CREB-binding protein (CBP) and p300 contain modular domains that mediate protein-protein interactions with a wide variety of nuclear factors. A C-terminal domain of CBP (referred to as the SID) is responsible for interaction with the α-helical AD1 domain of p160 coactivators such as the steroid receptor coactivator (SRC1), and also other transcriptional regulators such as E1A, Ets-2, IRF3, and p53. Here we show that the pointed (PNT) domain of Ets-2 mediates its interaction with the CBP SID, and describe the effects of mutations in the SID on binding of Ets-2, E1A, and SRC1. In vitro binding studies indicate that SRC1, Ets-2 and E1A display mutually exclusive binding to the CBP SID. Consistent with this, we observed negative cross-talk between ERα/ SRC1, Ets-2, and E1A proteins in reporter assays in transiently transfected cells. Transcriptional inhibition of Ets-2 or GAL4-AD1 activity by E1A was rescued by co-transfection with a CBP expression plasmid, consistent with the hypothesis that the observed inhibition was due to competition for CBP in vivo. Sequence comparisons revealed that SID-binding proteins contain a leucine-rich motif similar to the α-helix Aα1 of the SRC1 AD1 domain. Deletion mutants of E1A and Ets-2 lacking the conserved motif were unable to bind the CBP SID. Moreover, a peptide corresponding to this sequence competed the binding of full-length SRC1, Ets-2, and E1A proteins to the CBP SID. Thus, a leucine-rich amphipathic α-helix mediates mutually exclusive interactions of functionally diverse nuclear proteins with CBP.

CBP and p300 interact with a wide range of DNA-binding transcription factors and their cofactors (1, 2). Recruitment of CBP and associated factors permits acetylation and methylation of histones and other proteins at gene promoters, leading to chromatin remodeling, RNA polymerase II recruitment, and transcription. The ability of CBP and p300 to form contacts with multiple diverse factors assembled at gene promoters such as the IFN-β enhancesome, facilitates synergistic activation of transcription (3). Conversely, competition between transcription factors for common binding sites on CBP/p300, which are in limiting concentrations in the nucleus, is likely to be important in negative cross-talk, as observed between nuclear receptors (NRs) and AP-1, NFκB, or STAT proteins (4, 5) or hypoxia-inducible factor (HIF1α) and CITED2 (6). Furthermore, CBP and p300 are important targets in viral infection, as they associate with viral proteins such as adenoviral E1A, SV40 large T antigen, and HTLV Tax (1, 2). Thus, CBP and p300 act as molecular integrators of signal transduction pathways regulating cellular processes such as proliferation, differentiation, apoptosis, and the response to viral infection.

The interaction of CBP/p300 with a large number of functionally diverse proteins is facilitated by a series of modular protein-binding domains. These include the cysteine/histidine-rich domains CH1 and CH3, also known as TAZ1 and TAZ2, which are major sites of protein interaction. The CH3/TAZ2 domain has a compact globular structure consisting of four α-helices and three HCCC zinc-binding motifs (7). A short sequence (TRAM) has been identified within CH3, which partly mediates the interaction of CBP with adenoviral E1A proteins (8). Although recent studies have confirmed that the CH1 and CH3 domains are structurally similar (9, 10), they appear to exhibit distinct, if overlapping, specificities for binding subsets of transcription factors. Some proteins such as E1A, p53 and Ets proteins, have been observed to interact with several discrete CBP domains (10). The KIX domain undergoes a conformational change upon association with the phosphorylated form of the kinase-inducible domain (KID) of CREB (11). In addition, other protein-interaction domains in CBP/p300 include the bromodomain, which mediates interaction of CBP with the acetylated p53, MyoD and other proteins (12), and three LXXLL motifs that mediate interactions with NRs (13, 14).

The p160 coactivators bind to a domain located near the C terminus of CBP and p300 (5, 10, 15, 17). We have characterized this sequence, termed the SRC1 interaction domain (SID), and mapped it to amino acids 2058–2130 of CBP (18). The SID domain interacts with amino acids 926–960 of SRC1, the transcriptional activation domain AD1, which is conserved among the p160 proteins (5, 15–18). The solution structure of this region of CBP in complex with the AD1 domain of ACTR has revealed that SID and AD1 polypeptides undergo a synergistic folding giving a structure resembling a four-helix bundle (19). In addition to binding p160 proteins, recent reports have demonstrated that other factors can interact with the SID including IRF-3, E1A, p53, Tax, Ets-2, and KSHV IRF-1 (20–22).

In this study we describe experiments which indicate that diverse nuclear proteins such as SRC1, E1A, and Ets-2 display mutually exclusive binding to the CBP SID both in vitro and in vivo. We show that these proteins share a sequence motif similar to an amphipathic α-helix in the AD1 domain of p160s, which is important for their interactions with the CBP SID.
Materials and Methods

Plasmid Expression Vectors—The following plasmids were used in this study and have been described previously: pBex LacZ, pMT-MOR (ER–596), pGEX-TRAM (CBP 1808–2130), pSG5 FLAG-hSRC1e (18) and pSG5 hSRC1e (29).

The following plasmids were generous gifts and have been described previously: pCDNA3 Flag-Ets-2, pCDNA3 Flag-Ets-2 (T722a) and UPA-Luc reporter (25, 26), pGEX-CBP-N (1–596), pGEX-TRAM (CBP 1808–1826) (8), pASV3, pASV3-mouse embryonic cDNA library (25), pMT-MOR (ERα) (40), pCDNA3 p53 (41), pC1 E1A12S, pC1 E1A12S ΔΔ–36 (32), pSERE-TATA-Luc (42), pJ7-lacZ (E. Kalkhoven).

cDNA sequences flanked by appropriate restriction enzyme sites were generated by PCR using Elongase (Invitrogen) and cloned in frame to a modified version of the pASV3 vector (23) to generate VP16-acidic activation domain (AAD) fusion proteins. Full-length Flag-Ets-2 cDNA was amplified from pCDNA3 Flag-Ets-2 and cloned into a modified pSG5 vector to create pSG5 Flag-Ets-2. The Flag-Ets-2 APTN deletion of amino acids 116–161) and Flag-Ets-2 ΔH5 deletion of amino acids 154–161) were constructed by PCR methods. All constructs generated in this study were verified by sequence analysis.

The yeast two-hybrid system was carried out essentially as described previously (23). Saccharomyces cerevisiae L40 cells carrying DBA-LacZ (1982–2163) were transformed with the pASV3 mouse embryo cDNA library using a modified lithium acetate transformation method (23). Transformants were initially selected for t-leucine and t-tryptophan prototrophy on dropout medium resulting in the recovery of 1.5 × 10^6 library clones. The library was then replated (10-fold) onto dropout medium with selection for t-leucine, t-tryptophan, and t-histidine prototrophy, and resistance to either 10 mg or 20 mg 3-aminoazoliole (3-AT), to select bait-interacting clones. Colonies capable of growth on 3-AT and showing strong activation of the secondary reporter (β-galactosidase) were subjected to further analysis. The 351 putative positives from the first round of selection were cultured in the presence of t-tryptophan to eliminate the bait plasmid, followed by rescue of pASV3 library (LEU2) plasmids in E. coli HB101 (lexAB+) by complementation on M9 minimal medium containing 100 mg/ml ampicillin, and lacking t-leucine. [35S]labeled positive clones were selected by retransformation of fresh L40 pBM116-CBP 1982–2163 and tested for 3-AT resistance and β-galactosidase activity.

Qualitative β-galactosidase assays were carried out in duplicate, in three separate experiments, as previously described and reporter activities are expressed as nmol of substrate transformed/minute/mg protein extract (18, 23). The expression of fusion proteins in cell-free extracts was monitored by Western blotting using antibodies (Autogen BioClear) directed against VP16 (sc-7545), or LexA (sc-7544) or Flag-M2 (Sigma) epitopes.

Cell Culture and Transient Transfection—CO-1 and HEK293 cells were maintained in Dulbecco’s Modified Eagle’s Medium, (DMEM, Invitrogen) supplemented with 10% fetal calf serum (Invitrogen). For transient transfections, cells were grown to 70–80% confluence and transfected with 15 μg of either pCDNA3–Flag vector (Control), pCDNA3 Flag-Ets-2 or pSG5 Flag-SRC1C in addition to 15 μg of pSG5 CBP, pCMV HA-p300, or empty vectors. After 20 h, cells were lysed in 1 ml of Extraction Buffer I (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40), and protease inhibitors at 0 °C for 15 min. The cell nuclei were pelleted and resuspended in 1 ml of Extraction Buffer II (50 mM Tris-HCl, pH 8.0, 420 mM KCl, 1% glycerol; 2 mM diithiothreitol; with protease inhibitors). After 15 min at 0 °C, the lysate was centrifuged at 10 k rpm, 4 °C, for 10 min, and preclarified for 30 min at 4 °C with 20 μl of protein-A/G PLUS-agarose beads (Agilent BioClear, sc-2003). Flag-tagged proteins were purified using 20 μl of packed volume α-Flag M2-agarose affinity gel (Sigma, A-2220) on a rotating wheel overnight at 4 °C, followed by three washes in 0.5 ml of Extraction Buffer II.

Proteins bound to the beads were separated by SDS-PAGE (8% acrylamide for p300 or CBP, 12% acrylamide for Flag-tagged proteins), transferred to nitrocellulose and detected by Western blotting. Tagged-proteins were detected using a 1:5000 dilution of α-Flag M2 monoclonal antibody (Sigma, F-3165) followed by a 1:2000 dilution of secondary α-mouse IgG-HRP (Autogen BioClear, sc-2954). For detection of co-purified HA-p300, a 1:500 dilution of α-HA (Autogen BioClear, sc-7392) antibody was used, in conjunction with a 1:2000 dilution of α-mouse IgG-HRP. Similarly for CBP detection, a 1:500 dilution (mix) of CBP (A-223 and CBP (C-20) rabbit polyclonal antibodies (Autogen BioClear, sc-583 and sc-369, respectively) was used, in conjunction with a 1:2000 dilution of anti-rabbit IgG-HRP (Autogen BioClear, sc-2004). Bound peroxidase-coupled antibodies were revealed using the ECL Plus system (Amer sham Biosciences).

Results

The PNT Domain of Ets-2 Mediates Its Interaction with CBP SID—To isolate proteins that interact with the CBP SID domain, we carried out a yeast two-hybrid screen of a VP16 AAD-fused mouse embryonic cDNA library (43), using a DBD LexA-CBP 1982–2163 clone as bait (18). From two million individual clones screened, five bona fide positive clones were identified. Sequence analysis and database searches revealed that one of these (clone 1–01) encoded the N terminus (amino acids 1–273) of the mouse Ets-2 protein, with an additional 54 codons of in-frame sequence upstream of the proposed start codon derived from 5’UTR sequence. This comprises the entire transactivation domain of murine Ets-2 including the conserved PNT domain (amino acids 60–170). Removal of the codons derived from the 5’UTR sequence (AAD-Ets-2 (1–273)) did not affect the interaction with the bait sequence, confirming that this sequence was not required for the interaction with CBP (Fig. 1A).

As the boundaries of the bait sequence (1982–2163) were extended beyond the minimal SID, we used a series of LexA-CBP fusion proteins to define the minimal sequences capable of binding Ets-2 (1–273). As shown in Fig. 1B, the minimal CBP sequence required to bind to the AAD-Ets-2 (1–273) corresponded to the previously characterized SID domain (CBP 2058–2130). A previous study reported interactions of Ets-2 with the CH1/TAZ1 domain and also a large C-terminal portion of CBP...
(amino acids 1678–2370) containing the CH3/ZZ-TAZ2 and SID domains (24). We assessed the binding of in vitro translated full-length Ets-2 to GST-CBP fusion proteins GST-CBP-N (1–596) or GST-SID (2058–2130). Similar amounts of the GST fusion proteins and control (GST alone) were used as indicated (Fig. 2A, lower panel). Ets-2 was found to bind strongly to the CBP SID domain, whereas a weaker interaction was detected with CBP N terminus containing CH1 under similar conditions (Fig. 2A, upper panel). This result indicates a strong interaction between Ets-2 and the SID domain of CBP in vitro.

To determine the boundaries of the Ets-2 sequence required for binding to the CBP SID domain, we generated a series of VP16 AAD-Ets-2 fusion constructs. Reporter assays were carried out on extracts from yeast L40 cells co-expressing LexA-CBP (1982–2163) with VP16 AAD clone 1–01 isolate, and AAD-Ets-2 (1–273). A schematic representation showing domain structure of Ets-2, clone 1–01 isolated in the yeast two-hybrid screen and AAD-Ets-2 (1–273) is shown. Clone 1–01 contains an additional 54 codons of 5′-UTR sequence that are translated in-frame with Ets-2 (1–273). The PNT and ETS domains are indicated. B, yeast two-hybrid interaction of AAD-Ets-2 (1–273) with a series of DBD LexA-CBP fusion proteins. A schematic representation of the CBP sequence 2058–2163 is shown, indicating four α-helices H1 to H4 (black boxes) and the QPG(M/L) repeat sequences (black arrows). CBP sequences are represented schematically, and the central black box denotes the minimal SID as described previously (18).

Effects of Amino Acid Substitutions in the CBP SID on Recruitment of SRC1, Ets-2, and E1A—In addition to binding
proteins and Ets-2, the SID domain has been reported to interact with a variety of other cellular and viral nuclear proteins including the adenoviral E1A 12S protein (20, 22). To determine whether these functionally diverse proteins make similar contacts with the CBP SID domain, we examined the impact of amino acid substitutions in the CBP SID domain on its interaction with SRC1, Ets-2, and E1A. Full-length 35S-labeled SRC1e, Ets-2 and E1A proteins were generated by in vitro translation and assessed for binding to a panel of GST-SID mutants (18). Amino acid substitutions within the SID structure (19) are depicted schematically in Fig. 3A. As shown in Fig. 3B, and shown previously for SRC1, (18), substitution of the conserved leucine residues in helix H1 (L2071A/L2072A/L2075A, L2071P/L2072P, and L2071A/L2072A) only weakly reduced binding of E1A and SRC1 to the SID. However, Ets-2 binding to the SID was more sensitive to mutations in H1 (Fig. 3B). In addition, the F2101P and K2103P substitutions, which are predicted to disrupt helix H3 formation, ablated or strongly reduced the interaction of all three proteins with the SID (Fig. 3B). In contrast, amino acid substitutions that replace Lys-2103 or Lys-2108 with alanine, which would not be expected to disrupt H3 formation, had little effect on E1A or SRC1 binding, and only partly reduced the binding of Ets-2 to the SID. Similarly, amino acid replacements in the polyglutamine rich sequence (Q-loop) between helices H1 and H2 (Q2082R) or the QPG(M/L) repeat region (Q2113P/G2115P or Q2117A/P2118A)
had little or no deleterious effect on interactions with SRC1 or E1A, although substitution of three of the four glutamines in the region between H1 and H2 (Q2082P/Q2084P/Q2085G) resulted in reduced Ets-2 binding (Fig. 3B). These results suggest that SRC1, Ets-2, and E1A recognize a similar surface of the CBP SID domain, for which the integrity of H3 is critical. However, H1 appears to be specifically important for the docking of the Ets-2 protein.

To assess whether the SRC1 AD1 domain (900–970) and Ets-2 PNT domain (60–170) displayed similar sensitivities to SID mutations as the full-length proteins, we assessed the abilities of AAD fusion proteins containing these sequences to interact with a panel of LexA-CBP-SID proteins, in yeast two-hybrid experiments (Fig. 3C). The various LexA-CBP-SID proteins contained similar amino acid substitutions to those used in the GST pull-down experiments (Fig. 3B). Consistent with the in vitro binding data, replacement of Phe-2101 in H3 with a proline residue resulted in loss of binding to the AD1 and PNT domains. Similarly, the K2103P substitution resulted in loss of binding to AD1 and PNT. In contrast, replacement of Lys-2103 with alanine had little effect on AD1 and reduced the interaction with PNT/AD1 by 2-fold. Substitution of the conserved leucines in H1 (L2071A/L2072A/L2075A) had only a weak effect on reporter activation due to AD1 binding, whereas the interaction with PNT was strongly reduced (Fig. 3C). In addition, and in agreement with the in vitro binding data (Fig. 3B), SID proteins containing Q2082R, Q2117A/P2118A, and K2108A showed little or no impairment of their interactions with the AD1 or PNT domains. In summary, the results from these experiments are consistent and indicate that SRC1, E1A, and Ets-2 dock at a common site on the surface of CBP, None-theless, the differential sensitivity of Ets-2 to mutations in H1 compared with SRC1 and E1A suggests there are may be subtle differences between the various SID complexes.

**Mutually Exclusive Interactions of SRC1, Ets-2, and E1A with the CBP SID in Vitro**—The observation that different

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**Fig. 3. Effect of amino acid substitutions on recruitment of proteins to the SID. A, structure of the ACTR/CBP AD1/SID complex (19) highlighting α-helices in the AD1 (Aα1-Aα3) and SID (Cα1-Cα3) domains. The amino acids that were substituted in this study are indicated. B, in vitro binding experiments as in Fig. 2 showing the interactions of in vitro translated [35S]methionine-labeled Ets-2, E1A and SRC1e proteins with GST (lane 2) and GST-SID proteins (lanes 3–14). Ten percent of the input is shown in lane 1. The regions containing amino acid substitutions are indicated: H1 (or Cα1), H2 (or Cα2), H3 (or Cα3), the glutamine (Q) loop, and the QPGM/L repeat sequence. C, yeast two-hybrid assays were performed as in Fig. 1 to assess interaction of DBD LexA-CBP SID proteins with AAD-AD1 (900–970) and AAD-PNT (60–170). The SID is represented schematically; black boxes indicate H1, H2, and H3, and white circles indicate the relative positions of amino acid changes.**
nuclear proteins bind the CBP SID domain suggested that these interactions might be mutually exclusive. To test this, we performed competitive in vitro binding assays (38) using GST-SID or GST-CBP full-length proteins and in vitro translated [35S]methionine-labeled E1A, Ets-2, and SRC1 proteins. The amounts of Ets-2 or E1A proteins required to saturate aliquots of GST-SID beads were predetermined. Next, fixed amounts of E1A or Ets-2 were premixed with varying amounts of SRC1 protein prior to incubation with the GST-SID beads. The binding of E1A or Ets-2 protein to GST-SID was reduced in the presence of SRC1, in a dose-dependent manner (Fig. 4A, left panel) as confirmed by quantitative phosphorimager analysis (Fig. 4A, right panel). In similar experiments, E1A was found to reduce the binding of SRC1 to GST-SID (Fig. 4B). Similar assays were performed using GST-CBP full-length protein. As shown in Fig. 4C, the binding of Ets-2 to full-length CBP was partially reduced by SRC1, consistent with the presence of an additional binding site for Ets-2 at the N terminus of CBP. In conclusion, our results suggest that the interactions of SRC1, E1A, and Ets-2 with CBP SID are mutually exclusive in vitro.

Transcriptional Interference Between SRC1, Ets-2, and E1A in Vivo Due to Competition for CBP Binding—Competition for cofactors such as CBP in vivo has been proposed to account for the negative cross-talk observed between nuclear receptors, AP-1, and other DNA binding transcription factors (4, 5, 38). To test whether competition for binding to the CBP SID domain might lead to negative cross-talk in vivo, we examined the effect of co-expression of SID-binding proteins on their ability to activate reporter genes in transiently transfected cells. Initially, we examined the effect of Ets-2 co-expression on the transcriptional activity of the estrogen receptor (ERα), which is dependent on p160/CBP interactions (18, 27, 28). As shown in Fig. 5A, ligand-dependent reporter activation by ERα in COS-1 cells was enhanced by co-expression of SRC1, as previously reported (13, 18, 29). However, co-transfection of Ets-2 repressed the ligand-dependent reporter activity in a dose-dependent manner (Fig. 5A). Ets-2 also decreased ERα activity in the absence of exogenous SRC1, suggesting Ets-2 also competes with endogenous p160 co-activators (Fig. 5B). In a reciprocal experiment, Ets-2-mediated activation of the UPA-Luc reporter (26), which contains Ets-2 binding sites, was reduced by co-expression of Ets-2 with SRC1 (Fig. 5C). This inhibitory effect was not likely to be due to direct interaction of Ets-2 and SRC1 proteins, as we were unable to detect any interaction between these proteins in yeast two-hybrid or GST pull-down experiments (data not shown). Thus, the negative cross-talk between ERα/SRC1 and Ets-2 observed here is consistent with the hypothesis that the binding of p160s and Ets-2 to the CBP SID domain is mutually exclusive.

The adenoviral E1A 12S proteins interact with a number of key cellular proteins such as retinoblastoma (RB) and CBP/p300. Although cell transformation by E1A involves a number of cellular pathways, this function is dependent on the N terminus of E1A, which is the domain required for transcriptional repression and CBP/p300 interaction (30, 31). We tested whether E1A would interfere with Ets-2 activity in reporter assays. As shown in Fig. 5D, the transcriptional activity of Ets-2 was efficiently blocked by E1A, but not by E1A lacking the amino acids 2–36. Co-transfection with increasing amounts of recombinant CBP rescued the reduced reporter activity of Ets-2 (Fig. 5E), consistent with the hypothesis that E1A inhibition of Ets-2 may be due to competition for a limiting amount of CBP in vivo.

We also tested the ability of E1A to inhibit transcription by a construct consisting of GAL4 DBD fused to the AD1 domain of SRC1. This potent synthetic activator functions by recruitment of CBP via the SID domain (18). Our results showed that GAL4-AD1 activity was also blocked by E1A but not E1A2–36 (Fig. 5F), and that exogenous CBP rescued the reduced reporter activities in a dose-dependent manner (Fig. 5G). In control experiments performed in the absence of E1A, co-expression of Ets-2 and GAL4-AD1 with similar amounts of CBP did not significantly affect the level of reporter activity (data not shown).

A Conserved Amphipathic α-Helix in CBP SID-binding Proteins—Sequence comparisons revealed that the E1A N terminus and other SID-binding proteins contain a potential amphipathic α-helix similar to Aα1 in the AD1 domain of p160 proteins. The sequences within SID-binding proteins that share homology to the consensus sequence LLD/EQL(–2 X 3)LL are shown, and in some cases there are adjacent sequences similar to the LPEφ motif found in Aα3 (Fig. 6A). The PNT domain of Ets-1 consists of 5 α-helices that are conserved in Ets-2 (33). Sequence comparisons revealed that
Transcriptional interference between CBP SID-binding proteins in vivo. A, dose-dependent effect of Ets-2 on ERα/SRC1 transcriptional activity. Reporter activity in extracts from transiently transfected COS-1 cells cultured in the presence or absence of ligand (500 nM E2), as indicated. The cells were co-transfected with p3ERE-TATA-Luc, pJ7 lacZ, pMT MOR (ERα), pSG5 SRC1α, and pcDNA3 FLAG-Ets-2 as described under “Materials and Methods,” or as indicated (amount of plasmid expressed in nanograms). The luciferase reporter activity was normalized to the presence of the p300, and this interaction was reported to involve a short CBP sequence within the CH3 domain designated TRAM (8). Thus, as a control, we assessed the effect of the SRC1 AD1 peptide on the interaction of E1A protein with GST-TRAM. As shown in Fig. 6F, the AD1 peptide did not interfere with the binding of E1A to GST-TRAM (or GST-CH3, data not shown). As previous studies have focused on interaction of E1A with the CBP CH3 domain (8, 31, 32), we investigated whether the N terminus of E1A is required for binding to the CBP SID. As shown in Fig. 6G, deletion of amino acids 2–36 resulted in the loss of interaction of E1A with GST-SID in vitro. This result clearly shows that amino acids 2–36 of E1A are necessary for binding to CBP via the SID domain, as well as the CH3 domain as reported previously (8, 31, 32). Thus we conclude that E1A interaction with the CBP SID domain involves docking at a similar surface to that bound by the p160 AD1 domain. Taken together, our results show that diverse cellular and viral proteins employ a conserved leucine-rich amphipathic α-helix to dock with the CBP SID domain.

**DISCUSSION**

Docking at the SID Interface—The NMR structure of the ACTR AD1 domain in complex with the CBP SID showed that ACTR helix Aα1 occupies a hydrophobic groove between SID H1 and H3 (19). In addition, Aα3 helix of ACTR packs against the hydrophobic face of the SID H3. In this study we have characterized the interactions of three functionally distinct on Ets-2 reporter activation. Reporter activity in extracts from COS-1 cells co-transfected with pUPA-luc, pJ7 lacZ, pcDNA3 FLAG-Ets-2, and pSG5 SRC1α as indicated. The luciferase reporter activity was normalized to the β-galactosidase control, and expressed as fold induction over the activity of reporter alone in the absence of activators. D, dose-dependent effect of E1A or E1AΔ2–36 on reporter activation by Ets-2. E, dose-dependent CBP rescue of E1A-inhibition of Ets-2 reporter activation. F, effect of E1A Δ2–36 on activation of the GAL-ELB-Luc reporter by GAL4-AD1. G, dose-dependent CBP rescue of GAL4-AD1 reporter activation inhibited by E1A.

helix 5 (H5) of the PNT domain shares similarity with the AD1 Aα1 consensus sequence (Fig. 6A). We therefore constructed expression plasmids encoding mutant Ets-2 proteins lacking the entire PNT domain, or amino acids 154–161 encoding H5. In vitro binding experiments showed that the deletion of the PNT domain or the H5 sequence abrogated the interaction of Ets-2 with the SID domain (Fig. 6B) or full-length CBP protein (Fig. 6C), indicating a requirement for the PNT domain, and more specifically the H5 sequence, for CBP binding. Similarly, SRC1, Ets-2, or Ets-2 T72A proteins in whole cell extracts of transiently transfected COS-1 cells showed strong interaction with GST-SID, whereas binding of Ets-2 ΔPNT and Ets-2 ΔH5 was greatly reduced (Fig. 6D) Thus Ets-2 and p160s share an α-helical motif necessary for binding to the CBP SID domain.

We have previously reported that a short peptide comprising part of the AD1 sequence can disrupt the binding of full-length SRC1 to GST-CBP proteins in vitro (18). Based on the structure of the ACTR/CBP polypeptide complex (19), the SRC1 peptide used in our competition experiments (amino acids 925–958) encompasses helices Aα1 and Aα2 of the AD1 domain (see Figs. 3A and 6A). To examine the effect of this peptide on the recruitment of other SID-binding proteins, we assessed whether it would block the interaction of Ets-2, E1A, or p53 with GST-SID. As shown in Fig. 6D (left panel), the AD1 peptide efficiently competed the interaction of SRC1, Ets-2, and E1A with GST-SID. Recruitment of p53 to GST-SID was also reduced in the presence of the AD1 peptide, albeit less efficiently than SRC1, Ets-2 or E1A. These results were confirmed quantitatively by phosphorimager analysis (Fig. 6D, right panel).

E1A proteins also interact with the CH3 domain of CBP and p300, and this interaction was reported to involve a short CBP sequence within the CH3 domain designated TRAM (8). Thus, as a control, we assessed the effect of the SRC1 AD1 peptide on the interaction of E1A protein with GST-TRAM. As shown in Fig. 6F, the AD1 peptide did not interfere with the binding of E1A to GST-TRAM (or GST-CH3, data not shown). As previous studies have focused on interaction of E1A with the CBP CH3 domain (8, 31, 32), we investigated whether the N terminus of E1A is required for binding to the CBP SID. As shown in Fig. 6G, deletion of amino acids 2–36 resulted in the loss of interaction of E1A with GST-SID in vitro. This result clearly shows that amino acids 2–36 of E1A are necessary for binding to CBP via the SID domain, as well as the CH3 domain as reported previously (8, 31, 32). Thus we conclude that E1A interaction with the CBP SID domain involves docking at a similar surface to that bound by the p160 AD1 domain. Taken together, our results show that diverse cellular and viral proteins employ a conserved leucine-rich amphipathic α-helix to dock with the CBP SID domain.
nuclear proteins (Ets-2, E1A, and SRC1) with the SID. Our \textit{in vitro} binding experiments confirm that the structural integrity of SID H3 is critical not only for docking of the p160 protein SRC1, but also other SID-binding proteins such as Ets-2, E1A (Fig. 3, B and C; Ref. 18) and others (data not shown). Disruption of H3 by replacement of Phe-2101 or Lys-2103 with proline, resulted in loss of binding all three proteins tested (Fig. 3, B and C). In contrast, replacement of conserved lysine residues in SID H3 with alanines (K2103A and K2108A) did not alter the interactions with SRC1, and caused only a weak reduction in Ets-2 and E1A binding (Fig. 3, B and C). Thus, our results suggest that H3 integrity is essential for contacts with at least three of the proteins known to bind the SID, although lysine residues in H3, which are involved in hydrogen bonding interactions with the carbonyl backbone of ACTR AD1 (19), do not appear to be critical for the interactions of SRC1 AD-1 and CBP SID in these experiments.

Leucine residues in SID H1 pack against the hydrophobic face of ACTR A/H9251 (19), suggesting these residues may be important for p160 binding. However, our results show that replacement of Leu-2071, Leu-2072, and Leu-2075 in H1 with alanines only slightly reduced AD1 or SRC1 binding to the SID, and similarly, had little effect on the interaction with E1A (Fig. 3, B and C). In contrast, Ets-2 interaction with the SID was impaired by the H1 mutations (Fig. 3, B and C), suggesting that PNT may make additional contacts with the SID via H1.

Fig. 6. A conserved amphipathic \(\alpha\)-helix in CBP SID-binding proteins. A, sequence alignments of SID-binding proteins. The positions of helices \(\alpha\alpha_1\), \(\alpha\alpha_2\), and \(\alpha\alpha_3\) in the ACTR AD-1 domain (38) are indicated. The shaded areas denote sequences homologous to \(\alpha\alpha_1\) and \(\alpha\alpha_3\), and the consensus LXXLXXLL motif is indicated. B, interactions of \textit{in vitro} translated Ets-2, Ets-2 \(\Delta\text{PNT}\), and Ets-2 \(\Delta\text{H5}\) with GST or GST-SID. C, interactions of \textit{in vitro} translated Ets-2, Ets-2 \(\Delta\text{PNT}\), and Ets-2 \(\Delta\text{H5}\) with GST-CBP full-length protein. D, Western blots of whole cell extracts of transiently transfected COS-1 cells showing detection of FLAG-SRC1, Ets-2, Ets-2 \(\Delta\text{PNT}\), and Ets-2 \(\Delta\text{H5}\) and Ets-2 T72A proteins (input). SRC1e was detected using \(\alpha\)-FLAG-M2 antibody, whereas Ets-2 proteins were detected using \(\alpha\)-Ets2 antibodies (Sigma). Equal amounts of each extract were loaded onto GST-SID beads and bound proteins detected by Western blotting (lower panel). E, peptide competition experiments showing the binding of full-length SRC1e, Ets-2, E1A, or p53 proteins to GST-SID in the presence of various amounts of a competitor peptide corresponding to amino acids 925–958 of the SRC1 AD1 domain. The right panel shows the data quantitated using phosphorimager analysis. F, effect of the AD1 competitor peptide on the interaction of E1A with the GST-TRAM motif (CBP amino acids 1808–1826). G, \textit{in vitro} interactions of E1A wild-type (WT) and E1A (\(\Delta\text{2–36}\)) proteins with GST-SID.
Five glutamine residues that form a loop between H1 and H2 in the SID (Q-loop; Fig. 3B) have been suggested to be important for alignment of H1 with ACTR Aα1 (19). We have previously shown that mutation of individual (Q2082P/Q2084P/Q2085G) in this hinge had little effect on the binding of AD1 or full-length SRC1, consistent with our observation that H1 leucines are not critical for SRC1 binding (Ref. 18; Fig. 3, B and C). However, the Q2082P/Q2084P/Q2085G mutation reduced the efficacy of SID binding to Ets-2 (Fig. 3B). Thus, the differential effects of this mutation are consistent with the additional requirement of H1 integrity for Ets-2 binding.

The sequence C-terminal to H3 contains repeats of the sequence QPG(M/L) that appears to stabilize the interaction of SRC1 (18) and Ets-2 (Fig. 1B) with the SID. However, SID proteins containing Q2113P/Q2115P and Q2117A/P2118A mutations, showed no decreased binding of SRC1, Ets-2, or E1A (Fig. 3, B and C). Thus further analysis will be required to determine how this sequence stabilizes interactions with SID-binding proteins. In summary, the structural integrity of H3 is important for the binding of diverse proteins to the SID domain, whereas Ets-2 shows an additional requirement for the conserved leucine residues in H1 and a glutamine loop region for docking at this CBP surface.

**Competition between Activators and Coactivators for Binding to CBP/p300**—The ability of diverse nuclear proteins to associate with the CBP SID suggested that the complexes formed might be mutually exclusive. The results from our *in vitro* binding experiments are consistent with this model, as we observed competition between SRC1, Ets-2, and E1A for binding to GST-CBP proteins *in vitro* (Fig. 4). In addition, we observed reciprocal transcriptional repression between SID-binding proteins in reporter assays. The Ets-2 activator was found to inhibit the activity of Ets-2, and similarly, SRC1 inhibited Ets-2 reporter activity in a dose-dependent manner (Fig. 5, A–C). Negative cross-talk between nuclear receptors and Ets proteins has been reported previously (34–38).

For example, matrix metalloproteinase genes (MMPs) are down-regulated by steroid hormones, and androgen receptor (AR) down-regulation of the MMP-1 gene was shown to be mediated through effects on the Ets factor ER (34). Similarly, MMP-1 gene expression is repressed by all-trans retinoic acid in breast cancer cells (35). The peroxisome proliferator-activated receptor (PPARγ) was reported to inhibit Ets-1-mediated expression of the c-Met tyrosine kinase gene (36), and negative cross-talk was also observed between Fli-1 and retinoid or steroid receptors (37). Moreover, it has been shown that negative cross-talk between nuclear receptors and other DNA-binding factors, such as AP-1, Ets, or STAT proteins, involves competition for binding to CBP/p300 (4, 5, 38). Our results indicate that the negative cross-talk observed between ERα and Ets-2 in this study involves competition between p160 coactivator SRC1 and Ets-2 for CBP binding. Similarly, a recent report has shown that competition for binding to the CH1 domain of CBP underlies the negative regulation of HIF1α activity by the coregulator CITED2 (6).

The interaction of E1A with CBP and p300 is mediated by its N-terminal region, of which amino acids 1–25 and 60–80 have been proposed to form a bipartite binding domain (31, 32). Deletion of the amino acids 2–36 in E1A leads to a loss of interaction with CBP and p300 in co-immunoprecipitation experiments (32), and a concomitant loss of E1A repressor function and cell transformation (30, 31). Previous studies have demonstrated that nuclear receptor activity is blocked by E1A, and that E1A inhibits the assembly of p160/CBP complexes *in vitro* (38). Our results show that E1A interacts with the SID, and can repress transcription mediated by SID-binding proteins such as Ets-2 (Fig. 5D), or the synthetic activator GAL4-AD1 (Fig. 5F), and that this repression is dependent on amino acids 2–36 of E1A. The ability of exogenous CBP to rescue this activity (Fig. 5, E and G) provides further evidence that E1A repression involves interaction with the CBP SID. Thus, as the N-terminal 32 amino acids of E1A have been shown to contact both the CH1 and SID domains of CBP/p300, it is possible that discrete CBP domains form a composite docking site for the E1A protein.

**Ets-2 Interactions with CBP**—Previous reports have identified interactions of the N terminus of Ets-1 with the CH1 and CH3 domains of p300 (24, 26). In this study we identified a strong interaction between the Ets-2 N terminus and the C-terminal SID domain of CBP in a yeast two-hybrid screen (Fig. 1). Our interaction domain mapping experiments indicate that the PNT domain, which defines a subset of Ets proteins, is both necessary and sufficient for the binding of Ets-2 to the CBP SID (Figs. 2B and 6, B and D). The interaction requires a conserved α-helix (H5) in the PNT domain that shares homology with the Aα1 helix in the AD1 domain of p160s (Fig. 6A). An adjacent sequence encoding α-helix H4 has been reported to function as an ERK2 docking site, facilitating phosphorylation of a conserved threonine residue outside the PNT domain (39). Mutation of this threonine residue (which is also a target for JNK and Akt kinases) in Ets-2 (T72A) resulted in the loss of Ras-induced superactivation of a reporter gene (26). Our results indicate that the Thr-72 phosphoacceptor site of Ets-2 is not essential for CBP SID binding, as a PNT T72A mutant retained strong interaction with the SID *in vitro* and in yeast two-hybrid experiments (Figs. 2B and 6D). This is consistent with the observation that the phosphorylation status of the corresponding residue (Thr-38) did not influence folding of the Ets-1 PNT domain (33). Thus, while phosphorylation of Thr-72 may be important for the regulation of Ets-2 activity in *vivo*, it does not appear to influence the PNT/SID interaction.

**An Amphipathic α-Helix Constitutes a CBP SID-binding Motif**—Having established that diverse proteins compete for binding to the SID, and that a peptide encompassing SRC1 Aα1 and Aα2 domains has blocked the interaction of SRC1, Ets-2, and E1A with the SID (Fig. 6E), we were prompted to look for sequence motifs resembling p160 AD-1 in SID-binding proteins. As shown in Fig. 6A, sequences similar to the LLD/EQLXXL motif of AD-1 Aα1 were identified in E1A, Ets-1, Ets-2, IRF-3, Tax, and CAS. The interaction of CBP and the nuclear exportin CAS2 will be reported elsewhere. The E1A sequence (19–28) is contained within the N-terminal region required for binding to CBP SID (Fig. 6G) and CH3 domains (30–32). Similarly, deletion of the H5 α-helix of the PNT domain, which is positioned favorably for interactions with other proteins (33), abrogated the interaction of Ets-2 with CBP SID in similar experiments (Fig. 6B–D). Some SID-binding proteins contain sequences similar to the p160 Aα3 helix, which contains a LPEΦ motif (Fig. 6A). We note that a similar sequence (LPEL) is important for the interactions of CITED2 and HIF1α with the CH1 domain (6). Further studies will be required to investigate whether these motifs are important for the interaction of proteins with the CBP SID domain.

Leucine-rich amphipathic α-helices, such as the canonical LXXLL motifs, are known to mediate protein-protein interactions between transcription factors and co-regulators (21). We have shown here that another such sequence motif is important.

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*J. C. Harries, K. B. Kindle, C. Ryan, and D. M. Heery, unpublished results.*
for the association of diverse nuclear proteins including DNA-binding transcription factors, viral proteins, co-activators, and nuclear transport proteins with a conserved domain at the C terminus of CBP. In addition, our results suggest that p160s and other proteins such as Es factors compete for binding to CBP, and that such mutually exclusive interactions may contribute to the negative cross-talk observed between different signaling pathways in vivo.

Acknowledgments—We thank the following colleagues for generous gifts of materials: P. Chambon, A. Harel-Bellan, E. Kalkhoven, T. Kouzarides, X. Liu, D. Latchman, M. Ostrowski, and M. Parker.

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A Conserved α-Helical Motif Mediates the Binding of Diverse Nuclear Proteins to the SRC1 Interaction Domain of CBP

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J. Biol. Chem. 2004, 279:14055-14064.
doi: 10.1074/jbc.M310188200 originally published online January 13, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M310188200

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