E2F Transcriptional Activation Requires TRRAP and GCN5 Cofactors*

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The E2F family of transcription factors regulates the temporal transcription of genes involved in cell cycle progression and DNA synthesis. E2F transactivation is antagonized by retinoblastoma protein (pRb), which recruits chromatin-remodeling proteins such as histone deacetylases and SWI/SNF complexes to the promoter to repress transcription. We hypothesized that E2F proteins must reverse the pRb-imposed chromatin structure to stimulate transcription. If this is true, E2F proteins should recruit proteins capable of histone acetylation. Here we map the E2F-4 transactivation domain and show that E2F-1 and E2F-4 transactivation domains bind the acetyltransferase GCN5 and cofactor TRRAP in vivo. TRRAP and GCN5 co-expression stimulated E2F-mediated transactivation, and c-Myc repressed E2F transactivation dependent on an intact TRRAP/GCN5 binding motif. The transactivation domain of E2F-4 recruited proteins with significant histone acetyltransferase activity in vivo, and this activity required catalytically active GCN5. E2F-4 proteins with subtle mutations in the transactivation domain exhibited a positive correlation among transcriptional activation and GCN5 and TRRAP binding capacity and associated acetyltransferase activity. We conclude that E2F stimulates transcription by recruiting acetyltransferase activity and the essential cofactors GCN5 and TRRAP. These results provide a mechanism for E2F transcription factors to overcome pRb-mediated dominant repression of transcription.

Consistent with this, enforced expression of E2F proteins drives quiescent cells into S phase and transforms cells in conjunction with activated Ras (2–6). Furthermore, E2F binding sites are found in the promoters of numerous genes whose expression is necessary for the initiation of S phase and DNA replication (7–9). DNA binding activity of the E2F transcription factor family is composed of two subunits, E2F and DP, which form heterodimeric complexes with a high affinity for the DNA sequence 5’-TTTCGCG-3’ (7–9). Currently, the mammalian E2F family consists of six E2F and two DP genes. The DNA binding, heterodimerization, and marked box domains are highly conserved among all E2F family members. E2F-1, -2, and -3 have an extended N-terminal region containing a nuclear localization signal and binding sites for cyclin A, the transcription factor Sp1, and the E3 ubiquitin ligase complex SCF-SKP2 (10–17). E2F-4, -5, and -6 lack these sequences. E2Fs 1–5 have an acidic transactivation domain at the C terminus that is overlapped by the binding site for members of the pRb family (18–22).

The transactivation capacity of the E2F family members is primarily controlled through interactions with the retinoblastoma (pRb)1 family of tumor suppressors. The cell cycle machinery regulates pRb repression of E2F transcription factors by targeting pRb for phosphorylation by cyclin-dependent kinases (7–9). In quiescent cells, pRb proteins are hypophosphorylated and bound to the transactivation domain of E2F family members in the nucleus. The association of pRb with E2F heterodimers inhibits transactivation by both physically masking the E2F transactivation domain and by acting as a dominant transcriptional repressor (21, 23–29). pRb/E2F complexes actively repress transcription by recruiting chromatin-remodeling machinery to the promoter, including histone deacetylases and SWI/SNF complexes (30–36). Histone deacetylation is associated with transcriptional repression via a closed chromatin structure of promoter regions (37, 38). As cells re-enter the cell cycle, pRb becomes hyperphosphorylated and releases “free” E2F transcription factors. The cell cycle-dependent phosphorylation of pRb disrupts interactions with E2F transcription factors and chromatin-remodeling complexes and thereby relieves both passive and active repression of E2F-responsive promoters. Relief of pRb transcriptional repression is necessary for the expression of E2F-responsive genes and cell-cycle progression.

The regulation of transcription factors by acetylation and

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PCAF-related transcriptional co-activator/acyetyltransferase GCN5 is a component of the SAGA complex in yeast (y) (reviewed in Ref. 45). The human homologues of the SAGA complex consist of three distinct complexes, i.e., TFTC, PCAF, and STAGA complexes (46, 47). These complexes are related to TFIIID but are distinguished by various combinations of other proteins involved in transcriptional regulation. Common components of the ySAGA, TFTC, and PCAF complexes are the GCN5 or PCAF acetyltransferases and the cofactor yTral or TRRAP (48–50). TRRAP has been shown to bind the transcription domain of E2F-1, implicating a SAGA-like complex in E2F transcriptional activation (51).

This work characterizes the protein interactions of the transcriptional domains of E2F-1 and E2F-4 as archetypes of the E2F family. We show that GCN5 and TRRAP are components of E2F-1- and E2F-4-transactivating complexes and stimulate E2F-dependent transcription. Furthermore, E2F-4 recruits proteins capable of significant acetyltransferase activity that correlates perfectly with the ability to bind GCN5 and stimulate transcription. The E2F-4-associated acetyltransferase activity depends on catalytically active GCN5. GCN5 and TRRAP may contribute to E2F-dependent transcriptional stimulation by reversing the pRb-histone deacetylase-imposed chromatin structure to enhance access to the promoter for the general transcription machinery.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The human U-2OS osteosarcoma and primate Cos1 kidney SV40-transformed cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum. The human HepG2 hepatoma cell line was maintained in minimum essential media supplemented with 10% (v/v) fetal bovine serum.

**Plasmids**—M45-E2F-1 contains the cDNA of E2F-1 with a 13-amino acid epitope tag for the monoclonal antibody M45 (52) inserted between amino acids 2 and 3 in the eukaryotic expression vector pcDNA3 (In-vitrogen). To increase the stability of the E2F-1 protein, amino acids 14–76, which contain the E3 ubiquitin ligase binding site (17), are deleted in pcDNA3-M45-E2F-1. M45-E2F-4 contains the E2F-4 cDNA with the M45 epitope tag inserted 5′ of the E2F-4 natural ATG in pcDNA3. M45-E2F-4 truncation mutants T-406, T-390, T-378, and T-360, and T-215 were constructed by amplification of wild-type E2F-4 cDNA truncated after the indicated amino acid with a stop codon. The E2F-4 mutants, d361, d379, and d391, were constructed by PCR mutagenesis introducing an Nhel site 3′ to amino acid 215. Separately, E2F-4 coding regions for amino acids 361, 379, or 391 to the C terminus were amplified introducing an Nhel site 5′ to the designated amino acid and ligated to the Nhel site 3′ to amino acid 215. This created an in-frame fusion between amino acid 215 and the indicated amino acid with an intervening 2-amino acid linker. M45-E2F-4 mutants E370A, C403A, and E370A/T-406 were constructed by PCR amplification of E2F-4 designated coding sequences with primers that introduce convenient restriction sites. The PCR products were inserted into the pCMV-Myc46-GAL4 eukaryotic expression vector to form an in-frame fusion between the GAL4 DNA binding domain (amino acids 1–147) and the E2F transcription domain or transcriptional domain fragments. The identities of all clones were confirmed by DNA sequencing.

The CMV-DP1 expression vector was described elsewhere (53). The E2a-chloramphenicol acetyltransferase, C305-Luc, E2F-1 pro-Luc, c-Myc Luc, and c-Myc (mE2F) Luc reporter constructs were previously described (54–57). The GAL-Myc, GAL4-MBII1, and Cj-FTRRAP expression plasmids were described elsewhere (51, 58). pCMV-Flag-p300 eukaryotic expression plasmid encoding the entire human p300 protein was kindly provided by D. Livingston (59). pCI-FLAG-PCAF expression vector contains the human PCAF-coding sequence with an N-terminal FLAG epitope and was kindly provided by Y. Nakatani and co-workers (60). pHaH-PCAF was cloned by PCR amplification of the entire PCAF-coding sequence with primers that introduce convenient restriction sites. The PCR product was inserted into pCMH6KR expression vector (61) to form an in-frame fusion between an epitope consisting of hemagglutinin epitope and a six-histidine linker and the PCAF ATG. The CjS-GCN5 expression plasmid contains the entire coding sequence for human GCN5 and was kindly provided and cloned by M. Cole. Cata-
FIG. 2. Subtle mutations disrupt E2F-4 transactivation capacity. U-2OS cells were co-transfected with 0.1 μg of E2F1 pro-Luc reporter and 50 ng of pDNA3-E2F-4 constructs as indicated. The results are the average of three independent experiments in duplicate with the S.E. indicated. The promoter activity of E2F1 pro-Luc was set as 1.

TRANSCITATIONS AND TRANSCRIPTIONAL ASSAYS—HepG2 cells were transfected by calcium phosphate coprecipitation as described (53) 24 h after subculturing. 16–18 h after transfection cells were washed twice with Tris-buffered saline, and fresh media was added. Whole cell extracts were prepared 24 h later, and chloramphenicol acetyltransferase enzymatic activity was assayed using a fluorescent chloramphenicol substrate (FAST-CAT; Molecular Probes, Eugene, OR). Chloramphenicol acetyltransferase activity was quantified with a PhosphorImager. U-2OS cells were transfected with Fugene 6 (Roche Molecular Biochemicals) and collected 24 h later for luciferase and β-galactosidase assays per the manufacturer’s instructions (Promega).

Immunoprecipitations, Western Blots, and Acetyltransferase Assays—Whole cell extracts were prepared as described (62), and equal amounts of protein were subjected to immunoprecipitation with mouse monoclonal antibodies directed against M45 epitope, hemagglutinin epitope, or E1A protein. Precipitated proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) with 15% or 7.5% gels and immunoblotted using standard techniques. The following primary antibodies were used: mouse monoclonal anti-M45, mouse monoclonal anti-hemagglutinin 12CA5 (Roche Molecular Biochemicals), mouse monoclonal anti-E1A M73 (Labvision), goat polyclonal anti-TRRAP T-17 (Santa Cruz), goat polyclonal anti-GCN5 N-18 (Santa Cruz), and rabbit polyclonal anti-p300 N-15 (Santa Cruz). Immunoreactive bands were detected with an ECL kit (Amersham Pharmacia Biotech).

Immunoprecipitation acetyltransferase assays were performed using a modification of the assay previously described (63). Immunoprecipitated proteins were washed twice in lysis buffer and twice with IPH buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% (by volume) Nonidet P-40, 10 mM butyric acid, and 0.1 mM phenylmethylsulfonyl fluoride). After the final wash, the buffer was aspirated down to 100 μL. 5% of the precipitant slurries were resolved by SDS-PAGE and immunoblotted with anti-M45 antibodies, and immunoreactive bands were quantified using AttoPhos alkaline phosphatase substrate (Promega) and a PhosphorImager. The percentage of wild-type protein immunoprecipitated was used to normalize the acetyltransferase assay data. The remaining slurry was assayed for acetyltransferase activity by adding 2 μL of 20 μg ml⁻¹ calf thymus histones (Sigma) and 2.5 μL of [3H]Acetyl-CoA (1.85 MBq, 7.4 GBq mmol⁻¹, PerkinElmer Life Sciences). Reactions were incubated at 30 °C for 1 h, and histone acetylation was measured by the P-81 filter assay.

RESULTS

Transactivation Domain of E2F-4 Resides between Amino Acids 360 and 413, the C Terminus—E2F-1 and E2F-4 represent two structural subclasses of E2F family members. The transactivation domain of E2F-1 has been identified as amino acids 368–437 (64, 65), whereas the transactivation domain of E2F-4 has not been well characterized. To compare functional properties of E2F-1 and E2F-4, we mapped the transactivation domain of E2F-4. We established a transactivation assay with exogenously expressed wild-type and mutant E2F-4 proteins and an E2F-responsive reporter construct that contains the adenovirus E2a promoter (nucleotide −384 to +62) driving the chloramphenicol acetyltransferase gene (54). Endogenous E2F and pRb proteins complicate the mapping of E2F transactivation domains in vivo. Besides directly stimulating transcription, overexpressed E2F proteins may activate transcription indirectly by titrating pRb proteins from endogenous E2Fs or displacing E2F-pRb complexes from the promoter. The transactivation levels of an E2F-4 mutant with a deletion of the entire pRb binding site (T-391) did not dramatically differ from wild-type E2F-4 and established that E2F-4 mutants do not activate transcription by sequestering repressing pocket proteins away from endogenous E2F heterodimers (Fig. 1B). This showed that pocket protein binding is not necessary to activate transcription in our transient assays. A DNA binding-deficient E2F-4 mutant did not activate transcription above the levels of the reporter alone and established that pRb sequestration is not significant in these assays (data not shown). The difference between the level of transcription from the reporter alone and an E2F-4 mutant that contains only the DNA binding and dimerization domains (T-215) may have been due to displacement of endogenous repressing E2F-pRb complexes from the reporter (Fig. 1B).

We analyzed mutant E2F-4 proteins to map the transactivation domain (Fig. 1B). Truncating E2F-4 at amino acid 360 (T-360) diminished transactivation potential to the basal level as established by the T-215 mutant that lacks the entire C-terminal domain. The weak transactivation activity is not a unique property of mutant T-360 since other mutants with truncations N-terminal to amino acid 360 showed the same level of activity (data not shown). Internal deletion of amino acids 215–360 (d1361) did not lead to transactivation levels significantly different from that seen with wild-type E2F-4. Deletions from amino acid 215 to beyond amino acid 360 showed significantly reduced transactivation potential. Similar mapping results were obtained from adenoviral E2a and cellular E2F-1 and c-Myc promoter constructs in both HepG2 and U-2OS cell lines (data not shown). Gel shift analysis of the E2F-4 mutants shows that DNA binding activity was not compromised (data not shown), and protein expression was similar across the panel of mutants (Fig. 1C). These results show that the transactivation domain of E2F-4 is contained within amino acids 360 and 413.

To directly assess E2F transactivation potential, we analyzed the minimal homologous portions of the E2F-1 and E2F-4 transactivation domains fused to a DNA binding domain of a heterologous protein (GAL4). The E2F-1 (379–438) and E2F-4 (360–413) segments contain potent transactivation domains, and deletion of either the C-terminal or N-terminal half of the E2F-1 or E2F-4 transactivation domain severely disrupted transcriptional capacity (data not shown). We conclude that these segments contain most, if not the entire, transactivation domains of E2F-1 and E2F-4 and activate transcription independent of other portions of the wild-type proteins and a DP binding partner.

Analysis of E2F-4 Transactivation Domain Mutants—We have constructed and analyzed a panel of site-directed mutants in the E2F-4 transactivation domain that compromise transactivation capacity. Deletion of the C-terminal seven amino acids (T-406), alanine-substitution mutants at glutamic acid 370 (E370A), or cysteine 403 (C403A) marginally reduced E2F-4 transactivation (Fig. 2). However, the double mutant E370A/T-406 showed the same low activity as when the entire trans-
Coactivators p300 and PCAF Are Not Components of E2F-transactivating Complexes—To explain the loss of transcriptional capacity by the E2F-4 mutant E370A/T-406, we addressed whether reported E2F-1 transactivation domain-associated protein interactions were disrupted in vivo. We were unable to establish in vitro E2F-1 or E2F-4 interactions with p300 or PCAF despite the fact that these proteins were readily apparent in control immunoprecipitations with the adenovirus E1A 12S protein (Fig. 3, A and B). Furthermore, a truncated p300 protein (amino acids 1256–2414) that contains the E2F-1 binding region, as determined by in vitro GST pull-down experiments (19), did not co-precipitate with E2F-1 or E2F-4 (data not shown).

Consistent with these results, coexpression of p300 or PCAF failed to stimulate E2F-dependent transcription (Fig. 3, C and D). The reporter E2F-1 pro-luciferase contains the E2F-responsive murine E2F-1 promoter (nucleotide −176 to +36), driving the luciferase gene (56). E2F-1 transactivation was squelched by overexpression of p300, whereas E2F-4 activity was not affected (Fig. 3C). This is consistent with previous work (66). PCAF coexpression consistently repressed E2F-mediated transcriptional activation (Fig. 3D). This suggested that overexpressing PCAF sequestered an essential component of E2F-transactivating complexes. Similar results were obtained from the E2F-responsive c-Myc promoter (data not shown).

E2F-4 Transactivation Domain Binds TRRAP and GCN5—TRRAP has been implicated in the recruitment of acetyltransferase activity to promoters by transcriptional activators E2F-1 and c-Myc (49, 51). To observe potential E2F-4/TRRAP complexes in vivo, immunoprecipitated E2F-4 proteins were analyzed by immunoblotting for TRRAP and various histone acetyltransferases. Endogenous TRRAP specifically immunoprecipitated with wild-type E2F-1 and E2F-4 (Fig. 4A). The E2F-4 mutants E370A and T-406 immunoprecipitated TRRAP at the same levels as wild-type E2F-4, whereas the mutants E370A/T-406 and E370A/T-360 did not bind TRRAP. The same pattern was observed when the E2F precipitates were immunoblotted for endogenous GCN5 (Fig. 4A). Mutation C403A, which was modestly reduced in transcriptional activity (Fig. 2), bound TRRAP and GCN5 at reduced levels compared with wild-type E2F-4. This suggests that TRRAP and GCN5 act in concert as components of E2F-transactivating complexes.

Western blot analysis showed that both wild-type and mutant proteins were expressed at similar levels (Fig. 4A). Similar binding patterns were obtained from E2F precipitates when epitope-tagged TRRAP or GCN5 were cotransfected with the E2F expression plasmids in Cos1 and U-2OS cells (data not shown). Thus, the transactivation domain of E2F-4 interacts with TRRAP and the acetyltransferase GCN5. Since the TRRAP and GCN5 binding patterns of the E2F-4 mutants correlate well with their ability to stimulate transcription (Fig. 2), these results imply that E2F transcription factors recruit a SAGA-like complex to the promoter to stimulate transcription.

TRRAP and GCN5 Function in E2F-dependent Transcription—If TRRAP association with E2F transactivation domains is essential for transcriptional activation, then overexpressing TRRAP should stimulate E2F-dependent transcription. Wild-type E2F-1 and E2F-4 transactivation of the E2F-1 promoter was stimulated by TRRAP overexpression (Fig. 4B). TRRAP overexpression did not significantly influence the basal level of the reporter. However, E2F-1- and E2F-4-mediated transcription was stimulated 2- and 3.1-fold, respectively, by co-expression of TRRAP. TRRAP transcriptional stimulation was dependent on a functional E2F transactivation domain, since co-expression of TRRAP with the E2F-4 transactivation domain-deficient mutant (T360) did not significantly activate transcription above basal levels. TRRAP also stimulated E2F-4 transactivation of the c-Myc promoter dependent on an intact E2F binding site (Fig. 4C). The reporter c-Myc-luciferase contains the E2F-responsive human c-Myc promoter (nucleotide −140 to +340), driving the luciferase gene (57). E2F-4 transactivation and TRRAP stimulation of E2F-4 activity required the wild-type E2F consensus site lacking in the c-Myc (mE2F) Luc reporter. These results establish TRRAP as an essential component of E2F-transactivating complexes.

GCN5 stimulated E2F-responsive reporters independent of exogenous E2F proteins (Fig. 4, D and E). To confirm the involvement of E2F in GCN5 transactivation, we overexpressed GCN5 in the presence of increasing amounts of a dominant-negative E2F-4 mutant (T-215) that binds DNA but lacks transcriptional capacity (Fig. 1B) and the ability to bind GCN5 (data not shown). Increasing concentrations of T-215
repressed GCN5 activation of the E2F-1 pro-Luc reporter in a dose-dependent manner (Fig. 4D), suggesting that T-215 displaced endogenous active E2F-GCN5 complexes from the promoter. Similarly, overexpression of GCN5 stimulated the c-Myc promoter dependent on a wild-type E2F binding site (Fig. 4E). Since GCN5 activity is not completely squelched by T-215 overexpression or mutation of the E2F consensus site, it remains possible that GCN5 functions through other transcription factors in addition to E2F. However, these observations show that GCN5 is an important cofactor for E2F-mediated transcription.

The N Terminus of c-Myc Represses Transcription Activity of E2F-4 Complexes—If TRRAP is an essential cofactor in E2F-transactivating complexes, then proteins that bind TRRAP might compete for binding to TRRAP and reduce E2F-dependent transcription. To test this idea, we used c-Myc as a competitor in E2F-4 transcription assays. c-Myc binds TRRAP via the conserved MBI domain (51). A truncated form of c-Myc was used in these assays that lacks the C-terminal DNA binding and dimerization domains but contains the N-terminal 262 amino acids encompassing its transactivating and transforming activities. The c-Myc DNA-binding/dimerization function was provided by the GAL4 DNA-binding/dimerization domain to avoid the complication of co-expressing a c-Myc heterodimerization partner (58). E2F-4 activated transcription to normal levels in the presence of the GAL4 DNA binding domain, GAL0 (Fig. 5). The GAL4-Myc fusion repressed E2F-dependent transcriptional activation. Deletion of the c-Myc TRRAP binding domain (GAL4-MBI1A) abolished the repressive effect of GAL4-Myc on E2F-4 transactivation. GAL4 chimeric proteins were expressed at similar levels as seen by immunoblotting with GAL4 antibodies (data not shown). These results implicate TRRAP as an essential component of E2F-transactivating complexes that can be competed away by overexpressing c-Myc.

The E2F-4 Transactivation Domain Recruits Proteins Capable of Histone Acetylation—Since E2F transactivation is antagonized by Rb-associated histone deacetylases, we hypothesized that E2F recruits histone acetyltransferases to reverse the Rb-histone deacetylase-mediated repression. We assayed histone acetyltransferase activity of E2F-4-transactivating complexes assuming that TRRAP and GCN5 are part of a histone acetyltransferase/transcriptional coactivator complex. E2F-4 complexes were immunoprecipitated from Cos1 whole cell extracts and washed. Precipitated E2F-4 proteins were quantitated from 5% of the slurries, whereas the remainder was assayed for the ability to transfer radiolabeled acetyl groups from acetyl-CoA to partially purified histones. Acetyltransferase activity associated with the E2F-4 complexes was normalized based on the quantification of the immunoprecipitated proteins. Wild-type E2F-4 recruited a significant acetyltransferase activity that was lost in
the transactivation domain-deficient mutant (T-360) (Fig. 6A). As with T-360, the transactivation-deficient E2F-4 mutant E370A/T-406 lacked acetyltransferase activity, whereas individual mutants retained a portion of wild-type acetyltransferase activity. The associated acetyltransferase activity of E2F-4 mutant displays a positive correlation with its ability to stimulate transcription (Fig. 2) and bind TRRAP and GCN5 (Fig. 4A).

Presumably the E2F-4-associated acetyltransferase is GCN5, since GCN5 is the only acetyltransferase that binds E2F-4. To test this, we co-expressed either wild-type or catalytically inactive GCN5 with E2F-4 and analyzed E2F-4-associated acetyltransferase activity (Fig. 6B). GCN5 mutants 575 EIV and 586 QVKGYG contain alanine substitutions for the indicated amino acids and destroy the catalytic site, as determined by the crystal structure of tetrahymena GCN5 (67). The E2F-4-associated acetyltransferase activity was similar to endogenous proteins and overexpressed wild-type GCN5. However, E2F-4-associated acetyltransferase activity was reduced in the presence of GCN5 mutants 575 EIV and 586 QVKGYG. Western blot analysis showed that both wild-type and mutant proteins were expressed at similar levels (data not shown). This established that GCN5 is the relevant acetyltransferase activity binding to E2F-4 in vivo. These results further support our hypothesis that the recruitment of acetyltransferase activity is essential for E2F transcriptional activation since the ability of the panel of E2F-4 mutants to bind GCN5 and stimulate transcription correlates well with the associated acetyltransferase activity.

**DISCUSSION**

In this report we have identified a potential mechanism whereby E2F transcription factors overcome the effect of pRb-histone deacetylase-imposed chromatin condensation. We have demonstrated two important points about the mechanism of transactivation by E2F family members. First, GCN5 and TRRAP are important cofactors of E2F transactivating complexes. Both E2F-1 and E2F-4 bound GCN5 and TRRAP in vivo. TRRAP stimulated transactivation from wild-type E2F-1 and E2F-4 proteins dependent on an intact transactivation domain and E2F consensus sites in the reporter. GCN5 activates E2F-responsive promoters dependent on endogenous E2F transcription factors and E2F binding sites. In contrast, co-expression of p300, CBP, or PCAF had no influence on or repressed E2F transactivation in our assays and did not bind E2F-1 or E2F-4 in vivo. This is surprising because acetylation by p300 and PCAF is known to enhance DNA binding activity, protein stability, and transactivation capacity of E2F-1 (19, 40, 41). To establish that p300 and PCAF stimulated E2F-1 transcriptional activation, synthetic reporters consisting of multiple E2F binding sites were used (19, 41), which may account for a higher specific activity than was masked in our assays with native E2F-responsive promoters. Furthermore, the cellular proto-oncoprotein c-Myc suppressed E2F-4 transactivation capacity dependent on an intact TRRAP binding domain, MBII. Similarly, PCAF repressed E2F-mediated transcription and suggested that overexpression of PCAF sequestered an essential component of E2F transactivation into nonfunctional complexes, e.g. TRRAP. Taken together, this work demonstrates that E2F-transactivating complexes consist of two components of human SAGA-like complexes, namely GCN5 and TRRAP, which stimulate E2F activity, are in limiting supply in the cell, and can be competed away from E2F heterodimers.

Second, although E2F-4 is not a target of acetylation (41), E2F-4 proteins recruit potent acetyltransferases. E2F-4 transactivation domain mutants that are deficient in transactivation capacity also lack significant associated acetyltransferase activity. Our results show that GCN5 is responsible for the E2F-4-associated acetyltransferase activity, since E2F-4 mutants that lack associated acetyltransferase activity do not bind GCN5 (Fig. 4A), and catalytically inactive GCN5 mutants squelched E2F-4-associated acetyltransferase activity (Fig. 6B). Furthermore, we demonstrated that p300 and PCAF do not form stable complexes with E2F-1 and E2F-4 in vivo, although p300 and PCAF proteins are readily associated with the adenovirus E1A protein in parallel co-immunoprecipitations (Fig. 3, A and B). Two previous reports show in vivo interactions between E2F-1 and p300 or PCAF using overexpressed proteins (40, 68). The conflicting results may reflect the transient nature of p300- or PCAF-E2F interactions where overexpression of both test proteins may allow detection of weak protein-protein interactions. Alternatively, these differences may reflect cell line-specific E2F-acetyltransferase interactions. In our experiments with two cell lines, E2F-1 and E2F-4 associated with endogenous GCN5 and TRRAP proteins. This work is the first demonstration that transcriptional stimulation by E2F family members requires functional GCN5 that is capable of histone acetylation.

The correlation among the ability of the E2F-4 transactivation domain mutants to stimulate transcription, bind TRRAP, and GCN5 and recruit acetyltransferase activity indicates an essential role for establishment of an E2F-TRRAP-GCN5 ternary complex in E2F-mediated transactivation. Since E2F-4 is not a known substrate for acetyltransferases, the recruitment of active GCN5 to the promoter must stimulate transcription by acetylating downstream effectors such as histones or the general transcription machinery. This provides an important mechanism by which E2F transcription factors antagonize the dominant inhibitory effect of pRb-histone deacetylases repression.

Transcriptional activation domains are thought to function by either recruiting transcription machinery or complexes capable of reorganizing chromatin to the promoter (69). Previously, E2F transcription factors were thought to stimulate transcription by stabilizing the RNA polymerase II pre-initiation complex through interactions with the general transcription machinery. There are several reports describing interactions between transcription machinery and E2F family members, e.g. TBP and p62 subunit of TFIIH (22, 70–72). These interactions were established by in vitro GST pull-down experiments, which do...
not address the physiological relevance of these interactions in transcriptional activation. We also observed interactions between GST-purified E2F-1 or E2F-4 and in vitro translated TBP (data not shown). However, such interactions were not observed in vivo using co-immunoprecipitation assays. In vitro transcription assays with highly purified general transcription factors and E2F heterodimers have established that E2F-4 stabilizes a TFIID-TFIIA complex at the promoter that is resistant to pRB disruption (27). Whether E2F proteins stabilize TFIIID-TFIIA complexes through interactions with TBP and whether E2F proteins affect RNA polymerase II processivity through interactions with TFIIH remain to be clarified. If E2F interactions with TBP and TFIIH are relevant in vivo, then a complete model of E2F transactivation mechanisms involves contacts among the transactivation domains and both the transcription machinery and acetyltransferase complexes. In this scenario, E2F transactivation domains stimulate transcription by recruiting components of the RNA polymerase II pre-initiation complex and proteins capable of modifying the nucleosome.

Histone acetylation is intimately linked to transcriptional activation. The acetylation state of a promoter is a dynamic situation regulated in part by the recruitment of acetyltransferase and deacetylase activities that control chromatin structure, various transcription factor activities, and, ultimately, transcriptional initiation or repression. With the identification of many transcriptional coactivators (e.g. p300 and SAGA) containing histone acetyltransferase activity, chromatin reorganization has become a important mechanism for regulating transcription. It has been proposed that TRRAP interfaces with transcriptional activators to recruit histone acetyltransferases to the promoter. The isolation of multiple acetyltransferase-transcriptional coactivator complexes containing TRRAP or the yeast homologue Tra1 supports this idea. The human TFTC, PCAF, and yeast SAGA complexes contain TRRAP, GCN5, or PCAF acetyltransferases, multiple TBP-associated factors (TAFs), Ada proteins, and Spt proteins (46, 47). We propose that E2F transcription factors recruit acetyltransferase-transcriptional coactivator complexes, similar to TFTC or ySAGA complexes, to the promoter to transactivate gene expression. In support of recruitment of GCN5 and TRRAP in toto as a SAGA-like complex, GCN5 and TRRAP exist in a complex in mammalian cells independent of transcription factors such as c-Myc (49).

Exploring E2F-activated transcription is complicated by multiple layers of regulation and because cell lines used to assay E2F function commonly have mutations that deregulate the endogenous E2F regulatory pathway. To circumvent these problems, we assayed the minimal transactivation domains of E2F-1 and E2F-4 in a controlled context. To that end, the GAL4-E2F chimeric proteins showed that the E2F-1 transactivation domain is much more potent than the E2F-4 transactivation domain (data not shown). This is consistent with previous work (73). Many reports suggest that E2F transcriptional activating and repressing activities are mediated by different family members. It is hypothesized that E2F-1, -2, and -3 are transcriptional-activating and cell cycle-promoting family members, whereas E2F-4 and E2F-5 are repressing and cell cycle-inhibiting members (reviewed in Ref. 7). However, this may be an oversimplification since E2F-4 retains a potent transactivation domain that is conserved among E2Fs 1–5 and the ability to bind co-activators and acetyltransferases. These observations suggest that E2F-4 transcription factors may stimulate transcription in a restricted developmental or cell cycle window through the GCN5-induced relaxation of chromatin structure at target promoters.

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