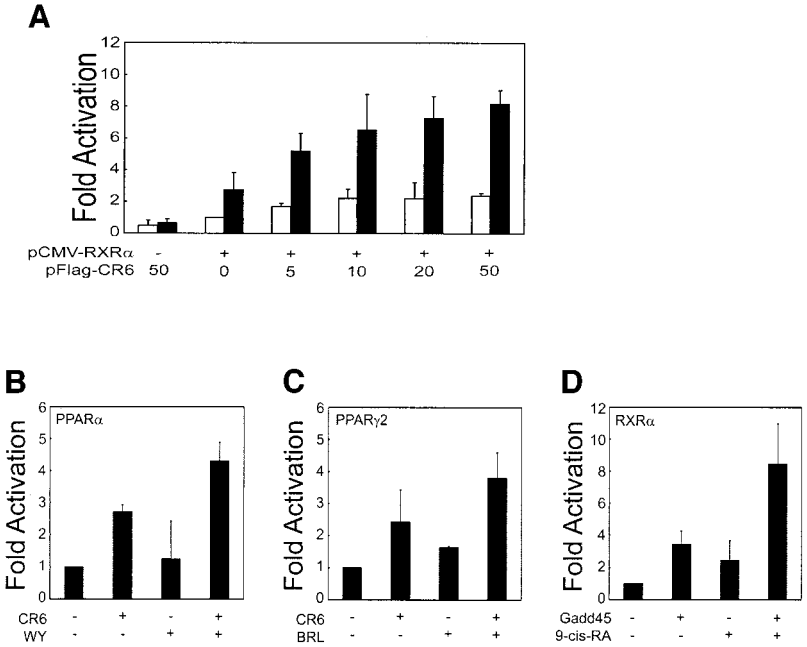


**FIG. 2.** Effects of Gadd45 family proteins on the nuclear hormone receptor-mediated transactivations. (A) NIH/3T3 cells were transfected with pCMV-RXR$\alpha$ and pSV-PPAR$\gamma$2 expression vector and increasing amount of CR6 expression vector (pFlag-CR6) along with a reporter gene PPRE-tk-Luc, as indicated. Relative luciferase activities from triplicated samples are presented. Open bars indicate no ligand added. Black bars indicate the presence of 1 $\mu$M 9-*cis*-RA. (B and C) CR6 coactivates PPAR$\alpha$ and PPAR$\gamma$2. NIH/3T3 cells were cotransfected with pFlag-CR6 and pmyc-PPAR$\alpha$ or pmyc-PPAR$\gamma$2 in the presence or absence of 0.1 $\mu$M of WY14643 or BRL49653, respectively. (D) Coactivation of RXR$\alpha$ transcriptional activity by Gadd45. NIH/3T3 cells were cotransfected with pFlag-Gadd45, pCMV-RXR$\alpha$ and pSV-PPAR$\gamma$2 in the presence or absence of 1 $\mu$M of 9-*cis*-RA. Ligands were treated 24 h after transfection for another 24 h. The data shown indicate the mean $\pm$ SD of triplicates.

Gadd45, -MyD118 and -CR6. *In vitro* translated luciferase protein were used as a negative control and found not to bind GST or GST-Gadd45 family proteins *in vitro* (data not shown).

### *Gadd45 Family Proteins Activates the Transactivation Function of Nuclear Receptors*

To assess the functional consequences of these interaction, we utilized the luciferase reporter systems in combination with RXR$\alpha$ and PPAR$\alpha$ or PPAR$\gamma$2 expression vectors. The presence of CR6 alone does not induce transcriptional activity of PPRE-tk-Luc vector (Fig. 2A). However, co-transfection of NIH/3T3 cells with CR6 in combination with RXR$\alpha$ moderately activated the reporter gene expression in the absence of ligands in dose-dependent manner (Fig. 2A). In the presence of ligand (1 $\mu$M of 9-*cis*-RA), CR6 still enhanced the expression of the reporter gene up to about 4-fold. CR6 protein also activated the PPAR$\alpha$- and PPAR$\gamma$2-mediated transcription about 2 to 3-fold respectively (Fig. 2B and 2C). This activation by CR6 is also in dose-dependent manner (data not shown). Gadd45 also activates the RXR$\alpha$ activity as similar manner (Fig. 2D). Like many other coactivators, maximal transactivation by Gadd45 or CR6 was observed when appropriate ligands added to the reactions.

Although hormone binding had little or no effect on NR-Gadd45 family protein interactions *in vitro,* optimal transcriptional response in transient transfection assay was seen when appropriate ligands were added. Similarly, ligand-independent bindings of PGC-1 to NRs (32), CBP to androgen receptor (33), and activating signal cointegrator 1 (ASC-1) to NRs (34) were reported. One of the plausible explanations is simultaneous, ligand-dependent docking of another coactivators, such as SRC-1, CBP, or others. It is possible that the binding conditions used for GST pulldown assays do not mimic the *in vivo* conditions that may be required for ligand-mediated effects of other coactivators. Another possibility is that ligand-dependent competitive binding between Gadd45 family proteins and other coactivators. As recently reported, although ASC-1 bound to NRs in a ligand-independent manner *in vitro,* the interaction between ASC-1 and thyroid hormone receptor was in a ligand-dependent manner when SMRT was added in the *in vitro* binding reactions (34). This implicated the complexity of mechanisms in interaction between coactivators and NRs.

### *Gadd45 Family Proteins Contain Intrinsic Transcriptional Activity*

To define the mechanism by which Gadd45 and CR6 act as coactivators, we sought to determine the existence of their intrinsic functions of transcriptional activation. The determination of this property is essential for the characterization of a coactivator. For

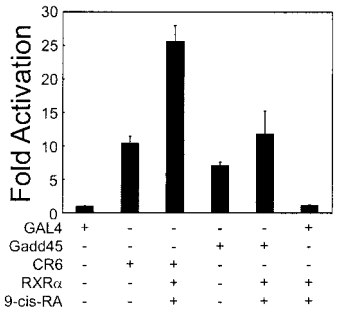


**FIG. 3.** Gadd45 and CR6 have autonomous transcriptional activation functions. NIH/3T3 cells were transfected with pFA2-Gadd45 or -CR6 proteins in the absence or presence of pCMV-RXR$\alpha$ as indicated. Transcription was assayed with pFR-Luc containing five copies of the UAS linked to luciferase. Ligand for RXR$\alpha$ (9-*cis*-RA) was treated 24 h after transfection for 4 h. The data shown indicate the mean $\pm$ SD of triplicates.

example, SRC-1 contains the intrinsic transcriptional activity, while PCAF does not (35). To determine whether Gadd45 and CR6 protein contain this property, each gene was fused to the DNA binding domain of GAL4 DNA binding domain and transfected into the NIH/3T3 cells with pFR-Luc reporter plasmid. The intrinsic transcriptional activity by GAL4-Gadd45 or CR6 was found and their induction folds were about 7 or 10 fold, respectively (Fig. 3). Transcriptional activation function of coactivators activates target gene expression through either disruption of the nucleosome structure or by modulating the preinitiation complex (36–38), resulting in an increased rate of transcription. Recently, it has been reported that Gadd45 directly associated with mononucleosomes that have been altered by histone acetylation or UV radiation and this interaction resulted in modification of DNA accessibility to cellular proteins (39). Since one of major role of coactivator is enhancing the accessibility of other transcription factor to DNA, these results suggest that Gadd45 family proteins might play as transcriptional coactivators.

### *Synergism between GAL4-Gadd45 Family Proteins and RXR$\alpha$*

To investigate the effect of nuclear hormone receptors on GAL4-Gadd45 family protein-mediated transactivation, NIH/3T3 cells were transiently cotransfected with pFR-Luc reporter construct, pCMV-RXR$\alpha$, and pFA2-Gadd45 or pFA2-CR6. RXR$\alpha$ enhanced GAL4-Gadd45 family proteins-mediated transactivation about 10 to 25-fold, but had no effect on the GAL4 DNA binding domain alone (Fig. 3). Although these conditions were not physiological with respect to the way in which Gadd45 family proteins and RXR$\alpha$ are normally anchored to the promoter, the data nevertheless indicate that tethering Gadd45 family proteins to the DNA is sufficient for synergism via protein-protein interaction.

In conclusion, we have demonstrated that Gadd45 family proteins are coactivators for nuclear hormone receptors via direct interaction and enhancement of RXR or PPAR-mediated transcription activity. The demonstration of this function has been achieved using transiently transfected reporter constructs. It would perhaps be more pertinent to examine the effect of Gadd45 family proteins using natural hormone responsive promoters, in their normal chromosomal context to truly assess its significance. However, it is clear that Gadd45 family proteins, in our system, appear to be as important as other coactivators are. And thus it is worthy of further investigation into their mechanism of action(s) as coactivators.

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