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9. Mutations in CBP and E1A were generated with the polymerase chain reaction. Small regions spanning each deletion or point mutation were confirmed by sequence analysis and transferred into the wild-type cDNA backbone. At least two independent clones of each mutant were analyzed for expression and function. For transient transfection assays of CBP and E1A function, we plated CV1 or HeLa cells in 24-well plates and transfected them with luciferase reporter genes and CBP or E1A expression vectors using $CaPO_4$ (*19*). Salmon sperm DNA was used as a carrier to balance the total amount of transfected DNA. Cells were treated with the RAR-specific ligand TTNPB {(E)-4-[2-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydro-2-napthalenyl)-1-propenyl] benzoic acid} (0.1 μM) or interferon-γ (100 u/ml) for 24 hours or 6 hours, respectively, before determination of luciferase activity.
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11. Full-length CBP and CBPΔC/H3 were expressed in SF9 cells with a baculovirus vector and contained a COOH-terminal FLAG epitope, which permitted purification on a matrix of FLAG antibody. GST fusion proteins were expressed in *Escherichia coli* and purified on a glutathione agarose affinity matrix. In studies with full-length CBP, SF9 whole-cell extracts containing FLAG-tagged CBP were incubated with GST-E1A fusion proteins produced in bacteria and captured on a glutathione affinity matrix. After they were washed, protein complexes were resolved by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by protein immunoblotting with a monoclonal antibody to FLAG. In studies examining the interaction of p/CIP and E1A with specific domains of CBP, [^{35}S]p/CIP or [^{35}S]E1A proteins were produced by translation in vitro and incubated with GST-CBP fusion proteins. After they were extensively washed, specifically bound proteins were resolved by SDS-PAGE and detected by autoradiography. For Far Western analysis of E1A-CBP interactions, 12*S* E1A and 13*S* E1A cDNAs were introduced into a vector providing an in-frame phosphorylation site for protein kinase A. After phosphorylation with ^{32}P, radiolabeled 12*S* E1A and 13*S* E1A were used to probe nitrocellulose membranes containing CBP fragments as previously described (*12*).
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17. To assess the effects of E1A mutants on retinoic acid–dependent differentiation of P19 cells, we cotransfected human kidney carcinoma 293 cells with 1.5 μg per 6-cm dish of pCL-7 helper-free retroviral packaging vector and pCL1-E1A, pCL1-H3N, and pCL1 vector as indicated, using the $CaPO_4$ method. Media was changed 8 hours after transfection. After 12 hours the supernatant was used in 1:100 dilution for infecting P19 cells. Retroviral vectors and packaging plasmids that produce helper-free retrovirus with 1×10^6 to 5×10^6 titers (*19*) were used for E1A and H3N overexpression. The supernatant of 293 cells infected with pCL-7, pCL1-E1A, or pCL1-H3N packaging constructs were used to infect P19 cells for 2 days. P19 cells were induced to differentiate with 0.5 μM retinoic acid in bacterial-grade petri dishes. Two days after infection and aggregation in the presence of retinoic acid, embryoid bodies were collected and plated again in bacterial-grade petri dishes in the absence of virus. Two days later, cells were transferred to poly-L-lysine–coated glass cover slips in media without retinoic acid (*19*). After 2 to 3 days cells were fixed with 4% formaldehyde and prepared for immunohistochemistry. Rabbit polyclonal antibodies to NF (Sigma) were diluted 1:100 in 0.5% blocking buffer (Boehringer Mannheim). Detection antibody fluorescein isothiocyanate–conjugated to goat antibody to rabbit $F(ab)_2$ fragments was used in 1:100 dilution. Fixed P19 cells were incubated with antibodies to NF for 2 hours at room temperature and rinsed before applying secondary antibody for two more hours at room temperature. Immunofluoresence-stained preparations were viewed with a Zeiss Axiophot photomicroscope. Photographic prints referring to comparative immunostaining were prepared under identical conditions.
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Transcription Factor–Specific Requirements for Coactivators and Their Acetyltransferase Functions

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Different classes of mammalian transcription factors—nuclear receptors, cyclic adenosine 3′,5′-monophosphate–regulated enhancer binding protein (CREB), and signal transducer and activator of transcription-1 (STAT-1)—functionally require distinct components of the coactivator complex, including CREB-binding protein (CBP/p300), nuclear receptor coactivators (NCoAs), and p300/CBP-associated factor (p/CAF), based on their platform or assembly properties. Retinoic acid receptor, CREB, and STAT-1 also require different histone acetyltransferase (HAT) activities to activate transcription. Thus, transcription factor–specific differences in configuration and content of the coactivator complex dictate requirements for specific acetyltransferase activities, providing an explanation, at least in part, for the presence of multiple HAT components of the complex.

Nuclear receptors exhibit ligand-dependent interactions with coactivators such as CBP/p300 (*1–3*) and p160 proteins (*4*), steroid receptor coactivator-1 (SRC-1)/NCoA-1 (*2*, *5*), TIF2/GRIP-1/NCoA-2 (*6*), and p300/CBP-interacting protein (p/CIP) (*7*, *8*). CBP/p300 interacts with other coactivators and with the p300/CBP-associated factor (p/CAF) (*9*), which is homologous to the yeast transcriptional adaptor GCN5 (*10*). Both CBP/p300 and p/CAF exhibit strong histone acetyltransferase (HAT) activities (*9*, *11*), whereas the p160 factors possess weak COOH-terminal HAT activity (*8*, *12*).

To determine whether p/CAF is recruited into the nuclear receptor coactivator complex (*2–5*, *13*), we performed coimmunoprecipitation assays with cell extracts. p/CAF exhibited ligand-dependent recruitment to the retinoic acid receptor (RAR) coactivator complex, and p/CAF binding was abolished by binding of an RAR antagonist (Fig. 1A). A minimal ligand-dependent interaction was observed in a yeast two-hybrid assay, and this interaction domain mapped to the NH_2-terminus (amino acids 1 to 351) of p/CAF (Fig. 1B). Using the avidin-biotin complex DNA assay (*14*) to assess protein interactions on DNA-bound receptors, we found that p/CAF bound to the RAR/RXR (retinoid-X receptor) heterodimer, but there was no detectable ligand-dependence for this association (Fig. 1C). The interaction between nuclear receptor and p/CAF was inhibited by the nuclear receptor corepressor (NCoR) (*15*)

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and was restored on ligand-induced elimination of NCoR from the receptor (Fig. 1C). This suggests that ligand-dependent association of p/CAF in cells depends on release of the NCoR complex (*16*). The interaction of p160 coactivators with RAR depends on a helical transactivation domain of RAR, referred to as AF2, through an allosteric mechanism induced by ligand binding (*4–7, 17*). However, p/CAF interaction with RAR was independent of the AF2 domain (Fig. 1C). Thus, the ligand-dependent recruitment of p/CAF to the activated nuclear receptor is distinct from the AF2-dependent mechanism of interaction between SRC-1–p/CIP and nuclear receptors.

Because *GCN5* was one of the genes identified by genetic selection, including also *ADA2* (*18*), that was required for function of certain classes of activation domains (*10*), we investigated the function of p/CAF in nuclear receptor–dependent regulation of gene transcription. Single-cell microinjection of highly specific blocking antibody against p/CAF (amino acids 465 through 832) [anti-p/CAF immunoglobulin G (IgG)] revealed that p/CAF was required for ligand-dependent activation, not only of RAR, but also of thyroid hormone– and estrogen receptor–dependent promoters (Fig. 1, D and E). A promoter that was under the control of multiple SP1 sites was unaffected by anti-p/CAF IgG (Fig. 1D), suggesting that p/CAF is not required for transcription of all promoters. When no specific antibodies were used, preimmune rabbit or guinea pig IgG was coinjected to identify the injected cells and to serve as a preimmune control (*2, 7*). The observed specificity of p/CAF function is consistent with observations concerning functions of GCN5 in yeast (*10*).

Glutathione S-transferase (GST) pull-down and yeast two-hybrid assays revealed that, in addition to the previously described interaction between p/CAF and the C/H3-E1A interaction domain of CBP (*9*), the NH_2-terminal region of p/CAF was capable of direct interaction with the NH_2-terminal region of CBP activity (Fig. 2, A and B). Consistent with the possible functional importance of this NH_2-terminal domain of CBP, as previously suggested for CREB (*19*), transcriptional activation by a GAL4-CBP(1-450) fusion protein was significantly inhibited by specific IgG against p/CAF, but not by specific IgG against either p/CIP or NCoA-1/SRC-1 (*20*). Both NCoA-1/SRC-1 (*2, 5, 12*) and p/CIP (*7*) can be coimmunoprecipitated with p/CAF that is present in cell extracts (Fig. 2C) (*8, 12, 20*). GST pull-down (*20*) and yeast two-hybrid assays (Fig. 2D) demonstrated that the NH_2-terminus of p/CAF mediated interactions with NCoA-1/SRC-1, whereas the most effective p/CIP interaction domain with p/CAF was localized to amino acids 649 to 725 (Fig. 2D), which corresponds to the conserved regions of yeast and human GCN5 that interact with ADA2 (*10*). Thus, there are multiple potential interaction interfaces between members of the coactivator complex (Fig. 2A).

To investigate the potential roles of specific enzymatic functions and interaction domains of these coactivators in transcription factor–specific gene activation events, we evaluated the roles of the acetyltransferase functions of p/CAF and CBP in RAR- and CREB-dependent transcription. On the basis of mutagenesis studies demonstrating that single amino-acid substitutions, particularly in the acetyl coenzyme A (acetyl-CoA)–binding region of acetyltransferases, resulted in loss of enzymatic activity (*21*), we generated a p/CAF mutant protein harboring a substitution of two conserved residues ($Tyr^{616}/Phe^{617} \rightarrow Ala^{616}/Ala^{617}$). This mutant has no intrinsic HAT activity (p/CAF_{HAT^-}) (Fig. 3A). Similarly, a two–amino acid mutation ($Leu^{1690}/Cys^{1691} \rightarrow Lys^{1690}/Leu^{1691}$) in CBP abolished its HAT activity (CBP_{HAT^-}) (Fig. 3A). The use of HAT^- proteins permitted an evaluation of the role of the HAT domains in transactivation function of specific classes of transcription factors. Blockade of both CBP and p/CAF activity by coinjection of both specific antibodies, which almost abolished the transcriptional activity of RAR or CREB, was completely reversed by coinjection of vectors expressing wild-type CBP and p/CAF (Fig. 3B). Conversely, expression

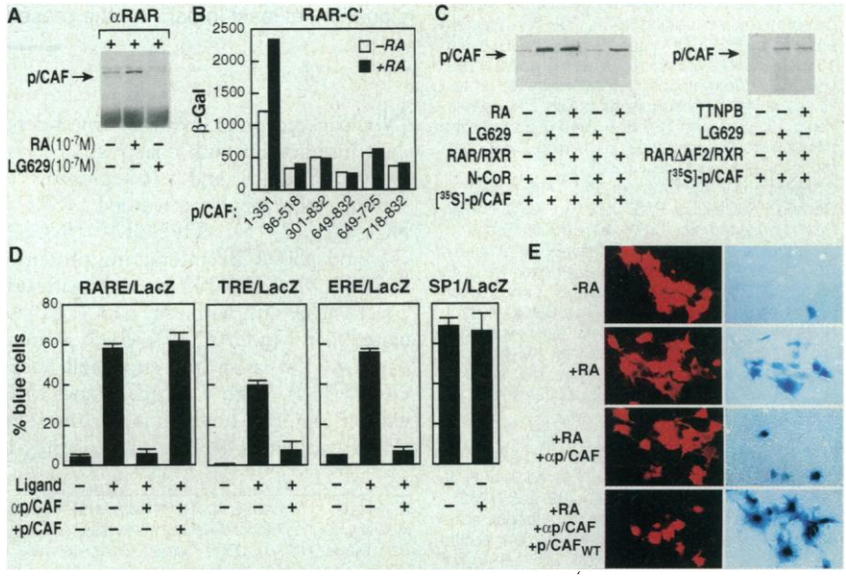

Fig. 1. p/CAF is present in a complex associated with nuclear receptors and is required for transcriptional activation induced by retinoic acid (RA). (**A**) Immunoprecipitation (*25*) with anti-RAR antibodies of nuclear extracts from HeLa cells transfected with Flag-tagged p/CAF and treated with all-*trans*-RA (10^{-7} M) or an antagonist, LG629 (10^{-7} M), reveals ligand-dependent coimmunoprecipitation of p/CAF and RAR, detected with monoclonal anti-Flag IgG. (**B**) A yeast two-hybrid assay (*26*) showed specific interaction between the indicated p/CAF fragments (denoted by ranges of residue positions) and the COOH-terminal domain of RAR (RAR-C') in the presence or absence of RA. Duplicates differed by less than 10%. (**C**) Interaction between p/CAF and RAR was tested in a DNA-dependent assay (avidin-biotin complex DNA) for protein-protein binding (*14*). Bacterially expressed RAR or RARΔAF2 (*17*) and retinoid-X receptor (RXR) (*14*) were bound to biotinylated oligonucleotides corresponding to a retinoic acid–responsive element (RARE), DR5, immobilized on streptavidin-agarose and incubated with ^{35}S-labeled p/CAF in the presence of pan-agonist 9-*cis*-RA (10^{-6} M), agonist (E)-4[2-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydro-2-naphtalenyl)-1-propenyl]benzoic acid (TTNPB) (10^{-6} M), or antagonist, LG629 (10^{-6} M), and analyzed by SDS-PAGE. Bacterially expressed NCoR (1 μg) (*15*) was incubated with receptor-DNA complexes before p/CAF addition. (**D**) Microinjection (*27*) of purified anti-p/CAF IgG (αp/CAF) blocked retinoic acid–, thyroid hormone (TR)–, or estrogen receptor (ER)–dependent activation of promoters containing the corresponding response elements (RARE, TRE, or ERE, respectively) in Rat-1 cells, but not SP1-driven reporter expression. Coinjection of p/CAF expression plasmid (p/CAF) reversed the blocking effect of anti-p/CAF IgG. All results are representative of experiments performed in triplicate, in which more than 1000 cells were injected for each experimental condition. (**E**) Photomicrographs of rhodamine-fluorescence (red) and Lac-Z (blue) staining of Rat-1 cells with the RARE reporter gene corresponding to the experiment in (D).

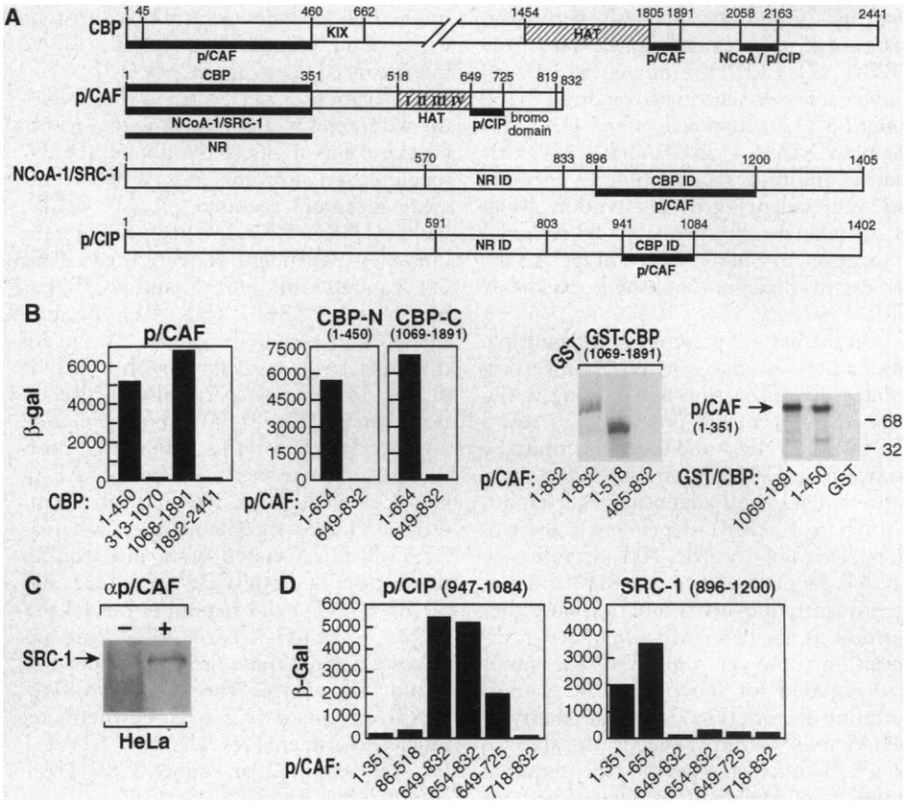

Fig. 2. Mapping of interactions between p/CAF, nuclear receptor, coactivators, and CBP. (**A**) Schematic of CBP, p/CAF, SRC-1, and p/CIP, and their interaction domains. KIX, interaction domain with kinase-inducible domain of CREB; NR ID, nuclear receptor interaction domain; CBP ID, CBP interaction domain. (**B**) (Left) A yeast two-hybrid interaction assay (*26*) using p/CAF(1-654) and CBP fragments, revealed two independent interaction domains (amino acids 1 to 450 and 1068 to 1891) of CBP (CBP-N, NH_2-terminal fragment; CBP-C, COOH-terminal fragment); (right) GST pull-down assays with the CBP COOH-terminal interaction domain and fragments of ^{35}S-labeled p/CAF (*28*). We incubated 3 μg of GST-CBP(1069-1891) protein with various ^{35}S-labeled p/CAF-derived fragments, and the specifically bound fragments detected above. GST pull-down assay of ^{35}S-labeled p/CAF(1-351) with NH_2- and COOH-terminal GST-CBP fragments. (**C**) Immunoprecipitations of HeLa extracts from cells cotransfected with Flag-tagged p/CAF and HA-tagged NCoA-1/SRC-1 expression vectors were subjected to protein immunoblot analysis employing anti-HA for detection. (**D**) Yeast two-hybrid interaction between p/CIP (947-1084) or NCoA-1/SRC-1 (896-1200) and fragments of p/CAF revealed a selective COOH-terminal p/CIP interaction domain and NH_2-terminal NCoA-1/SRC-1 interaction domains. Duplicate determinations differed by <10%.

of HAT^- factors mutated to abolish acetyltransferase function mutation (HAT^-), failed to effectively rescue activation (Fig. 3B). A failure to rescue RAR activity was also observed with expression of wild-type CBP and p/CAF_{HAT-} (Fig. 3B). However, expression of wild-type p/CAF in the presence of CBP_{HAT-} fully restored the ligand-dependent activation function of the RAR (Fig. 3B). In contrast, the HAT activity of CBP was required for CREB function, whereas that of p/CAF was of minimal importance (Fig. 3B). These transcription units served as internal controls, because both HAT^- coactivators were functional, with differences reflecting the distinct requirements for specific HAT activity by different classes of transcription factors.

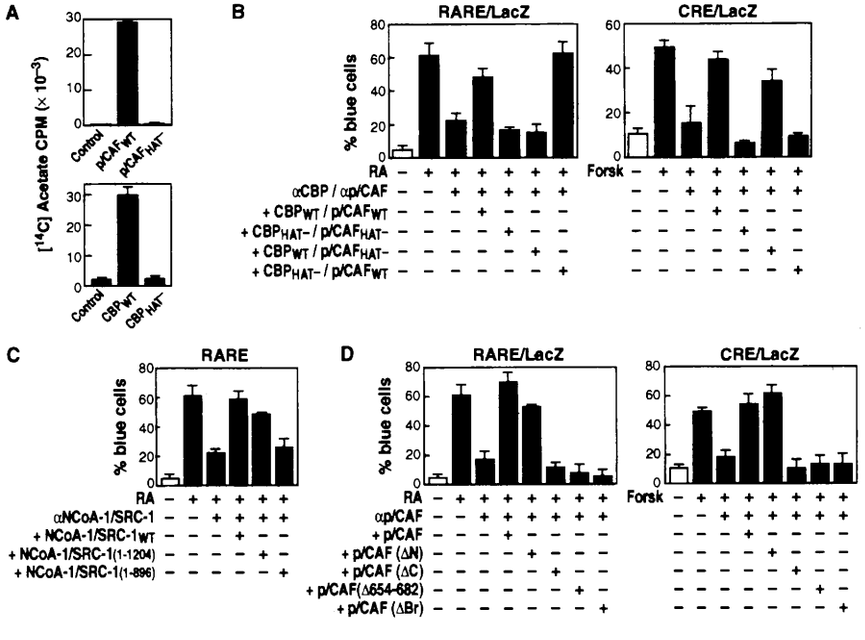

Fig. 3. Role of HAT and other domains in functions of the coactivator complex on RARE- and CREB-dependent gene activation. (**A**) Generation of mutations in the p/CAF (Tyr^{616}/Phe^{617} → Ala^{616}/Ala^{617}) (p/CAF_{HAT-}) or CBP (Leu^{1690}/Cys^{1691} → Lys^{1690}/Leu^{1691}) (CBP_{HAT-}) that abolish detectable acetylation of histones using [^{14}C]acetyl-CoA as substrate. Activity was determined by liquid HAT assay (*29*) using bacterially expressed p/CAF or baculovirus-expressed CBP. (**B**) Requirement for p/CAF and CBP acetyltransferase activity in RAR and CREB function tested in single-cell microinjection assays. In these experiments, specific IgGs against CBP and p/CAF were coinjected with vectors directing expression of wild-type (WT) or HAT^- mutant of p/CAF and CBP. The ability of retinoic acid (10^{-7} M) or forskolin (10^{-6} M) to activate the appropriate reporter gene was then determined. Similar results were obtained in three independent experiments of similar design. (**C**) NCoA-1/SRC-1 deleted in the COOH-terminal domain containing acetyltransferase activity (1-1204) remained effective for RARE-dependent gene activation, whereas further deletion of the CBP interaction domain (1-896) abolished its function. (**D**) The ability of wildtype p/CAF, p/CAF(ΔN) (amino acids 518-832), p/CAF(ΔC) (amino acids 1-654), p/CAF(Δ654-682) (missing residues 654 to 682) or p/CAF(ΔBr) (missing residues 745 to 832) to rescue RA-dependent activation of a RARE/Lac-Z reporter (left) or forskolin-dependent activation of the CREB-dependent reporter CRE/Lac-Z (right) in single-cell microinjection assays; similar results were obtained in three separate experiments.

We also tested, in an RAR activation assay, the requirement of the NCoA-1/SRC-1 (*7*) COOH-terminus, which encompasses a domain with reported HAT function (*12*). Removal of the HAT domain in NCoA-1/SRC-1 did not significantly diminish its function in retinoic acid–dependent gene activation (Fig. 3C), whereas further COOH-terminal truncation to remove the CBP/pCAF interaction domain abolished NCoA-1/SRC-1 activity.

On the basis of presence of the multiple interaction interfaces in p/CAF, we performed microinjection assays using RAR- or CREB-dependent promoters to assess the ability of NH_2- and COOH-terminally truncated p/CAF proteins to function in retinoic acid– and adenosine 3′,5′-monophosphate (cAMP)–dependent transcription. Deletion of the NH_2-terminus of p/CAF (amino acids 1 to 518) did not significantly impair its function on either retinoic acid– or cAMP-stimulated transcription; however, the COOH-terminus was required for function of both transcription factors (Fig. 3D). The ability of p/CAF to be recruited even in the absence of a CBP/nuclear receptor NH_2-terminal interaction domain is in agreement with the findings that the p/CAF interaction domain in CBP is not required for RAR function (*22*). Thus, alternative interaction interfaces appear to be used in recruitment of specific, required factors into the coactivator complex. p/CAF activity was also lost with deletion of the p/CIP interaction domain (Fig. 3D), consistent with the functional effects of the *ADA2* interaction domain in *GCN5* (*10*).

Activation of a given transcription factor may exhibit differential requirements for the components of the potentially dynamic coactivator complex. Whereas nuclear receptors required p/CAF, p/CIP, NCoA-1/SRC-1, and CBP, the protein kinase A–dependent activation of CREB (*1*) required CBP, p/CAF, and p/CIP, but not NCoA-1/SRC-1 (Fig. 4A). Because the COOH-terminal domain of p/CAF that selectively associates with p/CIP is distinct from the NCoA-1/SRC-1 interaction domain (Fig. 2), p/CAF may provide a molecular platform for differential positioning of components of the p/CAF–p/CIP–CBP/p300–SRC-1 complex in a transcription factor–specific manner. Whereas STAT-1 is associated with and requires the action of both CBP/p300 (*23*) and p/CIP, STAT-1 did require either p/CAF or NCoA-1/SRC-1, because blocking antibodies against these factors (*7*) failed to inhibit activity of the interferon-γ–responsive element (Fig. 4A). Furthermore, rescue experiments revealed that STAT-1 function required an intact CBP HAT activity (Fig. 4A).

We suggest that there are multiple possible configurations of the specific components of the coactivator complex recruited by different transcription factors. In the case of nuclear receptors, p/CAF and SRC-1/NCoA-1 bind receptors in a ligand-independent and ligand-dependent fashion, respectively, with p/CAF recruitment apparently dependent on dismissal of the NCoR complex (Fig. 4B). Our data also suggest a selectivity in the specific HAT activity required for function of distinct classes of transcription factors. The HAT activity of p/CAF, but not of CBP, appears to be indispensable for nuclear receptor activation, where CBP is likely to be recruited on the basis of interaction with a complex containing p/CAF and NCoA-1 (Fig. 4B). Conversely, the HAT activity of CBP, directly recruited by phosphorylated CREB (*1*), is required for transcriptional function of CREB and STAT-1 (*24*).

In concert with the finding of factor-specific interfaces at which E1A acts to block transcriptional function (*22*), our studies suggest transcription factor specificity in requirements for various functional domains (such as HATs) in components of the coactivator complex, which we speculate reflects the use of alternative interaction interfaces in coactivator complex assembly.

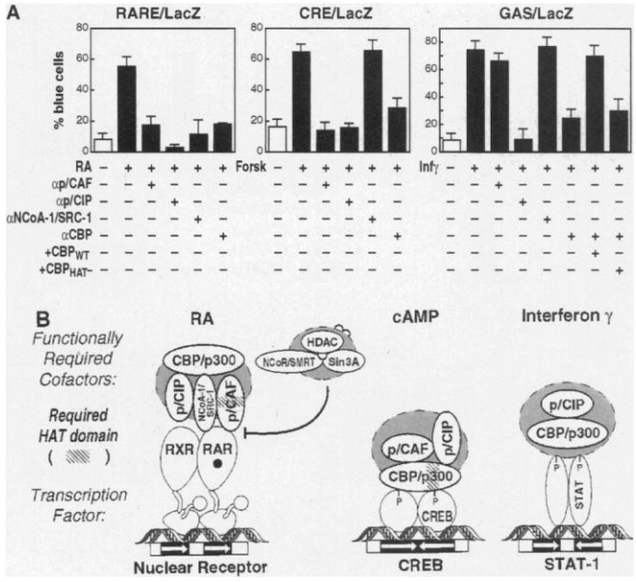

Fig. 4. Transcription factor specificity in required coactivator complex components. (**A**) (Left) Inhibition of RAR activity by microinjection of anti- p/CAF, anti-p/CIP, anti-NCoA-1/SRC-1, or anti-CBP IgG; (middle) inhibition of CREB activation by anti-CBP, anti-p/CIP or anti-p/CAF IgG, but not by anti-NCoA-1/SRC-1 IgG; (right) inhibition of interferon-γ (Inf-γ)–dependent activation of the interferon-γ activation sequence (GAS)–dependent promoter by anti-CBP or anti-p/CIP IgG but not by anti-p/CAF and anti-SRC-1. (**B**) Model of transcription factor–specific requirements for the CBP, p/CIP, p/CAF, and NCoA-1/SRC-1 in the coactivator complex. The functionally required components of the coactivator complex appear to be distinct for different classes of transcription factors, and the required HAT activity is also factor-specific. In the case of nuclear receptor, p/CAF and NCoA-1/SRC-1 appear to interact with liganded receptor by direct interactions, with p/CAF binding upon dismissal of NCoR from the receptor.

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25. Whole-cell extracts were prepared by lysing the cells in NET-N buffer containing 50 mM tris (pH 7.6), 5 mM EDTA, 0.3 M NaCl, 1 mM dithiothreitol, 0.1% NP-40, and protease inhibitors (2 mM phenylmethylsulfonyl fluoride and 10 μg/ml each of leupeptin, pepstatin, and aprotinin). The mixture was then centrifuged at 30,000*g* for 1 hour at 4°C, and the supernatant was stored at −80°C until use. For coimmunoprecipitation assays, 1 mg of cell extract was incubated in the presence of 2 μg of anti-p/CIP, anti-Flag, or anti-RAR IgG for 2 hours at 4°C. The immune complexes were then precipitated with protein A–Sepharose. Protein complexes were resolved by SDS–polyacrylamide gel electrophoresis (PAGE) [U. K. Laemmli, *Nature* **227**, 680 (1970)], protein immunoblotted, and probed using 1 μg/ml of anti-Flag, anti-p/CIP, or an anti-hemagglutinin (anti-HA).
26. The yeast strain EGY 48, the LexA-b galactosidase reporter construct (PSH 18-34), and the B42 parental vectors (PEG 202 and PJG 4-5) were all previously described [J. Gyuris *et al.*, *Cell* **75**, 791 (1993)]. Various p/CAF fragments or fragments of other coactivators were obtained by polymerase chain reaction or restriction digestion and subcloned into PEG 202 bait vector or PJG 4-5 prey vectors, respectively. EGY 48 cells were transformed with the *lac Z* reporter plasmid pSH 18-34 with the appropriate bait and prey vectors, and were plated out on Ura$^-$His$^-$Trp$^-$ medium containing 2% galactose. Isolated yeast colonies were then allowed to grow in the same liquid medium and were subsequently assayed for β-galactosidase, as previously described [F. M. Ausubel *et al.*, *Current Protocols in Molecular Biology* (Wiley, New York, 1995)]. All constructs were sequenced and functionally tested in yeast two-hybrid assays, showing interaction with other proteins as previously described (*2*, *7*). In the case of p/CAF constructs, each fragment interacted positively with at least one other coactivator except the fragment with amino acids 86 through 518.
27. Insulin-responsive Rat-1 fibroblasts were seeded on acid-washed glass cover slips at subconfluent density and grown in D-MEM/F-12 (Life Technologies) medium supplemented with 10% fetal bovine serum, gentamicin, and methotrexate. Before the injection, the cells were rendered quiescent by incubation in serum-free medium for 24 to 36 hours. Plasmids were injected into the nuclei of cells at a final concentration of 100 μg/ml. Immunoglobulin G specific for p/CAF was prepared from guinea pig serum raised against a bacterially expressed fragment (amino acids 466 to 832) of p/CAF; this IgG recognized the single band of p/CAF in protein immunoblot analysis. Either preimmune IgG or the appropriate specific antibodies directed against p/CAF, p/CIP, SRC-1, or CBP were coinjected and allowed the unambiguous identification of the injected cells (*7*). Preimmune controls were included in all experiments. Microinjections were carried out using an Eppendorf semiautomated microinjection system mounted on an inverted Zeiss microscope. Approximately 1 hour after injection, the cells were stimulated, where indicated, with the appropriate ligand. In the case of rescue experiments, the cells were stimulated with ligand 6 hours after injection to allow protein expression. After overnight incubation, the cells were fixed and then stained to detect injected IgG and β-galactosidase expression [D. W. Rose *et al.*, *J. Cell Biol.* **119**, 1405 (1992); (*2*)]. Injected cells were identified by staining with tetramethylrhodamine-conjugated donkey anti-rabbit IgG. All experimental results are expressed as the mean ± SEM of at least three experiments in which at least 1000 cells were injected.
28. GST-RAR and GST-CBP fragments were generated as described (*2*). We incubated 25 μl of GST-Sepharose beads containing 3 to 6 μg of the GST recombinant proteins with 5×10^5 cpm of ^{35}S-labeled p/CAF proteins generated by in vitro transcription and translation for 2 hours at 4°C. The complexes were washed five times with NET-N buffer, resolved by SDS-PAGE, and fluorographed.
29. Mutations in p/CAF and CBP were introduced by site-directed mutagenesis using the Quick-Change mutagenesis system (Stratagene) according to the manufacturer's instructions. Double-stranded oligonucleotides were designed such that the wild-type sequence corresponding to amino acids Tyr^{616}/Phe^{617} (acetyl-CoA–binding site) in p/CAF cDNA were substituted with alanines in order to generate a mutant of p/CAF lacking HAT activity (pCMV–p/CAF$_{HAT-}$). A similar strategy was used to obtain mutants of CBP. Mutants of p/CAF and CBP were expressed in bacteria and baculovirus, respectively, and tested for HAT activity in solution using histones as substrates [J. E. Brownell and C. D. Allis, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 6364 (1995)].
30. We thank R. Heyman for use of TTNPB and LG629, S. L. Berger for discussion and providing hGCN5 expression vector, and Y. Nakatani and X. J. Yang for providing a tagged p/CAF expression vector. We also thank C. Nelson for experimental assistance, L.-M. Phillips for excellent technical assistance, and P. Myer for expertise in figure preparation. E.K. is supported by a U.S. Army Medical Research Program Award, J.T. by the National Cancer Institute of Canada, E.M.M. by an NIH Postdoctoral Fellowship, D.W.R. by an American Diabetes Association Career Development Award, and L.X. by an American Heart Association Predoctoral Fellowship. Supported by grants from NIH to C.K.G. and M.G.R.

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Phosphorylation and Activation of p70^{s6k} by PDK1

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Activation of the protein p70^{s6k} by mitogens leads to increased translation of a family of messenger RNAs that encode essential components of the protein synthetic apparatus. Activation of the kinase requires hierarchical phosphorylation at multiple sites, culminating in the phosphorylation of the threonine in position 229 (Thr^{229}), in the catalytic domain. The homologous site in protein kinase B (PKB), Thr^{308}, has been shown to be phosphorylated by the phosphoinositide-dependent protein kinase PDK1. A regulatory link between p70^{s6k} and PKB was demonstrated, as PDK1 was found to selectively phosphorylate p70^{s6k} at Thr^{229}. More importantly, PDK1 activated p70^{s6k} in vitro and in vivo, whereas the catalytically inactive PDK1 blocked insulin-induced activation of p70^{s6k}.

The p70 ribosomal protein S6 kinase, p70^{s6k}, participates in the translational control of mRNA transcripts that contain a polypyrimidine tract at their transcriptional start site (*1*). Although these transcripts represent only 100 to 200 genes, they can encode up to 20% of the cell's mRNA (*2*), with most of these transcripts encoding components of the translational apparatus (*2*). The p70^{s6k} has been tentatively identified as a downstream effector of the phosphoinositide 3-kinase (PI3K) signaling pathway, but the upstream kinases linking PI3K with p70^{s6k} have not been identified (*3*). In addition, p70^{s6k} activity is controlled by the mammalian target of rapamycin (mTOR), which appears to protect the kinase from a phosphatase (*4*). Activation of p70^{s6k} is associated with increased phosphorylation of eight residues (Fig. 1A) (*4*). Of these, three appear to be essential in regulating kinase activation, including Thr^{229} in the catalytic domain as well as Ser^{371} and Thr^{389} in the linker domain (Fig. 1A) (*4*, *5*). All three sites are conserved in many members of the AGC (protein kinases A, G, and C) family of Ser/Thr kinases, including PKB and PKC. The sequence surrounding Thr^{229} is also highly conserved in the Ca^{2+}- and calmodulin-dependent protein kinase family (CaMK), the closest neighbor to that of the AGC family (Fig. 1A) (*6*). Recently, it was demonstrated that a newly described kinase, termed PDK1, phosphorylates the equivalent site to Thr^{229} in PKB, Thr^{308} (*7*). Further studies have indicated that although PDK1 is constitutively active, its ability to phosphorylate Thr^{308} is blocked by the NH_2-terminal pleckstrin ho-

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