p300 Functions as a Coactivator of Transcription Factor GATA-4*

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Transcription factor GATA-4 plays critical roles in controlling heart development and cardiac hypertrophy. To understand how GATA-4 functions under diverse conditions, we sought to identify its coactivators. We tested p300 as a coactivator in GATA-4-dependent transient transcription assays in NIH3T3 cells and found that p300 synergistically activated GATA-4-dependent transcription on both synthetic and natural promoters. Direct physical interactions between the N- and C-zinc finger domains of GATA-4 and the cysteine/histidine-rich region 3 (C/H3) of p300 were identified in immunoprecipitation and glutathione S-transferase pull-down experiments. Deletion of the C/H3 region of p300 abolished its coactivator activity indicating that the physical interaction was required for functional synergy. Through the use of a series of GATA-4 zinc finger mutants, the amino acids WRK in the C finger were identified as critical to the interaction. The adeno-viral E1A protein or a peptide encoding the C/H3 region of p300 could inhibit GATA-4-dependent transcription, presumably by competing for p300 binding. Furthermore, deletion of the region of p300 encoding the histone acetyltransferase activity abolished its effect on GATA-4-dependent transcriptional activity. These results establish that p300 acts as a GATA-4 coactivator and that the p300 histone acetyltransferase activity is necessary for the functional interaction.

Members of the GATA-4/5/6 subfamily of GATA-binding proteins participate in heart development (1–4), in the expression of genes in the adult heart, and in the adaptive response of the heart to pathological stresses (5–7). Since GATA-4 controls gene expression in many cellular environments, its activity is likely regulated by post-translational modifications such as phosphorylation or acetylation as has been shown for GATA-1 (8–10), or by interacting with other proteins (11–15).

GATA-4 mRNA is present in the heart at embryonic day 8, is expressed throughout development and is readily detectable in the adult mouse heart (12). In GATA-4 knockout mice the precardiac mesoderm fails to migrate to the ventral midline resulting in the formation of two aberrant heart tubes. Although cardiac myocytes form in GATA-4 null mouse embryos the role of GATA-4 in this process remains obscure because the level of GATA-6 mRNA is up-regulated in mutant embryos. In p19 embryonal carcinoma cells, inhibition of GATA-4 expression prevents the development of cardiac muscle cells while ectopic expression of GATA-4 accelerates cardiogenesis and increases the number of cardiac myocytes obtained by 10-fold (16). Thus, the possibility remains that GATA-4 may participate in the cardiogenic process. GATA-4 has been shown to interact with the homeobox protein Nkx 2.5/Csx which also expressed early in the developing heart (11). This interaction results in synergistic activation of the atrial natriuretic factor gene and activates cardiogenesis in P19 cells to a greater degree than GATA-4 alone (11, 12).

Several observations suggest that GATA-4 activity is involved in cardiac muscle hypertrophy associated with pressure overload. First, mutation of GATA-binding sites in the promoters of two hypertrophy responsive genes, encoding angiotensin receptor type 1a, and β-myosin heavy chain (MHC), diminish or ablate the pressure overload response (5, 6, 18). Second, GATA-4 was detected in nuclear extracts from pressure overloaded hearts but not normal hearts by gel shift experiments (5). Third, GATA-4 was shown to interact with NF-AT3 and overexpression of the calcineurin/NF-AT pathway was shown to initiate cardiac hypertrophy (7). Fourth, GATA-4 regulates the expression of a number of hypertrophy responsive genes including those encoding ANF and brain natriuretic peptide (19). These results suggest that GATA-4, either by itself or in concert with other regulatory factors, is a regulator of the hypertrophic response.

A potential GATA-4 co-activator is p300. p300−/− knockout mice die between 9 and 11.5 days of gestation from heart defects (20). Mutant embryos have enlarged heart cavities, significantly reduced trabeculation, and pericardial effusion. Interestingly, the expression level of the β-MHC gene (the major contractile protein in the fetal heart) is reduced. This gene is also regulated by GATA-4 (6). The adenovirus E1A protein, is known to interact with and inhibit the function of p300 and several other regulatory proteins. When overexpressed in cardiac myocytes, E1A has been shown to down-regulate many muscle-specific genes (21, 22). GATA proteins regulate a subset of these genes. These observations suggest a possible interaction between GATA proteins and p300. Furthermore, while this work was in preparation, it was reported that p300 and GATA-5 interact to regulate the ANF gene (23); however, the mechanism by which p300 coactivates GATA-5 was not determined.

p300 has been shown to increase the activity of several transcription factors including cAMP-response element-bind-

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1 The abbreviations used are: MHC, myosin heavy chain; AAs, amino acids; ANF, atrial natriuretic factor; BAP, bacterial alkaline phosphatase; C-finger, carboxyl-terminal zinc finger; C/H1, cysteine/histidine-rich region 1; C/H3, cysteine/histidine-rich region 3; EIa, adenoviral 12S E1A protein; GST, glutathione S-transferase; HAT, histone acetyltransferase; N-finger, amino-terminal zinc finger; PCR, polymerase chain reaction; DBD, DNA-binding protein; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin.
ing protein, p53, signal transducers and activators of transcription, HNF4, MyoD, GATA-1, GATA-5, and GATA-6, p300 possesses an intrinsic histone acetyltransferase (HAT) activity that can acetylate histones to modify chromatin structure as well as other transcription factors (24–29). In addition, p300 has multiple activation domains and interacts directly with TATA-binding protein and transcription factor IIIB (30–32). These studies demonstrate that p300 can influence transcription through multiple mechanisms.

In the present study, we investigate the potential interactions between GATA-4 and p300. p300 was found to synergistically enhance the transcriptional activity of GATA-4 on an artificial reporter containing regulatory GATA sites from both the ANF and α-MHC promoters in transient reporter assays. We mapped the regions in GATA-4 and p300 required for their interaction and demonstrate that mutations, which prevented the interaction, abolished the functional cooperation. Additionally, co-expression of either a peptide encoding the C/H3 domain of p300 or the adrenoviral EIA protein with GATA-4 alone or in combination with p300 blocks GATA-4-dependent transcriptional activity. Finally, we show that the p300 HAT domain is required for coactivation of GATA-4 transcription.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—pMT-GATA-4 (2) was obtained from Dr. David Wilson. CMV-p300HA, pGEM-E1A, and GST-E1A were obtained from Drs. D. Livingston, T. Leff, and D. Chakravarti, respectively. pFLAG-CMV-2 was from Sigma. Sequences encoding peptide fragments of GATA-4 fused to the GAL4 DNA-binding domain (DBD) (GAL4-GATA-4) were cloned into pM (CLONTECH). Clones encoding peptide fragments of GATA-4 fused to glutathione S-transferase (GST-GATA-4) were generated by subcloning GATA-4 PCR fragments from the appropriate GAL4-GATA-4 plasmids into the EcoRI and BamHI sites of pGEX4T-1 (Amersham Pharmacia Biotech). GATA-4 N-zinc finger (–1–294) and C-zinc finger (253–441) constructs and mutants thereof were derived from the full-length GATA-4 wild type and mutants by PCR and cloned into pRSET (Invitrogen) vector (N-zinc finger) or pcDNA6/myc/his (C-zinc finger). pFLAG-GATA-4 was made by subcloning the EcoRI GATA-4 fragment from pMT2GATA-4 into pFLAG-CMV-2. pFLAG-CMV-2-BAP encoding a FLAG-tagged bacterial alkaline phosphatase (BAP) was from Sigma. DNA sequences encoding p300 N1 (A1–537), N2 (Aas 598–1185), N3 (Aas 1186–1860), and C (Aas 1861–2414) were generated by PCR and cloned into pRSET, p300 (ΔC/H1) deleting sequences encoding of Aas 348 to 412 and p300 (ΔC/H3) encoding a full-length p300 except for a deletion of sequences encoding Aas 1737 to 1836 were purchased from Upstate Biotechnology. To construct the p300 HAT deletion mutant, p300 was cloned into pcDNA3.1 (NcoI/HindIII) and the 5’ end of the C/H3 domain was excised from this fragment. The vector containing the 3’ sequence was religated and used for PCR-based mutagenesis (Stratagene). Sequences encoding Aas 1415–1436 of p300 were deleted. The N5′ XbaI fragment was then cloned back to the mutated 3′ end via the XbaI site. pc1-EIA was produced by subcloning EIA from pGEM-EIA into the pc1 vector (Promega). EIA/AΔ2–36 was constructed by deleting Aas 2–36 of EIA. The Texas Red peptide plasmid pTATAurac was made by cloning an oligo containing the rat α-myosin heavy chain TATA sequence into the HindIII site of pGL2 basic (Promega). The sequence of the plus strand of the TATA oligo was 5′-ACCTGGTCCAGTATAAAGG-3′. pANFpGATA6iuc contains 6 GATA sites from the ANF promoter (position +120) cloned into the KpnI site of pTATAluc. The sequence of the plus strand of the ANF GATA oligonucleotide is 5’–CTGATAACTCTGATAACTCTGATAACTGGTAC-3’. The native ANF reporter plasmid (–638/Luc) was from Youngsook Lee (11). The reporter containing the α-MHC GATA sites has 3 copies of the oligonucleotide PRED (33) cloned into the SmaI site of pTATAluc. The β-MHC promoter was a gift from K. Ojama. The fragment containing the β-MHC promoter from the transcription start site to approximately +2200 was subcloned into pGL2 basic. The sequence of all clones was verified by DNA sequencing and by precipitation analysis.

Immunoprecipitation and Western Blot Analysis—COS7 cells were transfected with plasmids encoding FLAG-tagged GATA-4, HA-p300, or both. After 72 h, cells were harvested and lysed at 4 °C in lysis buffer A (1% Triton, 25 mM Tris-HCl, pH 7.4, 1 mM NaCl) and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μM pepstatin, 1 μM aprotinin). The lysates were incubated with FLAG-agarose for 2 h at room temperature, and the beads were washed four times with lysis buffer for 1 min at room temperature and eluted with SDS sample buffer. The samples were subjected to 8% SDS-PAGE and blotted with rat anti-HA antibody (Roche Molecular Biochemicals) and detected with ECL chemiluminescence reagents (Amersham Pharmacia Biotech). According to the protocol, NIH3T3 cells were transfected with pcdNA-His-GATA-4, Gal-DBD–1–80, 80–214, 214–441, and CMV-p300. The cells were lysed at 4 °C in lysis buffer B (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40) containing protease inhibitors (as above). Lysates were clarified by centrifugation at 18,000 × g for 10 min. The lysate proteins were immunoprecipitated overnight at 4 °C with anti-Gal-DBD antibody (Santa Cruz Biotechnology) and anti–T7 tag antibody (Novogen), followed by precipitation with 50 μl of protein A-Sepharose for 2 h at 4 °C. After 4 washes with lysis buffer B (described above), the immunoprecipitates were eluted by boiling for 5 min in Laemmli sample buffer. The resulting immunoprecipitates were electrophoresed on 4–20% sodium dodecyl sulfate-polyacrylamide gradient gels, transferred onto a nitrocellulose membrane, and immunoblotted with rat anti-HA antibody. The blots were developed with ECL chemiluminescence reagents.

GST Pull-down Assays—All GST fusion proteins were expressed in Escherichia coli BL21 cells. The bacterial cultures were induced by 1 mM isopropyl-thiogalactopyranoside for 4 h. The bacteria were then lysed by sonication in phosphate-buffered saline containing protease inhibitors (as above). The bacterial lysates were brought to 1% Triton and incubated for 1 h at 4 °C followed by centrifugation at 20,000 × g for 15 min. The pellet was discarded and soluble extracts were incubated with glutathione-Sepharose beads (Amersham Pharmacia Biotech) for 2 h at 4 °C before washing three times by inversion for 1 min at room temperature in phosphate-buffered saline containing protease inhibitors lysis buffer. The concentration of proteins immobilized on beads was quantified by SDS-PAGE and titrated against bovine serum albumin standards (Sigma) after Coomassie Blue staining. Binding assays were performed with radioactively labeled proteins synthesized in vitro using a TNT-coupled reticulocyte lysate system (Promega) in the presence of 35S-labeled methionine (Amersham Pharmacia Biotech). Equal amounts of immobilized GST fusion proteins were incubated for 2 h at 4 °C with 10 μl of 35S-labeled proteins in GST binding buffer (40 mM Hepes, pH 7.2, 50 mM Na acetate, pH 7.0, 200 mM NaCl, 2 mM EDTA, 5 mM dithiothreitol, 0.5% Nonidet P-40, protease inhibitors, and 2 mg of bovine serum albumin per ml). After four 1-min washes at room temperature in GST binding buffer, beads were boiled in SDS sample buffer, resolved by SDS-PAGE, and analyzed by autoradiography.

Transient Transfection Assay—NIH3T3 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 10% newborn calf serum, streptomycin (10 μg/ml), and penicillin (100 U/ml). All transfections were performed in 6-well plates with LipofectAMINE Plus reagents from Life Technologies, Inc. Cells were transfected with 0.3 μg of luciferase reporter constructs PREDuc, β-MHCuc, ANFuc, or G4-ANFuc, and 0.1 μg of pm2T2-GATA-4 or pm2T2CAT, 0.8 μg of pcdNA3-p300 or pcdNA3- p300HAT mutant, 0.6 μg of EIA wild type or E1A MutantΔ2–36. CMV-β-gal (20 ng in each well) was used as internal control. Luciferase activity was measured in a microplate luminometer (LKB696; Berthold), normalized to β-Gal activity. Fold activation represents a comparison of the ratio of luciferase/β-Gal activity for each condition with that of the reporter vector control which was arbitrarily set at 1. Each value presented is the average of triplicate samples and is representative of multiple independent experiments (n greater than or equal to 3). The data were statistically analyzed with Student’s t test.

RESULTS

GATA-4 Transcriptional Activity Is Enhanced by p300—It has been suggested that p300 can increase GATA-4 activity in reporter assays (34). To further characterize this functional interaction, we examined the ability of p300 to influence GATA-4 transcriptional activity in transient transfection assays in NIH3T3 cells. We first investigated the activity of p300 and GATA-4 on a functional GATA site from the ANF promoter. This GATA site, located at position +120 in the ANF promoter, was recently shown to support both GATA-4 and GATA-6-dependent transcription (35). From a reporter containing 6 copies of this GATA site upstream of a TATA sequence, GATA-4 alone increased activity 15-fold while p300 alone increased activity about 7.5-fold (Fig. 1A). Together they
increased activity 70-fold over the basal promoter alone, a level that was much more than additive, indicating a functional synergy between these two regulatory proteins. This functional synergy was also observed when a reporter driven by the proximal ANF promoter (−637 to +10) was used (Fig. 1B). With this natural promoter, GATA-4 alone gave 5-fold activation while p300 alone gave a 40-fold activation, presumably due to coactivation of other proteins that regulate this promoter. In combination GATA-4 and p300 activated the ANF promoter 95-fold over basal, a much greater than additive effect. These results confirm that a functional interaction can occur between GATA-4 and p300 in the context of a natural promoter.

We examined a second hypertrophy responsive promoter for activation by GATA-4 and p300. On the β-MHC promoter GATA-4 or p300 alone activated reporter activity 4-fold (Fig. 1D). In combination GATA-4 and p300 gave a 12-fold activation that was more than additive but less than multiplicative. These results indicate that p300 and GATA-4 functionally cooperate to activate transcription in a natural promoter context.

Finally we were interested in determining whether or not p300 would activate GATA-4 on a GATA site from a promoter that is not responsive to hypertrophic stimuli. To this end we made a reporter plasmid, containing six GATA sites derived from the α-MHC promoter upstream of a TATA sequence, which was co-transfected with the GATA-4 expression vector with or without p300 expression (Fig. 1C). In this assay GATA-4 alone increased expression from the artificial promoter nearly 9-fold while p300 alone increased 5-fold. The combination of GATA-4 and p300 increased expression 115-fold over the control (luciferase reporter alone), which represents a 12-fold increase over GATA-4 alone. In addition, p300 did not measurably influence the amount of GATA-4 produced from the expression vector as determined by Western blotting (data not shown). Thus, p300 appears to supplement GATA-4 activity on sites from both hypertrophy responsive and non-responsive genes. These results also indicate that GATA-4 and p300 can functionally cooperate to increase GATA-4-dependent transcription from GATA sites in both artificial and natural promoter contexts.

**GATA-4 Transcriptional Activity Is Inhibited by E1A**—The adenovirus E1A protein interacts with p300 and this interaction has been shown to inhibit the HAT activity of p300 (36, 37). To confirm that p300 acts as a coactivator of GATA-4, a clone encoding the E1A protein was co-transfected in selected assays with GATA-4 and p300 (Fig. 2). When E1A was present in these co-transfection assays, GATA-4/p300-dependent transcriptional activity was dramatically decreased (Fig. 2). Interestingly, E1A also inhibited the activity of GATA-4 alone indicating that endogenous p300 may play a role in the activation of GATA-4. To demonstrate that the decrease in GATA-4/p300 activity resulted from an interaction between p300 and E1A, we used a mutant of E1A (E1A(−2Δ−36)) from which AAs 2 to 36 were deleted. This E1A mutant is deficient in binding to p300 (36). As shown in Fig. 2, E1A(Δ2−36) was much less effective than wild type E1A in inhibiting the GATA-4/p300 functional cooperation. These results demonstrate that E1A can inhibit GATA-4/p300-dependent activity presumably by sequestration of p300. These results provide further evidence that GATA-4 and p300 can functionally cooperate to increase activity from GATA-dependent promoters.

**GATA-4 and p300 Physically Interact**—The functional synergy observed when these proteins were co-transfected into cells suggested that they physically interact. In order to determine if such an interaction could occur in vivo, we examined whether GATA-4 can form complexes with p300 in COS-7 cells. Lysates from cells transfected with FLAG-tagged GATA-4 and HA-tagged p300 were immunoprecipitated with the anti-FLAG antibody (Fig. 3A). The co-immunoprecipitated proteins were analyzed by immunoblotting with the anti-HA antibody. HA-tagged p300 co-precipitated with GATA-4 in cells transfected with GATA-4 and p300 (Fig. 3A), but not in cells transfected with either GATA-4 or p300 alone. These results indicate that GATA-4 and p300 interact in vivo. To more precisely determine
the region of GATA-4 that interacts with p300, NIH3T3 cells were co-transfected with plasmids encoding GATA-4 peptides fused to the Gal4-DNA-binding domain and HA-tagged p300. Complexes were immunoprecipitated with anti-Gal4-DBD antibody and Western blotted with anti-HA antibody (Fig. 3B). The result showed that GATA-4 AAs 1–80 and 80–214 were unable to precipitate p300 whereas AAs 214–441 bound p300. These results suggested that AAs 214–441 of GATA-4 contain the interaction domain for p300.

Mapping of the GATA-4 and p300 Domains Required for Interaction—In order to define the interacting domains of each protein, we prepared GST fusion proteins containing various peptides of GATA-4 and determined the ability of each of these to interact with peptides containing roughly equal portions of p300. GST-GATA-4 fusion proteins (shown schematically in Fig. 4A) were expressed, purified, and coupled to glutathione-Sepharose beads. p300 N1 (AA 1–597), N2 (AA 598–1185), N3 (AA 1186–1860), and C (1861–2941) were used in vitro translated in the presence of [35S]methionine. The in vitro translated fragments were run on a gel and compared to ensure equal input of protein (data not shown). Equal amounts of labeled p300 peptides were incubated with GST, GST-GATA-4, or GST-E1A coupled to glutathione-Sepharose beads. After pelleting and washing the beads, the protein remaining was separated by electrophoresis and visualized by autoradiography. The results are shown in Fig. 4, B and C. GATA-4 peptides 1–80, 80–214, and 294–441 did not significantly pull down any fragment of p300. These sequences encompass all of the GATA-4 protein except the zinc fingers. Peptides of GATA-4 containing the N-finger-(80–250) and C-finger-(241–378) interacted significantly with only the N3 fragment of p300. These results indicate that the N- and C-fingers of GATA-4 can interact with the N3 peptide of p300 independently. N3 contains AAs 1185–1860 that includes the cysteine/histidine-rich 3 (C/H3) region of p300. To further define sequences within the N3 region of p300 that interact with GATA-4, we generated in vitro translated peptides containing AAs 1200–1513, 1200–1587, and 1200–1817 of p300. After analysis to confirm equal input of protein, these peptides were used in GST pull-down assays with peptides of GATA-4 containing the N- and C-zinc fingers (Fig. 4D). p300 peptides containing AAs from 1200 to 1587 showed weak interactions with GATA-4, however, the interaction was greatly enhanced when the peptide included additional AAs from 1588 to 1817. Of the input p300 (1200–1817), ~25% interacted with GATA-4 (Fig. 4D). These results suggest that AA 1587–1817 is the region that interacts with each zinc finger domain of GATA-4 with high affinity. This corresponds to the C/H3 region of p300. The C/H3 region is known to interact with the adenovirus E1A protein (36). It is noteworthy that GATA-4 and E1A interact with the same region of p300 since we show above that E1A can block the GATA-4/p300 synergistic transcriptional activation. Our results suggest that the transcriptional inhibition might be due to direct competition between E1A and GATA-4 for binding to the C/H3 region of p300.

Zinc Finger Amino Acids Involved in Interaction with p300—Having identified the region of p300 that interacts with GATA-4, we wanted to determine the AAs in the zinc fingers of GATA-4 that are important for p300 interaction. We used zinc finger mutants of GATA-4 available in our laboratory to produce N- and C-finger mutations in plasmids encoding AAs 80–250 and 253–441 of GATA-4, respectively (Fig. 5A). The wild type and mutant GATA-4 peptides were synthesized in TNT reactions in the presence of [35S]methionine and used in pull-down experiments with GST-p300-(1587–1817) immobilized on glutathione-Sepharose beads. Deletion of the N-finger (Fig. 5C, Ndel) caused a marked reduction in the ability of the peptide to interact with p300. Surprisingly, none of the N-finger substitution mutations had any significant effect on p300 binding. Each of the mutant peptides bound as well as the wild type protein (Fig. 5C). This suggests that the amino acid substitutions used in this experiment did not disrupt contacts with p300 sufficiently to prevent binding. Either larger areas of substitution or different amino acid substitutions may be necessary to reveal the critical area for interaction. Similarly, as compared with the wild type C-finger peptide, most C-finger mutants retained the ability to bind to p300 (Fig. 5B). However, the one notable exception was the WRR to SSS substitution of the C-finger that abolished the interaction with p300 (Fig. 5B, lane 3). The C-finger deletion also diminished binding. This result demonstrates that the WRR residues in the C-finger are critical for p300 binding. It should be noted that the C-finger WRR mutant is transcriptionally inactive but this effect cannot be directly attributed to a loss of the ability to interact with p300 since this mutant also does not bind DNA (11).

Deletion of the C/H3 Domain, but Not the C/H1 Domain, of p300 Inhibits GATA-4 Transcriptional Activity—Since the C/H3 region is required for interaction with GATA-4, we expected that deletion of this region from p300 would result in
decreased transcriptional activation. Furthermore, since other regions of p300 are known to interact with several other proteins, including coactivators, we were interested to determine if a C/H3 deletion mutant of p300 might act as a negative regulator by sequestering other potential cofactors of GATA-4. If so, the activity of GATA-4 alone would be expected to be lower in the presence of the C/H3 deletion mutant. To address these issues, a p300 clone was constructed that lacked the coding sequence for a portion of the C/H3 region (AAs 1737 to 1836) (p300ΔH90041737–1836). As a control, we also prepared a clone encoding a p300 protein lacking amino acids from the C/H1 region (p300/H9004348–412) that is not involved in the interaction with GATA-4. GATA-4 was expressed in cells in combination with wild type or C/H3 or C/H1 deleted p300 and assayed for activity from an artificial promoter-reporter containing multiple GATA sites (PRED, Fig. 6). As shown previ-

FIG. 4. N- and C-zinc finger domains of GATA-4 interact with C/H3 region of p300. Radioactively labeled peptides of p300 produced by in vitro translation were incubated with GST fusion proteins encoding portions of GATA-4 as indicated. GST pull-down experiments were performed as described under “Experimental Procedures.” A, schematic representation of GATA-4 and p300 peptides used to localize the interaction domains and summary of results. B, GST-GATA-4 fusion proteins, as indicated, were incubated with 35S-labeled p300 AAs 598–1185 (N2) or AAs 1186–1860 (N3). GST-E1A was used as a positive control for binding to p300 N3 region. C, GST-GATA-4 fusion proteins, as indicated, were incubated with 35S-labeled p300 AAs 1–597 (N1) or AAs 1861–2414 (C). D, GST-GATA-4 fusion proteins (AAs 80–250 or 241–378) were incubated with 35S-labeled p300 peptides containing AAs 1200–1513, 1200–1587, or 1200–1817 as indicated. In B–D, the figure shows an autoradiogram of the p300 peptide, as indicated, that interacted with the GST-GATA-4 peptide indicated at the top of the figure.

FIG. 5. Identification of the amino acid residues in the C-finger of GATA-4 that are critical for interacting with p300. Radioactively labeled wild type or mutant peptides of GATA-4 including AAs 80–250 (N-finger) or AAs 253–441, produced by in vitro translation, were incubated with a GST fusion protein encoding the N3 region of p300. GST pull-down experiments were performed as described under “Experimental Procedures.” A, a schematic representation of the amino acid substitution mutants of the N- and C-fingers of GATA-4 used to map the p300 interaction region. B, 35S-labeled C-finger wild type and mutant peptides, as indicated, were incubated with GST-N3. C, 35S-labeled N-finger wild type and mutant peptides, as indicated, were incubated with GST-N3. In B and C, the figure shows an autoradiogram of the GATA-4 peptide, as indicated, that interacted with the GST-N3.
GATA-4-p300 Interactions

DISCUSSION

In this study we used multiple approaches to demonstrate both physical and functional interactions between GATA-4 and p300. Our results confirm a previous comment that GATA-4 might interact with CBP (34). They are also consistent with a recent study indicating that p300 can coactivate GATA-5 on the ANF promoter (23, 42). We extend these previous observations by: 1) demonstrating functional synergy between GATA-4 and p300 on artificial and natural cardiac promoters; 2) defining the regions of GATA-4 and p300 that are required for the physical interaction; 3) demonstrating that the mutation of amino acids WRR to SSS in the C-finger of GATA-4 prevent p300 binding to that finger; 4) demonstrating that a deletion mutant of p300 that lacks the GATA-4 interaction domain (C/H3) inhibits GATA-4-dependent transcription; 5) demonstrating that overexpression of the p300 C/H3 domain can inhibit GATA-4-dependent transcription; and 6) demonstrating that the HAT domain of p300 is required for p300 coactivation and support the interpretation that this acetyltransferase activity is not directed at GATA-4.

Our results establish that AAs 1587–1817, containing the C/H3 domain of p300 can inhibit GATA-4-dependent transcription (Fig. 6). Furthermore, deletion of the C/H3 region of p300 inhibits GATA-4-dependent transcription (Fig. 6) suggesting that binding of GATA-4 by p300 is required for activity. If the C/H3 interaction is required for the functional synergy between GATA-4 and p300 then overexpression of a peptide containing only the C/H3 region should be able to compete with exogenous and endogenous p300 for GATA-4 binding and thereby attenuate GATA-4-dependent transcriptional activity. To test this, a PCR product encoding the C/H3 region was cloned into an expression vector in-frame with 3 nuclear localization signals. This clone was co-transfected with the ANF promoter reporter and expression vectors encoding p300, HA, or both (Fig. 7). As shown in previous experiments, p300 increased the activity of GATA-4 on this promoter. However, when the plasmids encoding the C/H3 peptide and p300 were co-transfected the transcriptional activity was severely attenuated (from 8- to 3-fold). This inhibition was also seen in the absence of added p300, consistent with the E1A experiment (Fig. 2). These results establish that the C/H3 region of p300 can inhibit GATA-4 transcription presumably by binding to GATA-4 and preventing its interaction with wild type p300. They also lend further support to the hypothesis that p300 is required for GATA-4 transcription.

The HAT Domain of p300 Is Required for Activation of GATA-4 Transcriptional Activity—p300 has been shown to act alone and in complexes with other proteins to influence transcription. The intrinsic HAT activity of p300 can act directly to modify chromatin structure (38), however, p300 may also recruit other histone acetyltransferases to the transcriptional complex (39). To determine whether the intrinsic HAT activity of p300 is required for GATA-4 function, we generated a mutant that encodes a p300 protein deleted for 20 amino acids (AA 1415–1434) in the HAT domain (40). This mutant or wild type p300 were co-transfected with an HA-tagged GATA-4 expression vector in NIH3T3 cells transient transfection experiments (Fig. 8). It can be seen that with increasing amounts of wild type p300, HA-tagged GATA-4 was activated albeit to levels that was lower than native GATA-4. In contrast, increasing amounts of the p300 HAT mutant did not increase transcriptional activity of GATA-4. Protein extracts made from parallel plates in this experiment were used in immunoprecipitation experiments using anti-HA antibody. The precipitates were run on SDS-gels and Western blot analysis was performed with antibodies against acetylated lysine. These experiments failed to detect any acetylation of GATA-4 in any sample even though a similar experiment with GATA-1 could detect acetylation (data not shown). These results indicate that the HAT domain of p300 is required for GATA-4 activation and support the interpretation that this acetyltransferase activity is not directed at GATA-4.

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As shown in previous experiments, p300 increased the activity of GATA-4 on this promoter. However, when the plasmids encoding the C/H3 peptide and p300 were co-transfected the transcriptional activity was severely attenuated (from 8- to 3-fold). This inhibition was also seen in the absence of added p300, consistent with the E1A experiment (Fig. 2). These results establish that the C/H3 region of p300 can inhibit GATA-4 transcription presumably by binding to GATA-4 and preventing its interaction with wild type p300. They also lend further support to the hypothesis that p300 is required for GATA-4 transcription.
reported for GATA-1 that established that its zinc finger domain interacts with C/H3 domain.

The localization of the GATA-4-binding site in p300, reinforces the functional data and confirms an important role for p300 in GATA-4 transcriptional activation. E1A blocks GATA-4 transcriptional activation most likely by competing with it for binding to the p300 C/H3 domain. To confirm this interpretation, we used a C/H3 deletion mutant that has been shown to inhibit the activity of transcription factors known to interact with p300 through this site (49). The C/H3 deletion blocked GATA-4-dependent transcription (Fig. 6), indicating that p300 is essential for GATA-4 function. Furthermore, when the C/H3 domain was expressed in the presence of GATA-4 and p300 or GATA-4 alone, transcriptional activity was repressed (Fig. 7). Similar to the E1A protein, the C/H3 domain peptide presumably competes with co-transfected and endogenous p300 for GATA-4 binding. Taken together, our results establish that GATA-4 activity is dependent, in part, on a functional and physical interaction with the coactivator p300. The work presented here does not address the mechanism of this synergistic interaction.

p300 interacts with the zinc fingers of GATA-4 (Fig. 4). It joins the list of several other regulatory factors that interact with the zinc fingers of GATA-4 including Nkx 2.5 (11, 12, 15), NF-AT 3 (7), MEF-2 (17), GATA-6 (35), and serum response factor (13, 14) that interact with the C-finger and FOG-2 that interacts with the N finger (50, 51). p300 is unique in that it can bind to each finger individually. The ability of p300 to bind either zinc finger might prevent potential interference with the activity of additional transcription factors that might jointly interact with individual fingers.

Transcription of a class II gene by RNA polymerase II requires the assembly of general transcription factors and coactivators around the transcription start site in the promoter. When tethered to DNA, eukaryotic transcriptional activation domains can recruit the general transcription machinery in order to stimulate gene transcription (52). The general transcription machinery includes RNA polymerase II, the general transcription factors, and cofactors known as adaptors or coactivators (53). In many cases, p300 interacts with activation domains to influence function as in the case with VP16 and SP1 (38) and the sterol response element-binding protein (54). Although GATA-4 contains two activation domains (AA 1–74 and 130–177) (55), p300 did not directly interact with these regions of GATA-4. Furthermore, p300 did not enhance transcriptional activity of a mutant GATA-4(22–213) lacking these activation domains even though it could physically interact with the mutual.3 This suggests that p300 plays a role beyond that of histone acetylation in stimulating GATA-4-dependent transcription. One possible role for p300 is to help recruit or stabilize the interaction of other transcriptional regulatory factors with the activation domains. It is also possible that p300 competes with a corepressor, such as FOG 2, for GATA-4 binding.

GATA-4 is necessary for GATA-4-dependent transcription. p300 and its interacting protein P/CAF both possess intrinsic acetyltransferase activity, and this activity is involved in stimulating gene transcription (38, 56). Both P/CAF (43) and GATA-4 interact with the C/H3 domain of p300. Depending upon the nature of this binding, these factors may compete or cooperate for binding to p300. p300 can also activate transcription by a HAT-independent mechanism (32, 39, 57). Here we show that the p300 HAT activity is required for coactivation of GATA-4 transcription activity (Fig. 8). The HAT mutant was capable of binding to GATA-4, indicating that its inability to coactivate GATA-4 is not due to deletion of an interacting domain. Taken together, our results indicate that the recruitment of acetyltransferase activity of p300 by the zinc finger domains of GATA-4 is necessary for GATA-4-dependent transcription.

Acytation of the N-terminal portion of histones is one mechanism by which p300 stimulates transcription. p300 and p300 HAT mutant activity by the closed triangles.

\[ \text{Fold Activation} \]

![Graph](http://example.com/graph.png)

**Fig. 8.** The acetyltransferase activity of p300 is required for coactivation of GATA-4 transcription. NIH 3T3 cells were transfected with 0.3 µg of the PRED reporter alone (PRED) or in combination with CMV-Flag-GATA-4 (0.1 µg), and increasing amounts of pCMV-p300 (0 to 0.8 µg), or pCMV-p300 HAT mutant (0 to 0.8 µg) as indicated. pCMV-Flag-BAP (0.1 µg) was added with PRED as a control for CMV-Flag-GATA-4. Transient transfection experiments were analyzed as described under "Experimental Procedures." Wild type p300 activity is represented by the closed circles and p300 HAT mutant activity by the gray open circles.

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