Physical Interaction and Functional Synergy between Glucocorticoid Receptor and Ets2 Proteins for Transcription Activation of the Rat Cytochrome P-450c27 Promoter*

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We demonstrate that dexamethasone-mediated transcription activation of the cytochrome P-450c27 promoter involves a physical interaction and functional synergy between glucocorticoid receptor (GR) and Ets2 factor. Ets2 protein binding to a “weak” Ets-like site of the promoter is dependent on GR bound to the adjacent cryptic glucocorticoid response element. Coimmunoprecipitation and chemical cross-linking experiments show physical interaction between GR and Ets2 proteins. Mutational analyses show synergistic effects of Ets2 and GR in dexamethasone-mediated activation of the cytochrome P-450c27 promoter. The DNA-binding domain of GR, lacking the transcription activation and ligand-binding domains, was fully active in synergistic activation of the promoter with intact Ets2. The DNA-binding domain of Ets2 lacking the transcription activation domain showed a dominant negative effect on the transcription activity. Finally, a fusion protein consisting of the GR DNA-binding domain and the transcription activation domain of Ets2 fully supported the transcription activity, suggesting a novel synergy between the two proteins, which does not require the transactivation domain of GR. Our results also provide new insights on the role of putative weak consensus Ets sites in transcription activation, possibly through synergistic interaction with other gene-specific transcription activators.

Cytochrome P-450c27 (CYP27) is a multifunctional enzyme

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§ The abbreviations used are: CYP27, cytochrome P-450c27; GR, glucocorticoid receptor; Dx, dexamethasone; GRE, glucocorticoid response element; Ets, Ets-binding domain of Ets2 protein; GRDBD, DNA-binding domain of glucocorticoid receptor protein; BME, β-mercaptoethanol; EMSA, electrophoretic mobility shift analysis for DNA-protein binding; kb, kilobase(s); PCR, polymerase chain reaction; CMV, cytomegalovirus; β-gal, β-galactosidase; CAT, chloramphenicol acetyltransferase; bp, base pair(s); DSP, dithiothreitol/succinimidylpropionate; sulfos-MBS, m-maleimidobenzyl-N-hydroxysulfosuccinimide ester; Adx, adrenodoxin; SREBP, sterol response element-binding protein; GBP, GA-binding protein.
interacting with other transcription factors, such as members of the Ap-1 family, Jun and Fos (16–19), RelA protein of the NFκB pathway (20–22), liver-specific factor HNF3 (23, 24), and Stat-5 (25). GR also modulates transcription activity through receptor-receptor interaction between proteins bound to tandem GRE sites (15, 26). Recently Zhang et al. (22) demonstrated a complex pattern of interaction between interleukin-6 response element-bound protein and GR bound to an adjacent GRE site. This interaction was suggested to help synergize transcriptional effects of the interleukin-6 response element and GRE sites through Stat-3, which acts as a potent coactivator of GRE-mediated transcription.

Some studies suggest that transcription activation by GR is also subject to regulation by Ets family transcription factors. Transient transfection studies showed that PU.1 and GR reciprocally modulate the activity of each factor, resulting in transcriptional repression (27, 28). The precise mechanisms of repression, however, remain unclear. Another study showed that mutations targeted to the Ets1-binding site, overlapping the GR binding motif of the tyrosine aminotransferase promoter, resulted in a 2-fold reduction in transcription activity, suggesting synergistic effects of the two factors (29). However, the precise mode of physical interaction between the factors was not investigated. In the present study we show that Dx-mediated activation of CYP27 gene expression involves GR binding to a variant GRE site and its synergistic interaction with factors binding to an adjacent "weak" consensus Ets site, referred to in this study as an Ets-like site. Interestingly, the activation appears to require a novel functional and physical interaction between the DNA-binding domain of GR and the transcription activation domain of the ubiquitously expressed Ets2 transcription factor.

**MATERIALS AND METHODS**

Plasmid Construction—The CYP27 promoter constructs were generated by PCR amplification of the −841/+23mCAT DNA from the rat CYP27 gene described before (6) and cloned into the promoterless and enhancerless basic CAT plasmid pCATBasic (Promega Corp., Madison, WI) in the HindIII and SalI sites. The −329/+23mCAT was generated by digesting the −841/+23mCAT DNA with PstI (at position −329) and SalI (at position +23), and the fragment was cloned into the same sites of pCATBasic DNA. The 5′ progressive deletions −329/+23mCAT, −233/+23mCAT, and −133/+23mCAT were digested the SalI-digested −841/+23mCAT DNA with SacI (−194), XhoI (−76), or SalI (−45) enzymes, respectively. All the fragments were gel purified, blunt ended with T4 DNA polymerase, and recloned into pCATBasic plasmid at blunt ended SphI and SalI sites. Mutations at the cryptic GRE, Ets-like, Ap-1-like sites within the −329 to −194 promoter sequence were introduced by overlapping PCR using primers containing appropriate substitutions and further amplified using sense primer containing a HindIII site and antisense primer containing a SalI site for cloning. The mutant DNA fragments were cloned in the HindIII and SalI sites of pCATBasic vector. The nucleotide sequence of all the constructs was verified by the dyeoxy chain termination sequencing (30).

The rat GR expression cDNA (p6RGR) encoding the full-length receptor protein and the truncated rat GR DNA-binding domain (GRBBD, amino acids 407–556 of the GR protein) expression plasmid (p6R-X556) were kindly provided by Dr. K. Yamamoto (31). Expression plasmid P5V_6Ets2, encoding the full-length Ets2 protein, and a dominant negative mutant P5V_6Ets2, expressing only the DNA-binding domain of Ets2 were described before (32). CMVets, encoding an intact murine Ets1 was obtained from Dr. Michael Atchison. A cDNA encoding the DNA-binding region of Ets1 (tEts1, amino acids 260–440 of murine Ets1) was generated by PCR amplification and cloned in the pCMV vector. A chimeric construct of the GRBBD and the transcription activation domain of Ets2 was generated by cloning the GRBBD cDNA (NdeI and SalI fragment, nucleotides 1206–1656 of 6GR cDNA) differently cut pGEM5z plasmid DNA. cDNA corresponding to the transcription activation domain of murine Ets2 (amino acid residues 40–280) was amplified using the sense primer containing a SalI site and antisense primer containing a SalI site and cloned into similarly digested 5z GRBBD plasmid DNA to produce a contiguous open reading frame. The resulting cDNA expressed a 26-kDa fusion protein containing the DNA-binding domain of GR with a C-terminal GRBBD (frame) transcription activation domain of Ets2. Finally, the chimeric cDNA construct was cloned into the mammalian expression vector PCMV4, and was designated as pGRBBD-Ets2. This clone was used to generate deletion constructs pGRBBDZF-Ets2 (lacking amino acids 455–485 of GRBBD) and pGRBBD-Ets223200 (lacking the N-terminal 200 residues corresponding to a standard or even PCR. A cDNA encoding GRDBD and the N-terminal 40–280 amino acid residues of murine Ets1 fusion protein was generated using a similar approach.

Cell Culture and Transfection—Mouse Balb/c 3T3 fibroblast cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 50 μg/ml gentamicin, and 50 μg/ml gentamicin at 37 °C. Murine NIH 3T3 fibroblast cells were maintained as described before (6). Cells were transfected in replicate plates with CsCl gradient-purified CYP27 promoter plasmid DNA by the calcium phosphate coprecipitation method or in some cases using the Fugene 6 reagent (Roche Molecular Biochemicals). The latter procedure routinely yielded transcription efficiency of >60%. Cells (100-mm plates) were transfected with 5 μg of test plasmid DNAs and 1.5 μg of CMV β-galactosidase expression plasmid, pCH110 (35). For cotransfection experiments, 5 μg of the reporter plasmid was cotransfected with either 2 μg of GR DNA, 0.8 μg of CMVets/Ets2 cDNAs, 1.5–3.0 μg of GRBBD-Ets2, GRDBDZF-Ets2, GRBDBEDs2, and GRBDB-Ets1 chimeric cDNA constructs, or 1 μg of GRBBD cDNA construct. The total DNA in each transfection was normalized to 15 μg with CMV DNA as the filler. In some experiments the medium was changed with that containing 2% fetal bovine serum after 40 h of transfection, and cells were exposed to 100 nM Dlx (Sigma) for 8–10 h before harvesting. Control cells not subjected to Dlx induction were harvested after 48–50 h of transfection. Cell extracts were prepared by standard procedure and used for assaying CAT activity (33) or by the colorimetric method using a CAT enzyme-linked immunosorbent assay kit from Roche Molecular Biochemicals. The β-galactosidase activity of each of the extracts was used as an internal control for normalizing the transfection efficiency (33).

Preparation of Nuclear Extracts and Proteins—Nuclear extract from NIH 3T3 cells were prepared by the method of Dignam et al. (34). Bacterially expressed purified GRBBD protein (apparent molecular mass of 17.0 kDa) was a generous gift from Dr. K. Yamamoto (35). Ets2 protein was overexpressed in BL21 cells by isopropyl-1-thio-β-D-galactopyranoside induction using the pET vector (Novagen, Inc., Madison, WI) and purified to near homogeneity by affinity binding to the nickel-agarose matrix (Qiagen Inc., Velenica, CA).

Northern Hybridization—RNA from transfected and mock-transfected Balb/c 3T3 cells were prepared by the guanidinium thiocyanate method (36). RNA (30 μg each) was resolved by electrophoresis through a formaldehyde-containing gel, blotted to Nytran membranes, and hybridized with the 32P-labeled rat CYP27 cDNA probe labeled by nick translation using the nick translation kit (Boehringer Mannheim) or oxo-

Gal Mobility Shift Assays—DNA-protein binding was assayed by gel mobility shift EMSA as described (37). Binding was carried out with a 32P end-labelled 135-bp DNA fragment from the glucocorticoid hormone-responsive region of the promoter (amino acids −329 to −194), which was generated by digesting pCAT-329/-23 DNA with PstI and SacI restriction enzymes. In some experiments, a shorter 65-bp DNA (amino acids −233 to −298) generated by PCR was used as a probe. Typically, binding reactions were run in 20-μl volumes and contained 0.1–0.2 ng of labeled DNA (10,000–15,000 cpm), 5–8 μg of nuclear extract, and 1 μg of poly(dI-dC) under conditions described previously (37). Binding reactions with the bacterially expressed purified GR derivative X556 (GRBBD protein) were performed in a similar way except that 100–800 ng of protein, digested in a zinc-containing buffer (10 mM Tris, pH 7.6, 40 mM NaCl, 15 μM ZnCl2), was used (38). DNA-protein complexes were resolved on 5% non-denaturing polyacrylamide gels in 0.25× TBE (1× TBE: 89 mM Tris base, 89 mM boric acid, and 2 mM EDTA, pH 8.0). For supershift studies, standard amounts of unlabeled competitor DNAs were preincubated with the assay mixture for 10 min, at 25 °C, prior to the addition of labeled probes. For antibody supershift assays, 1 μl of BuGR2 monoclonal antibody (Affinity Bioreagents, Neshanic Station, NJ) or preimmune IgG was added to the reaction and incubated with the protein for 10 min at room temperature before the addition of DNA probe.
Synergistic Interaction between Ets2 and GR Proteins

Immunoprecipitation and Immunoblotting—Varied amounts of bacterially expressed GRDBD protein (35) and Ets2 protein were incubated in phosphate-buffered saline (20 mM KH2PO4, pH 7.0, 150 mM NaCl) for 30 min at room temperature to facilitate protein-protein interaction and immunoprecipitated with Ets2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) by the protein A-agarose (Sigma) binding method (39). In some experiments nuclear extracts from cells transfected with various cDNAs were used for communoprecipitation by the same procedure. The antibody-antigen complexes were dissociated at 56 °C for 1 h in nonreducing Laemmli’s sample buffer (42), and proteins were resolved on 10% SDS-polyacrylamide gel and transferred to Nytran membrane. The blot was successively probed with monoclonal antibody to GR (BUGR2) and polyclonal antibody to Ets2 by using the Pierce Super-glo chemiluminescence kit (Pierce) and visualized with a Bio-Rad FluorS imager. The Ets2 antibody used in this study also cross-reacted with Ets1 protein.

In Vitro Translation and Chemical Cross-linking—Various cDNAs cloned in pGEM 7zf vectors (Promega) were used as templates for generating 35S-labeled in vitro translation products in the rabbit reticulocyte cell-free translation system (Promega) according to the manufacturer’s protocol. Adx cDNA was cloned in pcRMT II vector as described elsewhere (40). 35S-Labeled translation products, 20,000 cpm in the case of GRDBD and Adx proteins or 50,000 cpm in the case of Ets1 and Ets2 proteins, were used for protein cross-linking. Cross-linking was carried out for 10 min at room temperature for 30 min, and the reaction was quenched by adding Tris-HCl, pH 7.5, to a final of 20 mM (in the case of DSP) or 100 mM Gly containing 5 mM -mercaptoethanol (in the case of sulfo-MBS). Immunoprecipitation of cross-linked products was carried out essentially as described before (39), except that the buffer system contained 1 mM iodoacetamide (iodoacetate, IAA). The immunoprecipitates were dissolved in 1 mM -mercaptoethanol (BME) in the case of DSP cross-linked products, by heating at 95 °C for 5 min. The cross-linked products were resolved by electrophoresis on SDS-polyacrylamide gels, and the gels were scanned through a Bio-Rad Molecular Imager.

RESULTS

Localization of Dx Response Region of the Rat CYP27 Promoter—It is known that Dx induces the rat hepatic CYP27 mRNA, specifically the shorter 2-kb species, by 15–20-fold of control animals (7). In the present study, the putative hormone response region of the CYP27 promoter was mapped by progressive 5'-deletion of the −841/+23CAT promoter construct, which contains essential elements for the expression of 2-kb mRNA (6). For this purpose we used NIH 3T3 cells, which efficiently supported the activity of the CYP27 promoter in our previous study (6). Fig. 1A shows the CAT activity of cells transfected with the full-length and deletion constructs under different conditions. A physical map of the promoter indicating the location of sequence motifs with full or partial consensus for binding to various transcription factors has been shown in the bottom panel of Fig. 1A. The activity of the full-length −841/+23CAT promoter construct was induced by about 3-fold either by cotransfection with GR cDNA or addition of 100 nM Dx. A combination of these two treatments, however, induced the transcription activity by about 6.5-fold. The −329/+23 CAT construct yielded a similar pattern of induction by cotransfection with GR cDNA and addition of Dx. Deletion to amino acid −194, however, drastically reduced promoter activity to a level lower than the activities of the −75/+23 and −45/+23 constructs. These results suggest the occurrence of a negative enhancer element within the −194 to −75 sequence region. The −75/+23 construct yielded less than half of the activity of the full-length promoter, whereas the −45/+23 construct yielded further reduced activity, which was previously shown to be the basal promoter activity (6). Results also show that deletion to −194 and beyond completely abolished responsive ness to GR and Dx, suggesting that the GHR region is located between sequence −329 and −194. As shown before (6), the activity of the full-length −841/+23 CAT construct was >70-fold of the pCAT basic plasmid DNA. Fig. 1B shows the nucleotide sequence of the putative GHR region (sequence −329 to −194), which includes a cryptic GRE (TGCTGT), two weak Ets sites (TGGGAG), an AP-1-like site, and an upstream Sp1-like site.

Protein Binding Property of the Dx Response Region of the Promoter—The nature of proteins binding to the Dx response region was studied by EMSA using the 135-bp (sequence −194 to −329) or 65-bp (−233 to −298) DNA probes. As shown in Fig. 2A, the 135-bp DNA probe formed a major complex (complex B) and a minor slow migrating complex (complex A), both of which were competed by 20-fold molar excess of unlabeled cryptic
GRE DNA (CYP27 GRE). Further, a 20-fold molar excess of Ets-like DNA motif from the 135-bp region of the promoter (TTGAGAACG) and also a high affinity Ets DNA motif (TCGGAGAC) competed with complex A, with marginal effects on complex B. The AP-1-like motif, on the other hand, failed to compete with either of the complexes. Although not shown, the Sp1-like sequence from upstream of the Ets-like sequence also did not compete with either of the complexes. Furthermore, antibody to GR but not preimmune IgG caused a supershifted complex, suggesting that a GR-related protein indeed binds to the 135-bp DNA probe. For yet unknown reasons the preimmune IgG marginally affected the intensity of complex A. The Ets2 protein by itself did not bind to the DNA probe, whereas a combination of GRDBD and Ets2 proteins resulted in the formation of a slow migrating complex (complex a), in addition to the GRDBD-specific complex b. Interestingly, addition of 20-fold molar excess of Ets DNA effectively abolished complex a formation without affecting complex b. These results suggest that Ets2 protein binding to the Ets-like site of the DNA probe is conditional, depending on the presence of GRDBD. This possibility was further investigated by testing the effects of variable concentrations of GRDBD on the extent of Ets2 protein binding using a more restricted DNA probe containing only the cryptic GRE and the downstream Ets-like sites. The gel shift in Fig. 2D shows that addition of GRDBD protein alone results in the formation of complex b, whose band intensity increases with increasing levels of the protein from 0.2 to 0.8 μg. Formation of complex a was observed only in the presence of added Ets2 and GRDBD and the extent of its formation was directly proportional to the amount of added GRDBD. Furthermore, 20-fold molar excess of cryptic CYP27 GRE DNA inhibited both complexes, whereas the cryptic CYP27 Ets2 DNA selectively inhibited complex a. Notably, mutations targeted to the core sequences of both GRE-like and Ets-like motifs drastically reduced their ability to compete with either of the complexes. These results further support the possibility that nucleation of the GRE site by GRDBD (complex b) is essential for Ets2 protein binding and that the slow migrating complex a represents a higher order complex containing the Ets2 protein. Results also show that both GRE-like and Ets-like motifs of the promoter are needed.

**Fig. 2.** Protein binding property of the Dx response region DNA by gel mobility shift analysis. A, EMSA using the 135-bp DNA probe (0.1 ng, 20,000 cpm) and 6 μg of nuclear extract from NIH 3T3 cells. The cryptic GRE, Ets-like, and AP-1-like DNA sequences were as shown in Fig. 1B except that each motif contained EcoRI and BamHI sequence overhangs used for cloning. A 20-fold molar excess of unlabeled DNA in each case was used for competition. The Ets motif consisted of a CCGAAG core sequence flanked by EcoRI and BamHI sites. 1 μg each of preimmune IgG or anti-GR-IgG (1 μl of antibody) was added in some reactions as indicated. B, EMSA using the cryptic GRE DNA probe (0.1 ng, 25,000 cpm) described for A and 50 ng of bacterially expressed purified GRDBD protein. 1 μg each of preimmune IgG or anti-GR IgG was used. SS represents supershifted complex. C, EMSA using the 135-bp DNA as in A and 50 ng each of bacterially expressed GRDBD and Ets2 proteins. A 20-fold excess of Ets DNA (CCGAAG core sequence) described in A was used for competition. D, EMSA using the 65-bp DNA containing only the cryptic GRE and the downstream Ets-like sequence carried out under excess probe conditions. EMSA was carried out in the presence of variable amounts of bacterially expressed and purified GRDBD (0–0.8 μg) and Ets2 (0–0.5 μg) proteins as indicated. A 20-fold molar excess of cryptic GRE and Ets-like sequence of the CYP27 promoter shown in Fig. 1B and the respective mutant DNAs as shown in Fig. 3B were used for competition. Details of probe preparation and conditions of protein binding and gel electrophoresis were as described under “Materials and Methods.”
for the formation of the higher order complex a.

Functional Analysis of GR and Ets Sites in Dx-mediated Transcription Stimulation—The functional importance of the cryptic GR, Ets, and other potential protein binding motifs was tested by mutational analysis. Mutations targeted to different protein binding motifs are shown at the bottom of Fig. 3. The promoter activity of wild type and mutant constructs in the presence or absence of added Dx and coexpression with full-length GR construct (p6RGR) were tested in 3T3 cells. As shown in Fig. 3, the transcription activity of the wild type 2329/123CAT construct was induced by about 5.5-fold in cells cotransfected with GR cDNA and added Dx. Interestingly, co-transfection with Ets2 and GR cDNAs in the presence of added Dx yielded a further increase in activity of about 9-fold as compared with the control. Cotransfection with Ets2 cDNA alone yielded a modest, albeit significant increase of activity (1.7-fold). The reporter construct with mutated GR site (2329/123CAT-GRmut) did not respond to cotransfection with GR and Ets2 cDNAs or added Dx. Additionally, mutations targeted to the cryptic Ets site, downstream of the GR site, also abolished the response of the promoter to GR and added Dx. Interestingly, cotransfection with Ets2 and GR cDNAs in the presence of added Dx yielded a further increase in activity of about 9-fold as compared with the control. Cotransfection with Ets2 cDNA alone yielded a modest, albeit significant increase of activity (1.7-fold). The reporter construct with mutated GR site (2329/123CAT-GRmut) did not respond to cotransfection with GR and Ets2 cDNAs or added Dx. Additionally, the overall promoter activity was reduced by about 80% of the control. Interestingly, mutations targeted to the cryptic Ets site, downstream of the GR site, also abolished the response of the promoter to GR and added Dx. As expected, a construct containing both mutated GR and Ets sites showed similar effects. The Ap1mut construct, however, showed the overall activity as well as responses to GR, Ets2, and also added Dx similar to the wild type construct. Although not shown, mutations targeted to the upstream Ets site did not have any effect on the promoter activity or inducibility with GR, Ets2, and Dx. In support of the EMSA results in Fig. 2, these results show that both the cryptic GR and the downstream Ets sites are important for Dx-mediated stimulation of promoter activity and that protein factors binding to these two motifs functionally synergize the transcription activation.

In companion experiments, we also investigated the requirements for specific domains of the GR and Ets2 proteins and possible synergistic activation by tissue-specific Ets1 protein. The nature of the full-length GR construct expressing p6RGR and the GRDBD construct used in cotransfection, treatment with Dx, and assay of CAT activity were as described under “Materials and Methods” and in the legend to Fig. 1. Results represent the averages and ± S.E. (n = 3).
Synergistic Interaction between Ets2 and GR Proteins

Ets1 also failed to show any synergistic activation with GR and Dx in combination.

Results in Fig. 4B show that coexpression with the GRDBD cDNA yielded a 3.5-fold higher transactivation of the −329/+23 CAT promoter construct, which was further increased to about 8-fold of control by cotransfection with Ets2 cDNA. As expected, addition of Dx did not have any additive effect on transcription activity induced by a combination of GRDBD and Ets2 cDNAs. Notably, tEts2 and tEts1 cDNA constructs lacking the transcription activation domain but containing the DNA-binding domain (32) alone or in combination with GR and Dx reduced the activity of the promoter by about 80%, showing a dominant negative effect. These results demonstrate that constitutively active GRDBD in the presence of Ets2 yields transcription stimulation nearly similar to that obtained with intact GR and Ets2 in the presence of added Dx. These results also show that although the transcription activation domain of Ets2 is highly essential for the synergistic activation of the promoter (Fig. 5), the transcription activation domain of GR may not be critical for the activity. Furthermore, as with the intact GR, the tissue-specific factor Ets1 was unable to show any synergistic activation with truncated GRDBD (Fig. 4B). Although not shown, the ubiquitously expressed GA-binding protein α and β factors were ineffective in synergistic activation with GRDBD, suggesting specificity.

**Dominant Negative Effects of tEts2 on the Expression of Endogenous CYP27 mRNA**—We investigated the effects of Dx and overexpression of GR on the CYP27 mRNA levels in Balb/c 3T3 cells. These cells are known to respond to glucocorticoid hormone and also express low, albeit measurable levels of GR protein (41). As shown in the Northern blot in Fig. 5A, addition of 100 nM Dx alone resulted in a 5–7-fold induction of 2-kb CYP27 mRNA under in vitro culture conditions. Similarly, transfection with 1 and 2 μg of full-length GR cDNA (p6RGR) without added Dx resulted in 5–6- and 15-fold induction, respectively. Addition of 100 nM Dx to cells transfected with 2 μg of GR cDNA caused a further increase in mRNA level to about 23-fold of control. The 2.3-kb mRNA is expressed at very low levels in these cultured cells, and the bands were visible only when the blots are overexposed (results not shown). These results demonstrate that the endogenous mouse CYP27 gene is under the regulation of GR and glucocorticoid hormones. The Northern blot in Fig. 5B shows that cotransfection with tEts2 cDNA reduced the endogenous mRNA levels by about 60–70% of the control cell level. Furthermore, coexpression with tEts2 cDNA also nearly completely abolished the GR2 + Dx-mediated induction of CYP27 mRNA levels from near 25-fold to a mere 1.8-fold of control (Fig. 5B, last lane). The level of ubiquitously expressed cytochrome oxidase subunit IV mRNA (Fig. 5B, COX IV mRNA) level was affected only marginally (less than 20%) by transfection with various cDNAs. These results suggest a physiological role for the transcription activation domain of Ets2 factor in the glucocorticoid hormone responsiveness of the promoter.

**Physical Interaction of GR and Ets2 Proteins**—Physical interaction between Ets family proteins and GRDBD under in vitro and in vivo conditions was studied by coimmunoprecipitation and chemical cross-linking. As shown in Fig. 6, indicated amounts of bacterially expressed and purified GRDBD and Ets2 proteins were preincubated and immunoprecipitated either with preimmune IgG or Ets2 antibody. The immunoprecipitates were successively probed with polyclonal antibody against Ets2 (Fig. 6A, upper panel) and monoclonal antibody to GR proteins (Fig. 6A, lower panel) by immunoblot analysis. Results show that preimmune sera failed to immunoprecipitate either of the proteins from the incubation mixture. Antibody to Ets2, on the other hand, coprecipitated the 17-kDa GRDBD (Fig. 6A, lower panel). A 2-fold increase of GRDBD in the incubation mixture resulted in a correspondingly increased amount of GRDBD coimmunoprecipitation by Ets2 antibody.
Conversely, a 2-fold increase of Ets2 protein in the incubation mixture resulted in the increased level of Ets2 protein accompanied by a marginal increase in the level of GRDBD in the immunoprecipitate. Thus, the coimmunoprecipitation is specific and varies depending on the input levels of GRDBD as well as Ets2 proteins. Although not shown, Ets1 protein also physically interacted with GRDBD as seen by antibody pull-down experiments.

The *in vivo* interaction between these proteins was studied by coimmunoprecipitation using nuclear extracts from cells transfected with various combinations of cDNA constructs. Fig. 6B shows that GRDBD failed to interact with Ets2 transcrip-
Synergistic Interaction between Ets2 and GR Proteins

Transcription activation domain (amino acids 40–280 of murine Ets2, TA-Ets2) because antibody to Ets2 immunoprecipitated only the TA-Ets2 and GR antibody immunoprecipitated only the 17-kDa GRDBD. The latter protein, however, efficiently interacted with wild type Ets2 or the DNA-binding domain of Ets2 because both pairs of proteins were immunoprecipitated by GR antibody. These results show that GRDBD interacts with Ets2 protein under in vivo conditions and that the interaction requires the DNA-binding domain of Ets2 protein. The results also indicate that transiently expressed truncated GRDBD, tEts2, and TA-Ets2 proteins are translocated into the nuclear compartment.

In the second approach, we used chemical cross-linking to test the physical interaction between GRDBD and Ets2 or Ets1 proteins, using DSP or sulfo-MBS, which show a high preference for positively charged and sulfhydryl-containing side chains. Furthermore, DSP is sensitive to treatment with reducing agents, such as BME, whereas sulfo-MBS is not. 35S-Labeled Ets2 or Ets1 proteins were incubated with 35S-labeled GRDBD in the presence or absence of DSP, and the products were immunoprecipitated with antibodies to either Ets2 or GR and resolved on polyacrylamide gels. In some experiments human adrenodoxin (Adx, see Ref. 40 for details) was used along with either Ets1, Ets2, or GRDBD proteins as negative controls. In this case immunoprecipitation was carried out with antibodies to either Adx, Ets2, or GR. Results in Fig. 7A, using DSP as the cross-linking agent show that rabbit or mouse preimmune sera did not immunoprecipitate either Ets1 or GRDBD proteins. Similarly, Adx and Ets1 antisera failed to produce any cross-linked products as tested by immunoprecipitation with Adx antibody as well as Ets2 antibody. Immunoprecipitation of the reaction mixture containing GRDBD and Ets2 proteins without added cross-linker, with GR antibody, yielded only the 17-kDa GRDBD protein, and that with Ets2 antibody yielded only the 50-kDa Ets1 protein. Immunoprecipitation of reaction mixtures with DSP, in the absence of added BME, on the other hand yielded an ~67-kDa cross-linked product with both of the antibodies. It is noted that both GRDBD and Ets1 translation products yielded double bands, possibly because of translation initiation from alternate ATG codons. The 67-kDa species detected in the immunoprecipitate (Fig. 7A, last lane) indeed represents the cross-linked product because use of Laemmli’s sample buffer (42) containing 5 mM BME dissociated the product into 50-kDa Ets1 and 17-kDa GRDBD proteins. Fig. 7B shows that reactions with 35S-labeled Ets2 protein also yielded a 69-kDa cross-linked product, except that in the case of GR antibody, an additional species of about 48 kDa was also immunoprecipitated. This latter species might represent an unknown protein from the reticulocyte lysate cross-linked with GRDBD. These results suggest that both Ets1 and Ets2 can physically interact with GRDBD even when they are not bound to DNA. Although not shown, sulfo-MBS also yielded similar cross-linked products, except that they were resistant to treatment with BME.

Functional Interaction between Ets2 and GR and Possible Sharing of Domains—To test the roles of the DNA-binding domain of GR and transcription activation domain of Ets2 in the synergistic activation, we constructed fusion proteins consisting of the wild type or mutated GRDBD with C-terminally fused transcription activation domains from Ets2 or Ets1. These proteins were tested for transcription activation of wild type and various mutant promoter constructs. As shown in Fig. 8, the GRDBD-Ets2 fusion construct was able to stimulate the activity of the wild type −329/+23CAT promoter by about 8-fold, which is nearly similar to the synergistic activation obtained by cotransfection with GRDBD and Ets2 cDNAs. The GRDBD-Ets1 fusion construct, by contrast, yielded only about 2-fold activation. The fusion construct with a deleted zinc finger region of GRDBD (GRDBDΔZF-Ets2), which is known to affect its binding to GRE DNA (35, 43), also drastically reduced transcription activation. Furthermore, deletion of most parts of the transcription activation domain of Ets2 (GRDBD-Ets2Δ200) also reduced the transcription activation to near basal level. Furthermore, transactivation by the GRDBD-Ets2 fusion construct was completely abolished by mutations targeted to the cryptic GRE site alone or a combination of cryptic GRE and Ets-like sites of the promoter. In contrast, mutations targeted to the Ets-like site alone did not affect the extent of activation by GRDBD-Ets2 fusion construct. However, fusion constructs GRDBDΔZF-Ets2 and GRDBD-Ets2Δ200 with the mutated DNA-binding domain or transcription activation domains failed to induce transcription of the promoter above the basal activity. These results strongly suggest a novel physical association and domain sharing between DNA-bound GR and Ets2 factors. These results also suggest that transcription activation is highly dependent on the transcription activation
Fusion cDNA constructs (1 activation of the CYP27 promoter. The activation domain of Ets2 proteins in the transcription machinery, which includes the TFIIID complex and polymerase II holoenzyme complex (46, 47). In this paper we demonstrate that DxD-mediated transcription activation of the rat CYP27 gene involves a cooperative interaction between GR and Ets2 factors. Functional synergies between gene/tissue-specific and ubiquitous housekeeping varieties of sequence-specific DNA-binding transcription factors can be classified into the following categories. The first type involves ligand or physiological pathway-specific transcription factors and ubiquitous constitutive factors binding to the same sequence motif as heteromeric complexes. Examples of heteromorphic factors include myogenic factors MyoD and E2A (38), proteins belonging to the Ap1/Ap2/CAAT family (Jun/Fos, NFATc and CEBP) (48–50), and ligand-specific factors belonging to the steroid superfamily nuclear receptors (51). The second type involves cooperativity between the same class or homologous factors bound to tandemly duplicated DNA-binding sites leading to functional synergy. Transcription activation of promoter sites with duplicated binding sites by GR (51, 52) and Ets family GABP factors (53, 54) belong to this class. The third type involves synergy between transcription factors bound to spatially separated DNA sites possibly through interaction with common coactivator proteins. Synergistic activation of the low density lipoprotein receptor promoter by sterol response element-binding protein (SREBP) and the ubiquitous factor Sp1 through interaction with coactivator cAMP-response element-binding protein-polymerase-binding protein is a classical example of this type (52, 55). The fourth type of functional synergy is seen in the recruitment of factor Pu.1 interacting protein by DNA-bound PU.1 or E2A in the activation of immunoglobulin κ enhancer (56, 57). The association of Pu.1 interacting protein with the promoter sites appears to involve both protein-protein interaction with DNA-bound PU.1 or E2A (56, 57) and also direct interaction with DNA. Thus, the functional synergy between the GR and Ets2 proteins observed in this study shares similarities with the last two mechanisms described above.

Physical and functional analyses of the DxD-responsive region of the promoter by site-directed mutations (Figs. 1 and 3) suggest that the cryptic GRE motif and a downstream Ets-like motif are important for DxD-mediated transcription activation of the promoter. Cotransfection with GR and Ets2 cDNAs resulting in synergistic activation of CAT activity further substantiated the role of these cis-DNA elements (Figs. 1, 3, and 4). In support of these functional data, the cryptic GRE motif (TGCTGT) bound to proteins from nuclear extracts, which cross-reacted with GR-specific antibody as seen by a supershift in EMSA (Fig. 2A). Additionally, bacterially expressed purified GRDBD also bound to this motif (Fig. 2B). However, the Ets-like motif by itself failed to form a detectable complex with both purified Ets2 protein (Fig. 2C) and 3T3 nuclear extract (not shown). These results are in full support of general consensus in the field that the core sequences (G/T)GGAA(T/A) are weak
motifs for binding to ubiquitously expressed Ets2 and GABP (variably called NRF2) as well as tissue-specific Ets1 and other members of Ets family proteins (58–60). EMSA with the nuclear extract and 135-bp hormone response region DNA probe also yielded a slow migrating complex, which was competed by both cryptic GRE and Ets consensus DNA (Fig. 2, A and C), suggesting that GR binding to the cryptic GRE site helps recruiting an Ets family protein. Use of bacterially expressed purified proteins and competition with site-specific DNA indeed supported this possibility (Fig. 2C). Direct evidence for the functional role of Ets2 factor and the physiological significance of its in vitro functional synergy with GR is presented by experiments showing the dominant negative effects of tEts2 (only the DNA-binding domain of Ets2) on the activity of the promoter construct and also the endogenous CYP27 gene expression in 3T3 fibroblasts.

Ets2 factor binding to the hormone response region of the promoter is dependent on GR or GRDBD bound to the cryptic GRE motif as well as the downstream Ets-like sequence motif, the latter thought to be a weak consensus sequence for binding to Ets family proteins (58, 59). This conclusion is based on EMSA results showing that slow migrating complex a obtained with purified GRDBD and Ets2 proteins (Fig. 2, C and D) was effectively competed by Ets-like DNA but not by a mutant form. A DNA probe carrying mutations at the Ets-like sequence of the GHR region probe formed only complex b but not the higher order complex a even in the presence of added GRDBD and Ets2 proteins (results not shown). Similarly, the intact Ets-like sequence motif is required for the synergistic activation of the promoter by cotransfection with GR and Ets2 cDNAs (Fig. 3). Results of cotransfection also show that only the Ets2 protein with an intact DNA-binding domain, but not the deletion mutant lacking this domain, is able to induce transcription activation (results not presented). These results suggest the need for DNA binding by Ets2 protein as an essential part of the functional synergy with the adjacent bound GR. Thus, a novel finding of this work relates to the role of the putative weak Ets consensus site in the transcription activation. The putative weak Ets consensus sites are widely distributed on many promoters. In view of our results, the roles of the ubiquitously expressed Ets2 protein and the weak consensus Ets-like sites, particularly those located in the close proximity of other sequence-specific factor binding sites, need to be further evaluated.

It is known that Ets1 and Ets2 factors can interact with members of Jun/Fos and other AP-1 family proteins (16–19, 48) and also other leucine zipper proteins such as Myb (15, 32), modulating their transcription activity. Results of coimmunoprecipitation (Fig. 6) and chemical cross-linking experiments (Fig. 7) reported in this study for the first time demonstrate a direct physical interaction between GRDBD and Ets2 as well as Ets1 proteins. Furthermore, the interaction requires the DNA-binding domains of both proteins because the transcription activation domain of Ets2, lacking the DNA-binding domain, showed no detectable interaction with the GRDBD protein. Thus, it is likely that the two factors interact physically in their DNA-bound form on the hormone response region of the CYP27 promoter. It is known that GRDBD, which lacks the ligand-binding domain, has a constitutively activated DNA binding activity (61). It was, however, surprising that GRDBD lacking both the N- and C-terminal activation domains (62, 63) and the ligand-binding domain was able to synergistically activate the promoter with Ets2 (Fig. 4B). The possible requirement for physical interaction between GRDBD and Ets2 for functional synergy was further supported in experiments showing that a fusion protein containing GRDBD and the Ets2 transcription activation domain could efficiently activate the promoter. Interestingly, mutation of the weak Ets-like site did not affect the fusion protein-induced activity as long as the cryptic GRE site was intact. Notably, the GRDBD-Ets2 fusion construct with mutated zinc finger domains of GRDBDB showed vastly reduced transcription activation of the promoter. Based on these results, we hypothesize that the role of the GR or GRDBD bound to the cryptic GRE of the promoter is to help recruit the Ets2 protein to the complex. Interaction with GR may induce conformational change(s) in the Ets protein (64) such that it can bind to the weak Ets consensus site. Apparently, binding of Ets2 factor to the weak Ets site alone in the absence of adjacent bound GR is not strong enough for resolution of the complex through the gel matrix in EMSA. Thus, the requirement for physical interaction between GR and Ets2 for functional synergy is uniquely different from the synergistic activation by SREBP and Sp1, which does not involve intermolecular protein-protein interaction (55). The requirement for the transcription activation domain of only Ets2 but not that of GR for synergistic interaction is reminiscent of the synergy between Pu.1 and Pu.1 interacting protein, Fos, and Jun for the activation of immunoglobulin κ 3’ enhancer (65).

A current model on the mechanism of synergistic activation by SREBP and Sp1 suggests that the transactivation domains of both of the proteins are essential for transactivation (50). It is proposed that the transcription activation domain of Sp1 may be involved in interaction with the basal transcription machinery, whereas that of SREBP might be involved in recruiting factors with HAT activity (50). The synergy observed with GR and Ets2 factors, on the other hand, appears to be highly dependent on the transcription activation domain of the latter, without requiring the presence of the former. It should be noted that tissue-specific Ets1 factor also binds to the promoter DNA in a GRDBD-dependent manner (results not shown) and physically interacts with GRDBD as tested by coimmunoprecipitation and chemical cross-linking (Figs. 7 and 8). However, cotransfection with Ets1 and GR or GRDBD cDNAs failed to show any synergistic activation of the −329/+23CAT promoter. Similarly, cotransfection with GRDBD-Ets1 fusion protein failed to show significant transcription activation of the promoter. These results suggest that the transcription activation domain of Ets2 protein is critical for the synergistic activation. Although the DNA-binding domains of Ets1 and Ets2 proteins share over 90% sequence homology, the N-terminal 300-amino acid region comprising the transcription activation domains of the two proteins shares only about 35% sequence identity. Notably, the transcription activation domain of Ets2 protein contains higher hydrophobic helical content and higher glutamine contents. It is likely that the structural features of Ets2 protein may be essential for interaction with components of basal transcription machinery or with specific coactivator proteins. In summary, we describe a novel functional synergy between GR and Ets2 proteins in the glucocorticoid hormone-dependent activation of the P-450c27 promoter.

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Physical Interaction and Functional Synergy between Glucocorticoid Receptor and Ets2 Proteins for Transcription Activation of the Rat Cytochrome P-450c27 Promoter


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