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A CBP Integrator Complex Mediates Transcriptional Activation and AP-1 Inhibition by Nuclear Receptors

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Summary

Nuclear receptors regulate gene expression by direct activation of target genes and inhibition of AP-1. Here we report that, unexpectedly, activation by nuclear receptors requires the actions of CREB-binding protein (CBP) and that inhibition of AP-1 activity is the apparent result of competition for limiting amounts of CBP/p300 in cells. Utilizing distinct domains, CBP directly interacts with the ligand-binding domain of multiple nuclear receptors and with the p160 nuclear receptor coactivators, which upon cloning have proven to be variants of the SRC-1 protein. Because CBP represents a common factor, required in addition to distinct coactivators for function of nuclear receptors, CREB, and AP-1, we suggest that CBP/p300 serves as an integrator of multiple signal transduction pathways within the nucleus.

Introduction

Nuclear receptors are ligand-dependent transcription factors that regulate diverse aspects of growth, development, and homeostasis by binding as homodimers or heterodimers to their cognate DNA elements (Yu et al., 1991; Bugge et al., 1992; Kliewer et al., 1992; Leid et al., 1992; Marks et al., 1992; Zhang et al., 1992; Chambon, 1994; Tsai and O'Malley, 1994; Beato et al., 1995; Mangelsdorf et al., 1995). Transactivation by nuclear receptors depends on a conserved motif within the distal C-terminus, referred to as the AF2 domain. Several lines of evidence indicate that nuclear receptors must interact with additional factors dependent on a conserved distal C-terminal motif (AF2) to mediate both activation and repression of gene expression (Cavailles et al., 1994; Halachmi et al., 1994; Baniahmad et al., 1995; Hörlein et al., 1995; Chen and Evans, 1995; Kurokawa et al., 1995). Biochemical assays have identified 140 and 160 kDa proteins (p140 and p160) (Halachmi et al., 1994; Cavailles et al., 1994; Kurokawa et al., 1995) that associate with estrogen, retinoic acid, thyroid hormone, retinoid X, and potentially other nuclear receptors as the most prominent ligand-dependent putative coactivators, binding in an AF2-dependent fashion. In addition, a series of proteins exhibiting ligand-dependent interactions with the C-termini of nuclear receptors that may also function as coactivators have been identified using a yeast two-hybrid screen, including TRIP-1, a homolog of the SUG-1 (p46) protein (Lee et al., 1995), TIF1 (p120) (Le Douarin et al., 1995), SRC-1 (p115) (Oñate et al., 1995), and RIP140 (p140) (Cavailles et al., 1995), as well as other less well-characterized proteins.

Several members of the nuclear receptor family, including glucocorticoid receptor (GR), retinoic acid receptor (RAR), thyroid hormone receptor (T_3R), vitamin D receptor, and retinoid X receptor (RXR) (reviewed by Beato et al., 1995), have been reported to inhibit AP-1 activity (Jonat et al., 1990; Yang-Yen et al., 1990; Schüle et al., 1990; Lucibello et al., 1990; Tzukerman et al., 1991; Salbert et al., 1993) without inhibition of DNA binding in vivo (König et al., 1992). AP-1 activity is mediated by activation of various heterodimers, particularly Jun and Fos or related proteins (reviewed by Abate and Curran, 1990; Angel and Karin, 1991), involving molecular mechanisms similar to that involved in regulating the cAMP response element–binding protein (CREB) (Arias et al., 1994). CREB functions as a homodimer, in many cases requiring phosphorylation of Ser-133 in the kinase-inducible domain to serve as a transactivator (Gonzalez et al., 1991). These observations prompted an expression screening approach that identified a 265 kDa CREB-binding protein, referred to as CBP, that interacted strongly with phosphorylated CREB (Chrivia et al., 1993; Kwok et al., 1994). A protein highly related to CBP, p300, was independently identified based on interactions with EIA (Eckner et al., 1994). These two proteins share extensive regions of homology throughout, including regions of interaction with CREB (Chrivia et al., 1993), E1A (Lundblad et al., 1995; Arany et al., 1995), TFIIB (Kwok et al., 1994), and c-Fos (Bannister and Kouzarides, 1995), as well as a bromodomain (Arany et al., 1994). Several independent approaches, involving cotransfection assays or microinjection of anti-CBP antibody and reporter, have revealed that CBP is required for activation of both CREB and AP-1 (Kwok et al., 1994; Arias et al., 1994).

In this paper, we demonstrate that the ligand-binding domains of multiple nuclear receptors, including RAR and GR, interact strongly in the cell with a conserved domain in the N-terminus of CBP and p300 in a ligand-dependent manner. Further, the putative coactivator p160 was found to interact independently and specifically with a conserved C-terminal domain in CBP and p300. Expression cloning of p160 cDNAs based on nuclear receptor and CBP interaction surprisingly revealed their identity as variants of SRC-1. Several independent experimental approaches have suggested a central role for CBP in ligand-dependent activation of RAR and T_3R, comparable to its role for CREB and Jun/Fos. Our data

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indicate competition for limiting amounts of CBP may account for many of the inhibitory actions of both GR and RAR on AP-1 activation. We propose that CBP/p300 is a required component common to the coactivator complexes of diverse transcription factors, each utilizing specific additional coactivators, thus providing a mechanism for integrating in the nucleus diverse signaling pathways in the maintenance of homeostasis.

Results

Nuclear Receptors Directly Interact with CBP

To investigate possible direct interactions between nuclear receptors and CBP, a series of overlapping fragments of the 265 kDa CBP protein were bacterially expressed as glutathione S-transferase (GST) fusion proteins and tested for interaction with RAR, revealing a strong ligand-dependent interaction confined to a single N-terminal CBP region (Figure 1A). The ability of this interaction to occur in intact cells in a ligand-dependent manner was confirmed using the yeast two-hybrid system (Figure 1B). Further mapping revealed that the N-terminal 100 amino acids of CBP retained full interaction with RAR; attempts to subdivide further this region resulted in a complete loss of the capacity to interact.

Consistent with the ability of several nuclear receptors to inhibit the activated AP-1 transcriptional response, we found that estrogen receptor (ER), T_3R, and RXR also exhibited strong ligand-dependent interactions with the identical N-terminal domain of CBP, dependent upon a functional AF2 domain (Figure 1C). Intriguingly, a point mutation outside of the AF2 domain that conferred dominant-negative functions to the RAR (Gly-303→Glu) (Saitou et al., 1994) also abolished interactions with CBP (Figure 1C). Together, these data indicate that CBP binds to the C-terminal domain of ligand-occupied nuclear receptors. These interactions were demonstrated to occur on DNA using the protein-dependent gel mobility shift assay, in which addition of an N-terminal fragment of CBP (amino acids 1–450) caused a marked supershift of the RAR–RXR–DNA complex only in the presence of ligand (Figure 1D). The specificity of this interaction was established because the region of CBP interacting with phosphorylated CREB (amino acids 450–720) failed to supershift RAR–RXR–DNA complexes (Figure 1D).

To determine whether complexes between RAR and the CBP holoprotein occurred in the context of the intact cell, a vector encoding CBP with a C-terminal FLAG epitope was constructed and expressed in a variety of cell types. Potential complexes were immunoprecipitated using RAR-specific antisera, fractionated on SDS–polyacrylamide gels, and analyzed by Western blot using specific monoclonal anti-FLAG antibodies. These assays established that ligand-dependent interactions between RAR and CBP occurred in vivo (Figure 1E). Furthermore, specific in vivo interactions with GR and RXR were demonstrated (Figure 1E).

CBP Modulates Ligand-Dependent Transcriptional Activation

Based on the in vivo interaction between the nuclear receptors and CBP, it became of interest to evaluate the potential role of these interactions in ligand-dependent functions of nuclear receptors. Although CBP is widely expressed, its low levels are rate limiting for CREB-mediated transcription, which permitted cotransfection assays to evaluate a role for CBP in CREB-dependent transcription (Kwok et al., 1994). We therefore examined ligand-dependent transcription from a retinoic acid response element (RARE) containing promoter in the presence and absence of coexpressed CBP. Expression of CBP led to a 3-fold further enhancement of retinoic acid–dependent transcription, but had little or no effect on promoters lacking a RARE (Figure 2A). To acquire further, independent evidence for a potential role of CBP in RAR-mediated transcription, we performed in vitro transcription assays using nuclear extracts prepared from cells with varied levels of CBP. To be certain that the effects of CBP were exerted through the ligand-binding domain of nuclear receptors, we utilized a GAL4–T_3 receptor fusion protein (Baniahmad et al., 1995). The ability of this fusion protein to activate the tk promoter–containing GAL4-binding elements (17-mer upstream activating sequence [UAS]) was assessed using GAL4 protein as a control. T_3-induced transcription of this UAS-driven reporter was increased >5-fold by additional CBP, while altered levels of CBP had little effect on transcription of several control promoters (Figure 2B). Increased levels of CBP also markedly increased AP-1 site-dependent reporter expression, consistent with the proposed role of CBP in AP-1 transactivation events (Arias et al., 1994) and indicating that CBP is limiting in extracts used in in vitro transcription assays.

Based on these suggestive results, the role of CBP, or highly related factors, in ligand-dependent transcriptional activation was directly tested by evaluating the ability of microinjected anti-CBP immunoglobulin G (IgG) to inhibit ligand-dependent transcription from RARE-containing promoters in intact cells. In each experiment, >200 cells were injected with lacZ reporter plasmids and either control or anti-CBP IgG; cells were treated with all-*trans* retinoic acid and stained with X-Gal, and positive cells were counted. In these assays, addition of a ligand was required for effective expression of two independent RARE-dependent reporters, but did not affect other promoters not containing RAREs (Figure 2C). In three independent experiments, coinjection of increasing concentrations of anti-CBP IgG, but not control IgG, resulted in a progressive and specific decrease in ligand-dependent gene activation, producing >80% inhibition of ligand-induced activation. Even in the remaining 20% of cells that scored positive, there was often a reduced signal compared with levels in cells injected with control IgG. These data revealed that, in a concentration-dependent fashion, anti-CBP IgG specifically inhibited ligand-dependent activation of transcription units containing RAREs, without altering expression of other promoters.

The Nuclear Receptor–Associated p160 Coactivator Directly Interacts with CBP

Because CBP or its related family members (or both) were required for transactivation by retinoic acid and other nuclear receptors, we investigated the possibility that putative nuclear receptor coactivators p140 or p160

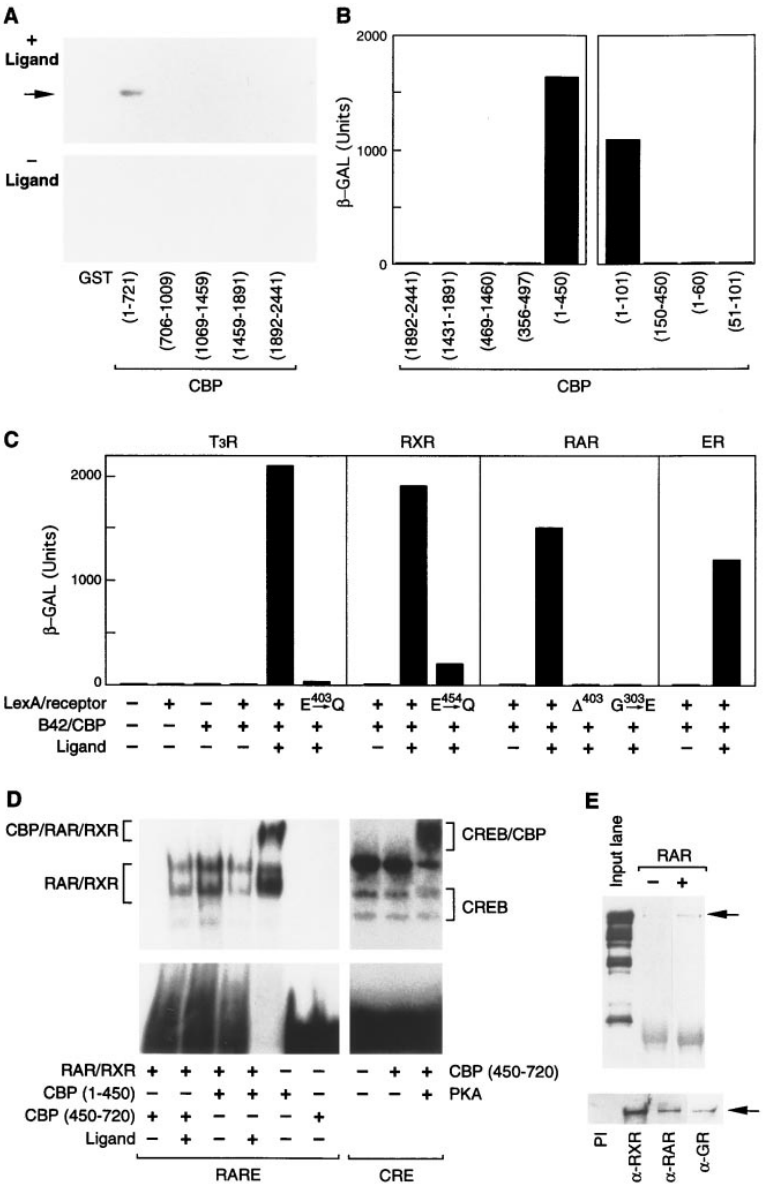

Figure 1. Ligand-Dependent Interactions between CBP and Nuclear Receptors In Vivo and In Vitro

(A) Specific regions of CBP interact with liganded RAR and ER C-terminal domains. A series of GST–CBP fusion proteins encompassing the entire CBP protein or GST alone were subjected to SDS–PAGE, transferred to nitrocellulose, and tested for their ability to interact with ^{32}P-labeled ER C-terminus in the absence or presence of 10^{-6} M β-estradiol. Equal amounts of protein were loaded. The arrow indicates the CBP fragment that interacts with ER.

(B) The yeast two-hybrid system reveals that CBP contains a domain that specifically interacts with RAR in the presence of ligand (10^{-7} M TTNPB). Fragments encompassing the entire CBP protein were used to generate in-frame fusions with the B42 activation domain. LexA human RARα (amino acids 143–462) was used as a bait. β-Galactosidase activity was assayed from Saccharomyces cerevisiae (EGY48) containing the pSH18-34 reporter plasmid to assess in vivo interactions (Gyuris et al., 1993).

(C) Several members of the nuclear receptor gene family interact with CBP in the yeast two-hybrid system. CBP N-terminus (amino acids 1–450) was used as a prey. Interaction of LexA fusion proteins encompassing the C-terminal ligand-binding domains of T_3R, RXR, RAR, and ER was measured in the presence or absence of the appropriate ligands. T_3R Glu-403→Gln contains a mutation in the AF2 region. RAR Δ403 has a C-terminal deletion at amino acid 403 of RARα (Damm et al., 1993). RAR Gly-303→Glu is a dominant-negative mutation (Saitou et al., 1994), and RXR Glu-454→Gln contains a mutation in the AF2 region that renders it incapable of gene activation. β-Galactosidase activity was determined as described above. Ligands used were TRIAC (10^{-7} M) for T_3R, TTNPB (10^{-7} M) for RAR, LG69 (10^{-7} M) for RXR, and β-estradiol (10^{-7} M) for ER.

(D) CBP exhibits ligand-dependent interaction with RAR–RXR heterodimers on DNA. Interaction on DNA was assessed using a ^{32}P-labeled synthetic oligonucleotide encompassing the β-RARE and bacterially expressed, purified CBP N-terminus (amino acids 1–450). Brackets indicate RAR–RXR-bound DNA and the CBP–RAR–RXR–DNA complex. No mobility perturbation was observed using a comparable amount of the CREB-binding domain of CBP (amino acids 450–720). Efficacy of the CREB-binding domain of CBP was confirmed by its ability to bind protein kinase A–phosphorylated, but not unphosphorylated, CREB.

(E) Ligand-dependent interactions between CBP and nuclear receptors in the intact cell. FLAG-tagged CBP was expressed by transient transfection in 293 cells. Anti-RAR, RXR, or GR antisera were used for immunoprecipitation of whole-cell extracts prepared from these cells in the presence or absence of 10^{-7} M all-*trans* retinoic acid, 9-*cis* retinoic acid, or dexamethasone (DEX). Coimmunoprecipitation of CBP was examined by Western blot analysis using anti-FLAG antibody. The arrows indicate FLAG–CBP. Note that only full-length CBP, containing the N-terminal nuclear receptor interaction domain, but not the breakdown products, is coimmunoprecipitated.

could themselves interact with CBP. A series of GST fusion proteins spanning the entire sequence of CBP were used in interaction assays with extracts from various cell types. Proteins interacting with CBP fragments were fractionated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), transferred to nitrocellulose membranes, and detected using ^{32}P-labeled ER C-terminus in the presence of ligand. As expected, the N-terminal fragment of CBP interacted directly with nuclear receptors. In addition, a specific interaction was identified between the C-terminal region of CBP and p160 (Figure 3A), but not with p140. These regions are distinct from the internal regions of CBP that interact with CREB, Fos, TFIIB, and E1A (Chrivia et al., 1993; Kwok et al., 1994; Arany et al., 1995; Bannister and Kouzarides, 1995; Lundblad et al., 1995). To define better the determinants of p160 interaction, a series of fragments were generated from the CBP C-terminus, revealing a region of 105 amino acids that was sufficient for interactions with p160 (Figure 3A).

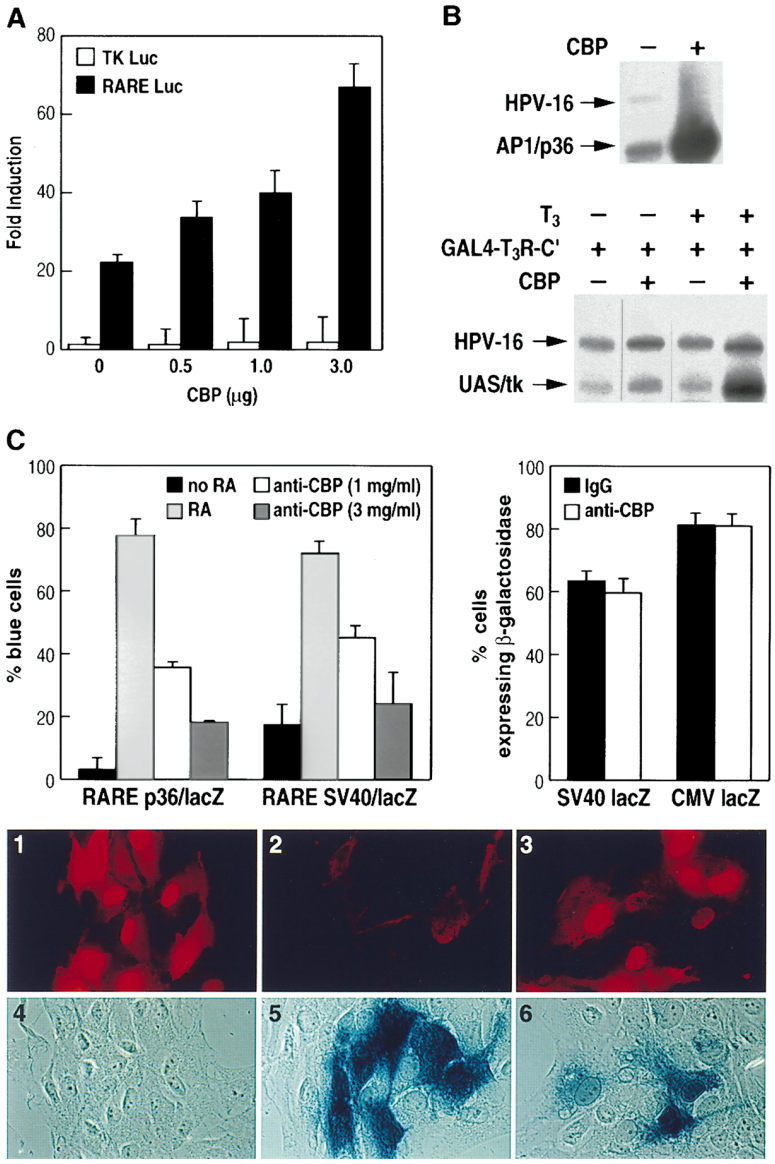

Figure 2. Role of CBP in RAR and T_3R Gene Activation Events

(A) Cotransfection of CMV–CBP plasmids in HeLa cells enhances RAR-mediated transactivation.

(B) Increased CBP levels potentiate hormone-dependent transactivation by a GAL4–T_3R C-terminus fusion protein. In vitro transcription assays were performed with luciferase reporters driven by 3× UAS(GAL)–tk, human papilloma virus 16 (HPV-16) or a 12-O-tetradecanoylphorbol-13-acetate (TPA) response element upstream of the prolactin minimal promoter (Drolet et al., 1991) (TRE–p36). Increased CBP levels greatly enhanced the TRE-driven reporter, consistent with published results. Elevated CBP levels also increased transcription of the UAS(GAL)–tk-driven reporter by GAL4–T_3R C-terminus fusion protein in the presence of hormone. In contrast, a viral promoter (HPV-16) containing an Sp1 site was not activated by elevated CBP levels.

(C) Microinjection of anti-CBP antibody abrogates ligand-dependent gene activation by the RAR. Plasmids consisting of a lacZ reporter under the transcriptional control of RAREs were injected into the nuclei of Rat-I cells. RARE simian virus 40 (SV40) lacZ contains three copies of the DR+5 element upstream of the SV40 minimal promoter, whereas the RARE–p36 lacZ contains two copies of DR+5 upstream of the prolactin minimal promoter (Drolet et al., 1991). Each construct was injected with preimmune IgG (no retinoic acid [RA]) or with affinity purified anti-CBP antibody and demonstrated a retinoic acid–dependent response. The expression of the reporter plasmids was monitored by X-Gal staining and quantitated based on the percentage of injected cells that stained blue. Coinjection of the same anti-CBP antibody did not inhibit expression of the reporters driven by either the SV40 or CMV viral enhancers. Photomicrographs of rhodamine-stained injected cells (1–3) and the corresponding phase-contrast picture (4–6) of X-Gal staining show a typical result. In the absence of retinoic acid (1 and 4), background expression of β-galactosidase is minimal. Addition of 10^{-6} M retinoic acid (2 and 5) results in a strong transcriptional response, which is significantly reduced in the presence of anti-CBP (3 and 6). X-Gal staining in some cells was sufficient to quench the rhodamine fluorescence (as in 2 and 5); therefore, the number of injected cells was counted as the sum of red or blue cells. All experiments were performed at least three separate times in which 200 or more cells were injected; error bars represent the SEM.

In addition, ^{32}P-labeled CBP C-terminus could detect p160 in a biochemical interaction assay (Figure 3B). These observations raised the possibility that RAR, CBP, and p160 could potentially form a ligand-dependent complex. Because both p160 and nuclear receptors interact with distinct domains in CBP holoprotein, it was possible to devise an assay that would unambiguously demonstrate the potential simultaneous interaction of nuclear receptors with both p160 and CBP. To achieve this, we used a GST fusion protein containing only the N-terminus of CBP to bind nuclear receptors in the presence of cellular extracts containing p160. The data shown in Figure 3C utilized the T_3R. Interacting protein was probed on Western blots using ^{32}P-labeled ER C-terminus. These experiments revealed that ligand-dependent simultaneous interactions between p160 and CBP-associated T_3R or RAR could occur (Figure 3C; data not shown).

cDNAs encoding the putative p160 were obtained by expression cloning based on the criterion that phage plaques exhibited interaction with both the CBP C-terminus and liganded nuclear receptors (Figure 4). As illustrated in Figure 4D, a series of cDNAs were identified that encoded variant forms of the SRC-1 protein reported to have a predicted molecular mass of 115 kDa (Oñate et al., 1995). The N-terminally extended variants that we identified included forms of 1465 and 1405 amino acids, with predicted molecular masses of 159 and 152 kDa. This additional N-terminal sequence has revealed a homology with the A region of the PAS domain characteristic of the PAS/helix-loop-helix (HLH) gene family (reviewed by Hankinson, 1995). In addition, related factors

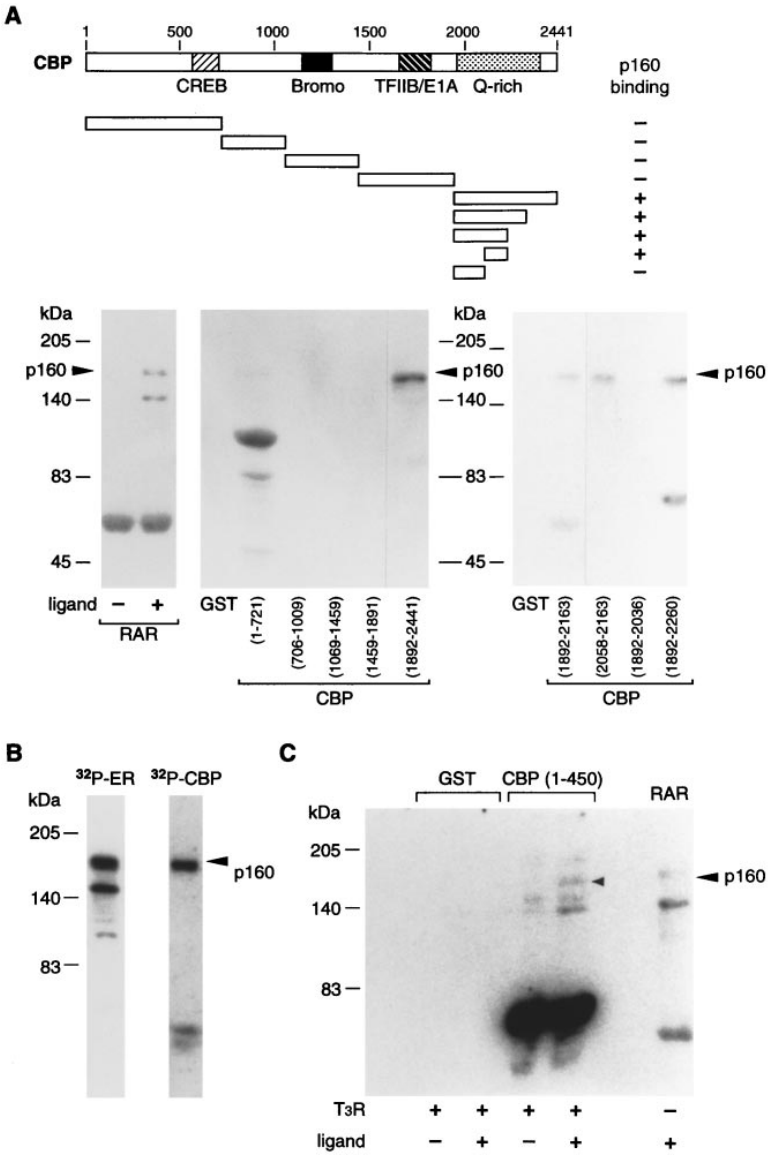

Figure 3. Interactions between CBP and the Nuclear Receptor–Associated p160 Coactivator

(A) p160 interacts with the C-terminus of CBP. The relative position of the CREB-binding domain, bromodomain, E1A, and TFIIB interaction domain and the glutamine-rich region in CBP are schematically represented. CV-1 whole-cell extracts were incubated with a series of GST–CBP fusion proteins, immobilized on glutathione–agarose. The whole-cell extracts were also incubated with GST–RAR C-terminus in the absence or presence of 10^{-6} M all-*trans* retinoic acid. After washing, specifically associated proteins were resolved by SDS–PAGE, transferred to nitrocellulose, and detected using a [^{32}P]GST–ER C-terminus in the presence of ligand. The numbers at the left indicate molecular size markers (kilodaltons).

(B) Whole-cell extracts were incubated with a ligand-treated GST–ER bound to glutathione–agarose, and associated proteins were tested for interaction as described for (A) with either [^{32}P]GST–ER in the presence of ligand or [^{32}P]GST–CBP C-terminus (amino acids 1892–2441).

(C) Nuclear receptor, CBP, and p160 can simultaneously form a ligand-dependent protein complex. A GST fusion protein containing only the N-terminus of CBP (amino acids 1–450) was incubated with bacterially expressed T_3R in the presence or absence of ligand (10^{-6} M TRIAC) and whole-cell extracts. After washing, specifically associated proteins were resolved by SDS–PAGE, transferred to nitrocellulose, and detected using [^{32}P]GST–ER in the presence of ligand as described. The control for identification of p160 was provided by analyzing proteins from the same whole-cell extract bound to GST–RAR in the presence of ligand.

exhibiting ~40% identity in the CBP and nuclear receptor interaction domains were identified (data not shown). Antiserum raised against an internal 50 kDa region common to all the recombinant SRC-1 variant proteins was found to interact specifically with a 160 kDa protein doublet in whole-cell extracts that specifically interacted with GST–RAR in a ligand-dependent fashion (Figure 4B). Immunodepletion experiments demonstrated that approximately 75% of the biochemically defined p160 could be removed from HeLa whole-cell extracts by this antiserum (Figure 4B), indicating that the l59 kDa variant of SRC-1 is the biochemically identified p160. The N-terminus of the cloned SRC-1 protein was capable of forming a ternary complex with CBP and a liganded nuclear receptor (Figure 4C); we have also observed association between SRC-1-related factors. Cotransfection experiments confirmed that the SRC-1 variant potentiated the activity of RAR (data not shown) in concert with effects of SRC-1 on several members of the nuclear receptor family reported by Oñate et al. (1995), indicating that p160 functions as a coactivator.

Both Nuclear Receptors and p160 Interact with Conserved Motifs on CBP and p300

While p300 was initially described based on its interaction with E1A (Eckner et al., 1994), recent data confirmed its ability to interact with both CREB and Jun/Fos; conversely, CBP interacts with E1A (Arany et al., 1995; Lundblad et al., 1995). Although the regions of CBP found to mediate interaction with nuclear receptors and p160 were not initially recognized as regions of high homology, comparison of the N- and C-terminal regions of CBP involved in these interactions with the corresponding region of p300 revealed multiple clusters of complete identity in both the nuclear receptor interaction domain (amino acids 1–101, CBP; amino acids 1–117, p300) and in the p160 interaction domain (amino acids 2058–2163,

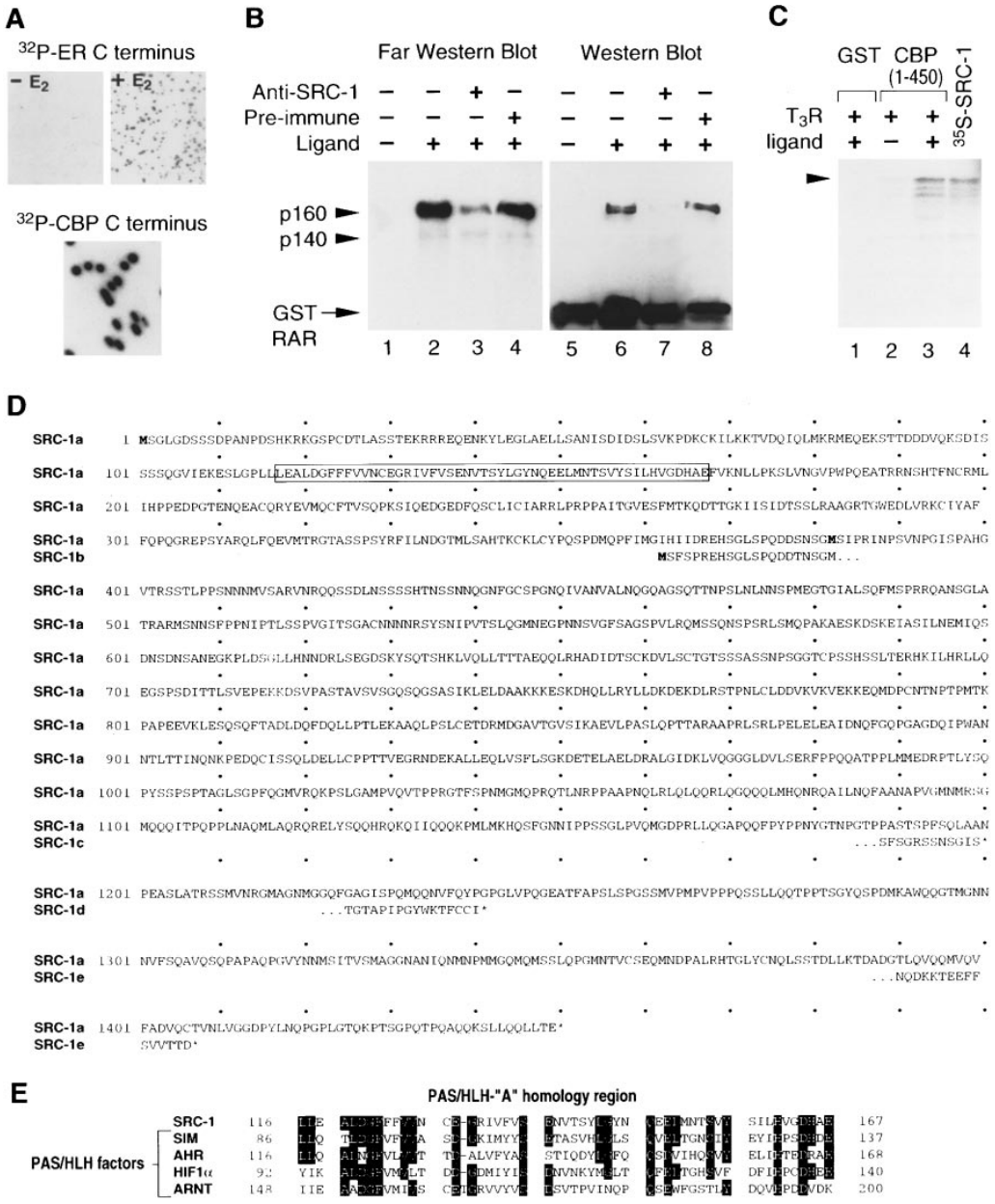

Figure 4. The p160 Gene Family: Interactions with CBP and Liganded Nuclear Receptors

(A) A mouse pituitary and a human macrophage λgt11 cDNA library (Lin et al., 1992) were screened for proteins interacting with ^{32}P-labeled ligand-bound ER C-terminus. We identified 11 positive plaques from 1.5 × 10^6 plaques evaluated, 7 of which also interacted with the [^{32}P]GST–CBP C-terminus (amino acids 2058–2163).

(B) p160 is antigenically related to SRC-1. A guinea pig polyclonal antiserum was raised against a GST fusion of a 500 amino acid region common to all SRC-1 variants, encompassing the nuclear receptor and CBP interaction domains. This antiserum was used to immunodeplete reactive proteins from HeLa whole-cell extracts (lanes 3 and 7). Preimmune serum was used as a control (lanes 4 and 8). Treated and untreated whole-cell extracts were incubated with GST–RAR bound to glutathione–agarose, in the presence or absence of all-*trans* retinoic acid, to purify putative coactivator proteins. Following extensive washing, specifically bound proteins and GST–RAR were resolved by SDS–PAGE and transferred to a nitrocellulose membrane. The membrane was first probed with liganded [^{32}P]GST–ER to detect p140 and p160 (lanes 2 and 6). Pretreatment of HeLa extracts with SRC-1 antiserum, but not preimmune serum, resulted in a marked decrease in the p160 band detected by labeled ER (compare lanes 2 and 3). The membrane was then stripped and probed with anti-SRC-1 IgG. This demonstrated near quantitative (>75%) removal of the p160 recognized by specific antisera during the initial immunodepletion (compare lanes 6 and 7).

(C) Nuclear receptors, CBP, and SRC-1 can form a ligand-dependent ternary protein complex. A protein–protein interaction assay was performed with GST–CBP (1–450), which contains only the nuclear receptor interaction domain, bacterially expressed His–T_3R, and in vitro translated ^{35}S-labeled SRC-1 in the absence or presence of 10^{-6} M TRIAC (lanes 2 and 3). GST alone was used as a negative control (lane 1), and 25% of SRC-1 input is shown (lane 4).

(D) A mouse cDNA encoding a 159 kDa variant of SRC-1. Sequencing of murine and human cDNA isolates revealed an open reading frame predicting a protein of 1465 amino acids, containing an N-terminal extension beyond the sequence of human SRC-1 (Oñate et al., 1995) with

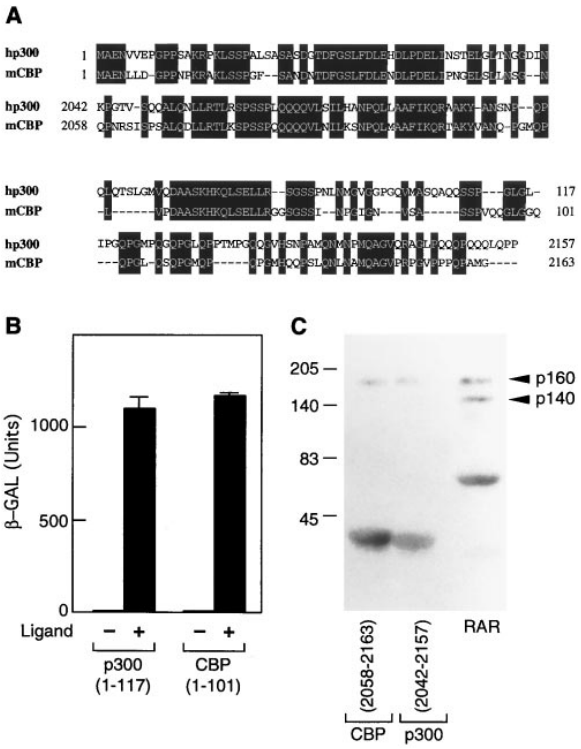

Figure 5. p300 Interacts with Nuclear Receptors and p160

(A) Comparison of the N- and C-terminal regions of CBP involved in nuclear receptor and p160 interactions with the corresponding region of human p300. Black background indicates identical residues.

(B) Interaction between the N-terminus of p300 (amino acids 1–117) and CBP (amino acids 1–101) with RARα C-terminus was assessed using the yeast two-hybrid assays as described in the legend to Figure 1B.

(C) p300 (amino acids 2042–2160) and CBP (amino acids 2058–2162) were expressed as GST fusion proteins, bound to glutathione–agarose, and incubated with whole-cell extracts. After washing, specifically associated proteins were resolved by SDS–PAGE, transferred to nitrocellulose, and then detected using [^{32}P]GST–ER in the presence of ligand as described for Figure 1A. The numbers at the left indicate molecular size markers.

CBP; amino acids 2042–2157, p300) (Figure 5). Consistent with these sequence homologies, the N-terminus of p300 interacted with the RAR in a ligand-dependent manner, while its C-terminus interacted with p160 (Figure 5). The strength of these interactions was comparable to those of the corresponding regions of CBP.

Role of CBP in Nuclear Receptor–Mediated Inhibition of AP-1 Activity

In parallel, we wished to explore a potential role for CBP in mediation of ligand-dependent inhibition of AP-1 function. To begin to test this hypothesis, we established a transfection assay in which liganded RAR or GR could effectively inhibit (~80%) phorbol ester–mediated activation of AP-1-dependent promoters. If competition for limiting quantities of CBP accounts for the inhibitory effect of nuclear receptors, then increased levels of CBP should restore AP-1-dependent activation. Indeed, the inhibitory effects of liganded RAR and GR were largely or completely abolished by cotransfection of vectors expressing CBP (Figure 6A). Cotransfection with vectors expressing p300 also diminished the AP-1 inhibitory effects of nuclear receptors, but appeared somewhat less effective than CBP (Figure 6A). Mutations in the RAR AF2 domain that inhibit binding of CBP and other coactivator proteins abolished AP-1 repression by nuclear receptors, consistent with previous data involving C-terminal receptor deletions (Desbois et al., 1991; Saatcioglu et al., 1994) (data not shown). The CBP-dependent inhibition of AP-1 activity could formally reflect either an allosteric effect whereby binding of nuclear receptors could preclude binding of the AP-1 complex to CBP or a competition for limiting amounts of CBP. However, the first possibility appears unlikely because we found that binding of nuclear receptors (RAR) and Jun could simultaneously occur on CBP (data not shown).

A recently described synthetic RAR ligand (LG550) exerts a potent anti-AP-1 effect, while LG629 serves as an antagonist (Figure 6B). Based on the model that CBP partitioning can mediate *trans*-repression of AP-1 by nuclear receptors, it would be predicted that LG550 might result in potent association with CBP. This possibility was initially assessed using GST–RAR fusion proteins, which revealed significantly enhanced binding of CBP N-terminus in the presence of LG550 compared with all-*trans* retinoic acid, while interaction was abolished by the antagonist, LG629. Further, using the yeast two-hybrid assay, LG550 was found to be a more potent stimulator of CBP–RAR interactions than all-*trans* retinoic acid, while LG629 abolished interaction (data not shown). Finally, interactions in intact cells were assessed by immunoprecipitation of CBP-containing complexes after addition of various ligands. As shown in Figure 6B, CBP–RAR interactions were markedly increased when LG550 was added compared with cells treated with all-*trans* retinoic acid, and the interaction was abolished by LG629. Together, these observations are consistent with the hypothesis that the interaction between RAR and CBP is responsible for some forms of repression of AP-1 activity. If this were the case, one would predict that CREB, which appears to have a very high affinity for CBP, might be able actually to inhibit nuclear receptor activation. This possibility was tested by transfection of a RARE-dependent reporter and cytomegalovirus (CMV)–CREB and retinoic acid–dependent induction after treatment of cells with 8-bromo-cAMP. Indeed, activated CREB reduced retinoic acid–dependent stimulation from >40-fold to ~5-fold (Figure 6C), without affecting the basal activity of the RARE-driven reporter.

high homology (~88% identity) over the rest of SRC-1. Additional variants of SRC-1 with distinct N-terminal (SRC-1b) or C-terminal sequences (SRC-1c and SRC-1d) reflecting alternative splicing events were detected.

(E) Homology between an N-terminal region of p160 and the A region of the PAS domain of PAS/bHLH proteins.

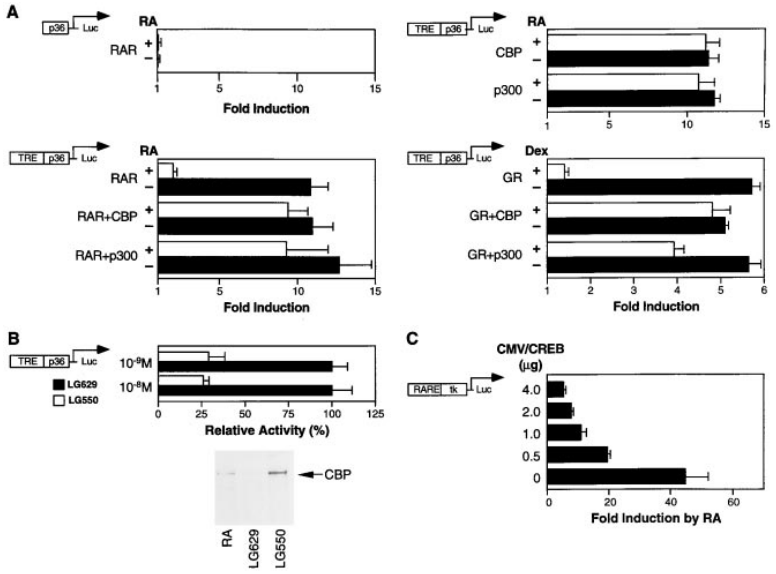

Figure 6. Role of CBP in *trans*-Repression of AP-1 by Nuclear Receptors

(A) Increased expression of CBP or p300 abrogates RAR- and GR-mediated repression of AP-1 activation in HeLa cells. Fold induction of the reporters by TPA was plotted. Both RAR and GR exhibited ligand-dependent repression of TPA-stimulated transcription of a promoter containing an AP-1 site. Induction by TPA was ~80% repressed by retinoic acid (10^{-7} M) or dexamethasone (10^{-7} M). The inhibition by retinoic acid was largely decreased or abolished when plasmids expressing either a CBP or p300 transcription unit were cotransfected (lower left). In a similar fashion, the repression by liganded GR was alleviated with cotransfection of CBP and partially recovered with overexpression of p300 (lower right).

(B) LG550 enhances interactions between RAR and CBP. Whole-cell extracts were prepared from 293 cells expressing FLAG–CBP and immunoprecipitated with RAR antibody (Hörlein et al., 1995) in the presence or absence of all-*trans* retinoic acid, LG550, or LG629 (10^{-7} M). Western blot analysis was performed using anti-FLAG antibody. Transient transfection experiments similar to that described in (A) were done with LG550 and LG629 at two concentrations. Activity in the absence of ligand was arbitrarily defined as 100%.

(C) Effects of overexpression of CREB on retinoic acid–stimulated gene expression. Increased CREB levels led to reduced retinoic acid stimulation of RARE-driven reporter in HeLa cells. Thus, the ~45-fold induction of the reporter by retinoic acid was gradually reduced to ~5-fold with increasing amounts of CMV–CREB cotransfected. In all cases, 8-bromo-cAMP (1 mM) was administered together with retinoic acid (10^{-7} M) to activate both pathways at the same time. The baseline expression of the reporter in the absence of retinoic acid and cAMP remained constant with increasing amount of cotransfected CREB. Similar results were obtained in P19 cells.

Discussion

Coactivator Complexes in Nuclear Receptor Transactivation Events

Based on the data presented in this manuscript, we have found, unexpectedly, that CBP/p300 appear to be required for effective ligand-dependent gene activation by nuclear receptors. A crucial role of CBP in ligand-dependent activation events is supported by the observations that ligand-dependent association between CBP and nuclear receptors occurs both in intact cells and on DNA-bound receptor complexes and that anti-CBP antibodies selectively inhibit the transcriptional activities of nuclear receptors in intact cells. Furthermore, increased levels of CBP selectively permit thyroid hormone–dependent transcription in vitro, indicating that this factor is rate limiting in nuclear extracts. Thus, the identical criteria that have linked CBP to transactivation by CREB and Jun/Fos (Arias et al., 1994; Kwok et al., 1994) have been fulfilled for ligand-dependent gene activation, indicating that CBP plays a comparable role in nuclear receptor transactivation events.

While these surprising results implicate members of the CBP/p300 family as required for transcriptional activation by nuclear receptors, they are not likely alone to be sufficient. A series of additional potential nuclear receptor coactivators have been identified and reported (Cavailles et al., 1994; Halachmi et al., 1994; Lee et al., 1995; Le Dourain et al., 1995; Oñate et al., 1995) that require a specific C-terminal domain (AF2) that plays a structural role in configuring the receptor C-termini (Bourguet et al., 1995; Renaud et al., 1995; Wagner et al., 1995). Because nuclear receptors function well in cells, such as HeLa cells, containing little p140 (Figure 4), our observations suggest that transcriptional activation by nuclear receptors is mediated by a complex that contains both CBP and p160 and probably other components. We have presented evidence that p160 represents a major form of a family of SRC-1-related proteins. Because CBP can interact independently with nuclear receptors, p160, and the related family members, it is likely that interactions between these proteins are involved in transactivation events. Thus, one function of a nuclear receptor coactivator, such as p160, could be to increase the specificity and strength of interaction of nuclear receptors with members of the CBP family, potentially providing a direct link to the core transcriptional machinery (Drapkin et al., 1993), analogous to the independent contacts proposed in synergistic interactions between transcription factors bound to distinct DNA sites (Hansen and Tjian, 1995). The existence of CBP and p300, and of multiple SRC-related nuclear receptor coactivators, provides a potential combinatorial code for imparting receptor, DNA site, and polarity specificity. The presence of a canonical bromodomain in CBP and p300, but not in p160/SRC-1 factors, suggests that a component of the requirement for CBP/p300 might reflect actions on chromatin structure comparable with the actions proposed in SWI/SIN control of gene expression (e.g., Peterson and Herskowitz, 1992; Winston and Carlson, 1992).

Partitioning of CBP/p300 in AP-1 Inhibition by Nuclear Receptors

The identification of CBP as an integral component of the activation complex for nuclear receptors obviously

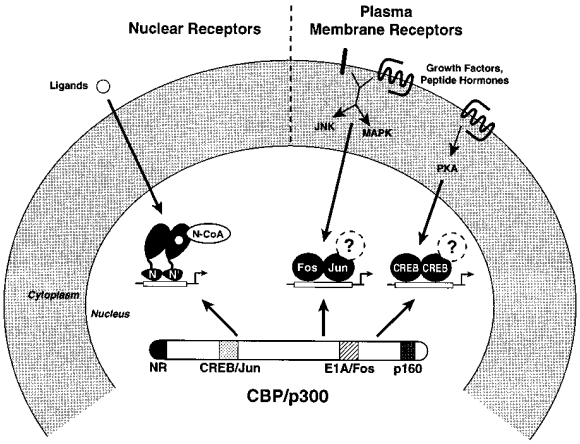

Figure 7. CBP/p300 Function as Cointegrators of Diverse Signaling Pathways

Both nuclear receptors and targets of plasma membrane receptors, including Jun, Fos, and CREB, associate with the CBP/p300 family. In the case of nuclear receptor heterodimers, such as RXR and RAR, liganded RAR associates with nuclear receptor coactivators (N-CoA), including variant forms of SRC-1, which recruit CBP into the complex. CBP/p300 serves as the cointegrator, partitioning between AP-1, nuclear receptor heterodimers, and other factors, accounting, at least in part, for antagonistic relationships between diverse signal transduction pathways.

suggests a molecular mechanism by which nuclear receptors might inhibit AP-1 action, based on a requirement for the relatively low intracellular levels of the CBP/p300 family proteins by both classes of transcription factors. Because increasing the intracellular levels of this family of coactivators serves to abolish RAR or GR repression of AP-1, and because AP-1 can effectively bind to RAR-associated CBP, it is likely that the limiting amount of the CBP family factors is partitioned by the alternative activation of nuclear receptors by ligand, or of AP-1 by phosphorylation. Although a competition model provides a mechanism for the observed antagonism between nuclear receptors and AP-1 on genes that contain binding sites for only one class of factor, they do not account for the more complex pattern of regulation observed for genes containing composite response elements, as studied most extensively in the case of the proliferin gene (Lefstin et al., 1994). In this case, nuclear receptors and AP-1 protein can act either cooperatively or antagonistically, depending upon the identity and conformation of the nuclear receptor (i.e., GR or mineralocorticoid receptor) and AP-1 complex (Jun/Jun or Jun/Fos) bound to the composite element. It is possible that on such an element, some combinations of coactivators promote, while others inhibit, the recruitment of CBP.

The CBP/p300 Family as Functional Integrators of Diverse Signal Transduction Pathways

The demonstration that CBP is required for transcriptional activation by CREB, AP-1, and nuclear receptors, and that these factors compete with each other to interact with limiting amounts of CBP within the cell, suggests that CBP functions as a nuclear integrator of multiple signal transduction pathways (Figure 7). Because the antagonistic effects of nuclear receptors and AP-1 can be abolished by raising the levels of CBP, regulation of CBP expression is likely to be of critical importance in determining the transcriptional consequences of simultaneous activation of multiple pathways. Indeed, evidence that CBP is physiologically maintained at a limiting concentration is strongly suggested by the observation that individuals heterozygous for a truncated CBP transcription unit exhibit severe developmental defects, the Rubinstein–Taybi syndrome (Petrij et al., 1995).

The observation that the interaction of CBP with nuclear receptors not only involves a direct contact with the nuclear receptor ligand-binding domain, but also a simultaneous interaction with p160, suggests that comparable coactivators will be required for other classes of transcription factors. For example, studies of CREB have indicated that, while necessary, interaction with CBP is not sufficient for transcriptional activation. In addition to phosphorylation of Ser-133 of CREB, which permits CBP interaction, an additional domain, referred to as Q2, is required for functional activity (Brindle et al., 1993). These observations suggest the existence of a CREB coactivator that is distinct from p160 (unpublished data), but functions in an analogous manner (Figure 7).

Analysis of the N-terminal sequence of the p160 protein reveals that it contains a region that is homologous to the PAS domain of the growing family of PAS/basic–helix-loop-helix (bHLH) factors (Hankinson, 1995). This family includes the dioxin receptor and hypoxia-inducible factor, which bind as heterodimers with ARNT to asymmetric DNA response elements. The PAS domain is established to be a bipartite dimerization interface, suggesting an intriguing linkage of p160/CBP integration to a second, structurally distinct family of heterodimeric transcription factors, of which at least one is ligand regulated. Further, these HLH factors may directly interact with CBP, completing the parallel to nuclear receptors. Thus, the same integrator complex partners may mediate transactivation by nuclear receptors and PAS/HLH factors.

We propose that the CBP/coactivator complexes be referred to as “integrators” based on their role in determining the relative transcriptional responses of a specific target gene in the face of activation of multiple signaling pathways. The relative abilities of coactivators to recruit limiting amounts of CBP into integrator complexes could account for aspects of the multifactorial control of biological processes under regulation of multiple signal transduction pathways. Thus, the precise cohort of genes activated in a given cell will depend on both the relative activity of diverse signaling pathways and the organization and relative affinity of the complex on various DNA-bound factors that utilize CBP as a coactivator (see Figure 7). Synergy might be expected on some promoters, while competitive interaction would be expected for others that contain only a subset of sites for interacting transcription factors. Thereby, the CBP/p300 family serves to integrate the signals of diverse growth factors, hormones, and intracellular ligands that combinatorially modulate homeostasis and proliferation.

Experimental Procedures

Yeast Two-Hybrid Assay and Interaction Assays

Yeast strain EGY48, the LexA–β-galactosidase reporter construct (pSH18-34), and the LexA and B42 parental vectors (pEG202 and pJG4-5) were as reported previously (Gyuris et al., 1993). PCR fragments of rat $T_3R\alpha1$ (122–410 and E403Q), human RARα (143–462 and 143–403; Δ403 and G303E), mouse RXRγ (227–463 and E454Q), and human ER (251–595) were subcloned into pEG202 bait vector. cDNA encoding the CBP holoprotein was isolated from a mouse pituitary cDNA library (Lin et al., 1992). DNA fragments encompassing the entire CBP protein were subcloned into the pJG4–5 prey vector. β-Galactosidase activity was determined as described previously (Ausubel et al., 1994). Ligands used in the yeast two-hybrid assays were 10^{-7} M TRIAC for T_3R, 10^{-7} M TTNPB for RAR, 10^{-7} M LG69 for RXR, and 10^{-7} M β-estradiol for ER. Protein–protein interaction assays using GST fusion proteins were performed as previously described (Hörlein et al., 1995). For gel mobility shift assays, oligonucleotides containing the consensus CREB-binding site (5′-AGAGATTGCCTGACGTCAGAGAGCTAG-3′) or the DR+5 RARE (5′-TCGACGAAGGGTTCACCGAAAGTTCACTCGCG-3′) were labeled with ^{32}P using T4 polynucleotide kinase (New England Biolabs).

In Vitro Transcription, Transient Transfections, and Reporter Assays

The cell-free transcription reaction with nuclear extracts was carried out as described previously (Gloss and Bernard, 1990). Where indicated, 100 ng of purified His–GAL4–human $T_3R\beta$ protein (amino acids 1–147 of GAL4 fused to amino acids 165–456 of $T_3R\beta$) was added to the reaction prior to addition of nuclear extract. Templates were 300 ng of 3× GAL4–tk luciferase (Hörlein et al., 1995), 50 ng of HPV-16 luciferase (Gloss and Bernard, 1990) or 100 ng of AP-1–p36 luciferase per reaction. Primer extension was carried out with SuperScript RNaseH$^-$ (GIBCO BRL) and the ^{32}P labeled primer CCAGGAACCAGGGCGTATCTCTTCATAGCC. For studying AP-1 repression by nuclear receptors, 2–4 hr before transfection the medium, calf serum, was replaced with DMEM containing 10% charcoal-stripped serum. Plasmid DNAs were transfected into HeLa cells with standard calcium phosphate precipitation procedure (Gorman et al., 1982). Typically, 1 μg of TRE-driven reporter, 1 μg of RSV-RXRα or GR, and 2 μg of CMV–CBP or CMV–p300 were used, the final DNA concentration was adjusted to 5 μg per 60 mm dish, and TPA (Sigma) was administered at a final concentration of 100 ng/ml in the presence or absence of all-*trans* retinoic acid, LG550, LG629, or dexamethasone at indicated concentrations.

Nuclear Microinjection, Staining, and Fluorescence Microscopy

Insulin-responsive Rat-1 fibroblasts were seeded on acid-washed glass coverslips at subconfluent density and grown in MNE/F12 medium supplemented with 10% fetal bovine serum, gentamicin, and methotrexate. Prior to the injection, the cells were rendered quiescent by incubation in serum-free medium for 24–36 hr. Plasmids were injected into the nuclei of cells at a final concentration of 100 μg/ml. Either preimmune rabbit IgG or anti-CBP antibody (Santa Cruz Biotechnology) was coinjected and allowed the unambiguous identification of the injected cells. Microinjections were carried out using an Eppendorf semiautomated microinjection system mounted on an inverted Zeiss microscope. Approximately 1 hr after injection, the cells were stimulated where indicated with 10^{-6} M all-*trans* retinoic acid (Sigma). After overnight expression, the cells were fixed with 3.7% formaldehyde and then stained to detect injected IgG and β-galactosidase expression (Rose et al., 1992). The latter was detected by incubation with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal). Injected cells were identified by staining with tetramethylrhodamine-conjugated donkey anti-rabbit IgG. Cells were viewed and the results were analyzed on a Zeiss Axiophot microscope. Photomicrographs were taken with the same equipment using Kodak Ektachrome 400 color film.

Isolation of p160 cDNA Clones

Expression screening was performed essentially as described previously (Kaelin et al., 1992), using λgt11 cDNA expression libraries constructed from human macrophage and mouse pituitary libraries, as described (Vinson et al., 1988).

Immunoprecipitation

EBNA cells (293) were transfected with pCEP4 CBP containing a C-terminal in-frame FLAG epitope, $(EYKEEEK)_2$. Where required, RSV-RAR or GR was cotransfected. Immunoprecipitation and detection were performed as previously described (Hörlein et al., 1995).

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