Transcriptional Coactivator p300 Stimulates Cell Type-specific Gene Expression in Cardiac Myocytes*

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Terminal differentiation is characterized by cell cycle arrest and the expression of cell type-specific genes. Previous work has suggested that the p300 family of transcriptional coactivators plays an important role in preventing the re-initiation of DNA synthesis in terminally differentiated cardiac myocytes. In this study, we investigated whether p300 proteins are also involved in the transcriptional activation of cell type-specific genes in these cells. Since p300 function can be abrogated through direct binding by the adenovirus E1A protein, we overexpressed E1A in cardiac myocytes using recombinant adenoviral vectors. The expression of transfected reporter genes driven by α- or β-myosin heavy chain promoters was markedly diminished by expression of the 12 S E1A protein. In contrast, the activity of a promoter derived from the ubiquitously expressed α-actin promoter was nearly the same as that driven by the α- or β-myosin heavy chain promoters to nearly the same extent as wild type 12 S E1A, transcriptional repression by a mutant defective for p300 binding was severely impaired. Furthermore, overexpression of p300 and, to an even greater extent, p300Δdel33, a mutant lacking residues required for binding by E1A, relieved E1A's repression of β-myosin heavy chain promoter activity while having no effect on the activity of the α-actin promoter. Thus, E1A's transcriptional repression of cell type-specific genes in cardiac myocytes is mediated through its binding of p300 proteins, and these proteins appear to be involved in maintaining both cell type-specific gene expression and cell cycle arrest in cardiac myocytes.

Cardiac myocytes are striated muscle cells whose structural and functional properties are especially suited to maintain normal blood flow. These cells differentiate from progenitors in the lateral plate mesoderm (Ref. 1; reviewed in Ref. 2) during mid-gestation (3). Beginning in mid-gestation, however, increasing proportions of these cells arrest irreversibly in G1 of the cell cycle. DNA synthesis is undetectable in most cardiac myocytes by the end of fetal life and occurs rarely, if at all, after the third postnatal week in the rat (4, 5). Although cardiac myocytes retain considerable plasticity to undergo further modifications in gene expression, morphology, and function (reviewed in Ref. 6), their identity as post-mitotic heart muscle cells is fixed following terminal differentiation. The molecular mechanisms that induce pre-cardiac mesoderm to differentiate into cardiac myocytes and subsequently maintain these cells in a differentiated state are poorly understood.

Clues about cellular proteins that regulate aspects of striated muscle differentiation have been provided by the effects of the adenovirus E1A oncoprotein in these cells. E1A blocks the differentiation of skeletal myocytes from their myoblast precursors (7–12). In addition, when expressed in already differentiated skeletal (13) or cardiac (14, 15) muscle cells, it stimulates cellular DNA synthesis and represses muscle-specific gene expression. E1A interacts with several cellular proteins including members of the p300 family of transcriptional coactivators and the retinoblastoma (Rb) family of pocket proteins (reviewed in Ref. 16). E1A mutants defective for binding these proteins are less efficient than wild type at stimulating DNA synthesis and repressing muscle-specific gene expression in cardiac myocytes (14, 15). These experiments suggest that E1A's effects on cardiac myocyte differentiation are mediated through its interactions with these cellular proteins.

Although analyses of E1A mutants offer a starting point from which to understand the roles of cellular proteins in cardiac myocyte differentiation, these studies are limited in two respects. First, they provide only correlative information. Second, the multifunctional nature of the E1A protein makes it difficult to attribute a given effect to a specific cellular protein. Therefore, the goal of the current study was to use a more direct approach to determine whether the p300 family of proteins is involved in maintaining the cardiac myocyte phenotype. We chose to focus on p300 because, in our previous work, the E1A mutant unable to bind this protein was most defective in its ability to stimulate DNA synthesis in cardiac myocytes (15). The p300 family of transcriptional coactivators have been noted to modulate many examples of enhancer-mediated transcription (17–35). The members of this family, which, thus far, include p300 and the CREB-binding protein (CBP),1 interact directly with components of the basal transcriptional apparatus (e.g. TFIIB (19, 26) and TBP (26,36)) and diverse enhancer-binding proteins (reviewed in Ref. 26). In skeletal muscle, MyoD and MEF-2 are included among the latter (26–29). Currently, nothing is known about which transcription factors

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1 The abbreviations used are: CBP, CREB-binding protein; MHC, myosin heavy chain; CAT, chloramphenicol acetyltransferase; m.o.i., multiplicity of infection; pfu, plaque forming units.
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FIG. 1. E1A differentially represses muscle-specific gene transcription in cardiac myocytes. Embryonic day 20 cardiac myocytes were co-transfected with 5 μg of α- or β-MHC or β-actin luciferase reporters and 1 μg of pRSVCAT. Twenty-four hours later, cells were infected with adenoviruses dl70-3 (not expressing E1A) or dl520E1B+ (expressing 12 S E1A) at m.o.i. 10 pfu/cell (panel A) and 1 pfu/cell (panel B). Activities of luciferase and CAT were determined 24 h after transfection. Relative luciferase activity (mean ± S.E.) represents luciferase activity corrected for CAT activity and is expressed as % relative to that in dl70-3-infected cardiocytes. The data show the combined results from three independent preparations of cells each performed in duplicate. The absolute luciferase activities for cardiac myocytes infected with dl70-3 at m.o.i. 10 pfu/cell is 2658 ± 469 for pα-MHCLuc, 9775 ± 2225 for pβ-MHCLuc, and 34454 ± 9246 for pβ-actinluc.

We demonstrate here that E1A differentially represses the expression of cell type-specific genes in cardiac myocytes and E1A's ability to bind p300 correlates with this function. Furthermore, E1A's repression is rescued partially by overexpression of this construct. In contrast, E1A's mild repression of a ubiquitously transcriptional activator is not relieved by overexpression of this domain. These data demonstrate that E1A's transcriptional repression of cell type-specific genes in cardiac myocytes is mediated through p300 protein(s) and suggest that this family of coactivators plays a role in the transcription of these genes in the absence of E1A.

MATERIALS AND METHODS

Adenoviruses—Mutant human type 5 adenoviruses lacking a functional E3 gene were used throughout these experiments. dl520E1B+ and its derivatives (Ref. 39; gift of Dr. S. T. Bayley, McMaster University, Hamilton, Ontario) contain an E1A gene in which the 5′ splice site needed to generate the 13 S transcript has been mutated; therefore, these viruses give rise only to the 12 S E1A transcript encoding the 243 residue protein. In addition, dl520E1B+ and derivatives lack a functional E1B gene. Derivatives of dl520E1B+ include dl1101/520E1B+ and dl1108/520E1B+ in which E1A residues 14–25 and 124–127 have been deleted, respectively. dl70-3 (Ref. 40; gift of Dr. F. L. Graham, McMaster University, Hamilton, Ontario) lacks functional E1A and E1B genes. Viral stocks were grown on 293 cells, purified on CsCl gradients, and titered by plaque assay on 293 cells (41).

Plasmids—pα-MHCLuc (42), pβ-MHCLuc, and pβ-actinluc (Ref. 14; gift of Dr. M. D. Schneider, Baylor University, Houston, TX) consist of the firefly luciferase (luc) cDNA driven by 2936, 3542, and 433 base pairs of rat α-myosin heavy chain (MHC), rat β-MHC, or avian cytoplasmic β-actin promoter sequences. pRSVCAT contains the bacterial chloramphenicol acetyltransferase (CAT) gene driven by Rous sarcoma virus long terminal repeat sequences (43). p300wt and p300del33 contain the cytomegalovirus promoter/enhancer fused to a full-length human p300 cDNA or one with an internal deletion in the E1A-binding domain as described previously (Ref. 17; gift of Drs. R. Eckner and D. Livingston, Harvard Medical School, Boston, MA).

Cell Culture, Transfection, and Infection— Cultures of primary rat cardiac myocytes were prepared from the hearts of embryonic day 20 Sprague-Dawley fetuses (Taconic Farms) as described (15). After cells were pre-plated for 1 h to minimize fibroblast contamination (typically 5–10% following this step), myocytes were plated at a density of 700/mm² onto 60-mm dishes (Primaria, Falcon) and cultured at 37 °C, 5% CO₂ in media containing 10% fetal bovine serum (Hyclone). Thirty-six hours after plating, transfections were performed using DEAE-dextran as described previously (44) with minor modifications. Briefly, cells were incubated for 1 h in serum-free media with DEAE-dextran (500 μg/ml) and the DNAs to be transfected (see figure legends for combinations and doses) following which they were incubated for 2 min with 10% dimethyl sulfoxide and for 3 h with 100 ng chloroquine. Twenty-four hours after transfection, cells were infected with adenovirus at a multiplicity of infection (m.o.i.) of 10 plaque forming units (pfu) per cell as specified in a volume of 1 ml for 60-mm plates at 37 °C, 5% CO₂ for 1.5 h (45). Twenty-four hours after infection (48 h after transfection), cells were harvested and luciferase and CAT activities were determined as described (46). The relative luciferase activity was calculated from the ratio of luciferase minus background/CAT minus background and expressed as the mean ± S.E. Groups were compared using the unpaired, two-tailed t test and differences considered significant if p < 0.05.

Immunoprecipitation—Extracts from primary cultures of cardiac myocytes were immunoprecipitated using either anti-human p300 monoclonal antibody (Upstate Biotechnology) or normal mouse serum in low stringency buffer (50 mM Tris (pH 7.4), 0.15 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 10 μg/ml aprotinin (Sigma) and leupeptin (Sigma), and 0.5 μM phenylmethylsulfonyl fluoride (Sigma)) for 16 h at 4 °C and incubated with protein A beads for 1 h at 4 °C. The precipitate was washed four times in the same buffer, resuspended in 50 μl of SDS lysis buffer (20 mM Tris (pH 7.5), 50 mM NaCl, 0.5% SDS, 1 mM diithiothreitol), heated to 95 °C for 2 min, electrophoresed by SDS-polyacrylamide gel (12%), transferred to Immobilon membranes, reacted with adenovirus E1A monoclonal antibody (Oneogene Science) which was subsequently detected using horseradish peroxidase-conjugated anti-mouse IgG. Signals were detected using the ECL Western blotting detection system (Amersham) according to the manufacturer's instructions.

RNA Analysis—Northern blot analysis of 10 μg of total RNA was performed as described previously (47). An isofrom specific antisense deoxyoligonucleotide complementary to nucleotides 5846–5869 of the rat 3′-untranslated region (48) was used to detect β-MHC mRNA as...
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RESULTS

E1A Differentially Represses Cardiac-specific Transcription—Previously, we demonstrated that expression of E1A is sufficient to stimulate DNA synthesis in embryonic day 20 (E20) cardiac myocytes, most of which had already undergone cell cycle arrest (15). This occurs in essentially all successfully infected cells within 24 h of the addition of adenovirus to cultures. E1A can also induce apoptosis in these cells; this process, however, is not evident until at least 36 h after infection. Previous studies in a variety of cell types, including immortal skeletal muscle lines and primary cultures of neonatal cardiac myocytes, have shown that expression of E1A represses the transcription of cell type-specific genes (7–14). To determine if it has a similar effect in our system, we transfected E20 cardiac myocytes with luciferase constructs driven by promoters derived from the α- or β-myosin heavy chain (MHC) genes, whose expression is limited to striated muscle cells, or the β-actin gene, which is ubiquitously expressed. A CAT reporter driven by the constitutively active RSV promoter was co-transfected to control for transfection efficiency. Twenty-four hours after transfection, plates were infected with one of two adenoviruses: dl520E1B−, which expresses the wild type 12 S E1A or dl70-3, which does not. These viruses are otherwise identical. Activities of reporter genes were assayed 24 h following infection. As shown in Fig. 1A, activities of α- and β-MHC promoters were 95 and 98% lower, respectively, in cardiac myocytes infected with dl520E1B− as compared with dl70-3 (both m.o.i. 10). In contrast, the activity of the β-actin promoter was ~35% lower in cells infected with dl520E1B− as compared with dl70-3 (both m.o.i. 10). Thus, E1A represses the activity of both cell type-specific and ubiquitously active promoters in E20 cardiac myocytes; the magnitude of repression, however, is considerably more marked in the case of cell type-specific promoters.

To investigate further whether β-MHC and β-actin promoters have different sensitivities to E1A’s transcriptional repression, we assessed whether differences in this parameter would be even more exaggerated when viruses were delivered at a lower m.o.i. We chose m.o.i. 1 because the percentage of cardiac myocytes exhibiting positive immunostaining for E1A following infection with dl520E1B− at this m.o.i., 80–90%, is similar to that observed at m.o.i. 10 (data not shown). At m.o.i. 1, β-MHC promoter activity was repressed 67% while that of the β-actin promoter was affected only minimally (Fig. 1B). Thus, the muscle specific β-MHC promoter is more sensitive than the ubiquitously active β-actin promoter to transcriptional repression by E1A.

E1A’s Repression of Cardiac-specific Promoters Correlates with Its Ability to Bind p300—Previously, we had observed that an E1A mutant defective for binding members of the p300 family was considerably less efficient than wild type 12 S E1A at inducing DNA synthesis in E20 cardiac myocytes (15). In contrast, a second E1A mutant that was defective for pocket protein binding was as active as wild type in this function. To determine whether the ability to bind p300 family members described previously (47). To detect β-actin mRNA, a 0.4-kilobase pair cDNA probe consisting of the HindIII fragment of human β-actin gene (49) was used.

analysis of the effect of E1A mutants on the expression of endogenous β-MHC and β-actin genes. RNA was harvested 24 h after infection with dl70-3 (lane 1), dl520E1B− (lane 2), dl1101/520E1B− (lane 3), and dl1108/520E1B− (lane 4) at m.o.i 10 pfu/cell. Rows labeled β-MHC and β-actin show representative autoradiograms following sequential hybridizations of the same blot with radiolabeled probes specific for β-MHC and β-actin transcripts. The 28 S rRNA bands on the ethidium bromide stained gel are shown to indicate the equivalency of loading.
also correlates with E1A's repression of cell type-specific promoters, we compared the effects of wild type 12 S E1A and each of the above mutants on the transcriptional activities of α- and β-MHC promoters in E20 cardiac myocytes.

To confirm that the mutants exhibited the appropriate p300 binding properties in cardiac myocytes, lysates of cells infected with each of the viruses were immunoprecipitated with an antibody that reacts with both p300 and CBP. Western blots of these immunoprecipitates were then reacted with an antibody monospecific for E1A (Fig. 2A). As expected, E1A co-immunoprecipitated with p300 proteins in plates infected with dl520E1B, encoding wild type 12 S E1A (lane 2), and dl1108/520E1B, encoding the mutant unable to bind pocket proteins (lane 4). In contrast, E1A was not present in plates infected with dl1101/520E1B, encoding the E1A mutant defective for p300/CBP binding (lane 3). E1A was also absent from plates infected with dl70-3, which does not produce an E1A protein (lane 1), and from plates infected with dl520E1B' when normal mouse serum was substituted for anti-p300 in the immunoprecipitation (lane 5). Thus, dl1101/520E1B', but not dl520E1B' or dl1108/520E1B', is defective for binding p300 family proteins in cardiac myocytes. Immunoblots were also performed on cellular lysates (without prior p300 immunoprecipitation) to confirm that similar E1A levels resulted from infection with each of the viruses (lanes 6–9). Since the infection efficiencies of each of the viruses in cardiac myocytes are also similar (data not shown), it follows that the levels of E1A per myocyte are similar.

Fig. 2B shows the effect of the various E1A mutants on the transcriptional activities of transfected α- and β-MHC promoters. The most striking finding is that dl70-3 or dl520E1B as indicated at m.o.i. 10 pfu/cell (panel A) or 1 pfu/cell (panel B). Activities of luciferase and CAT were determined 24 h after this. Relative luciferase activity (mean ± S.E.) represents luciferase activity corrected for CAT activity and is expressed as % relative to that in dl70-3-infected cardiac myocytes. The data presented in panels A and B show the combined results from five and three independent preparations of cells, respectively, each performed in duplicate.

p300 Constructs Relieve E1A's Repression of Cell Type-specific Genes—To test whether a causal connection exists between E1A's repression of cell type-specific promoters and its binding to p300 proteins, we assessed the effect of overexpression of p300 on this transcriptional repression. To augment p300 levels in the cell, we overexpressed either full-length p300 (p300wt) or a p300 mutant that lacks residues required for E1A binding and can, therefore, avoid sequestration by E1A (p300del33 in Ref. 17). While both p300 and p300del33 diminish E1A's repression of β-MHC promoter activity, the effect is more marked with p300del33 (Fig. 3). Relief of repression is incomplete when viruses are infected at m.o.i. 10; at m.o.i. 1, however, p300 effects nearly complete rescue and p300del33 stimulates β-MHC promoter activity to levels exceeding control. Increases in β-MHC promoter activity due to p300del33 occur in a dose-dependent manner. In contrast to its effects on β-MHC promoter activity, p300del33 has no effect on E1A's mild repression of the β-actin promoter. Thus, exogenous p300 can bypass E1A's transcriptional repression of cell type-specific promoters and this effect is independent of E1A binding. This result suggests that E1A's binding of p300 family member(s) is
causally related to its transcriptional repression of the β-MHC promoter and that p300 proteins positively modulate the activity of this promoter.

**DISCUSSION**

The major conclusion of this study is that E1A's transcriptional repression of cell type-specific genes in cardiac myocytes is mediated by its binding of p300 and/or related proteins. Previous work has demonstrated a correlation between the ability of E1A to bind p300 and to induce DNA synthesis efficiently in cardiac myocytes (14, 15). Taken together with the present study, these data support the hypothesis that member(s) of the p300 family play important role(s) in maintaining terminal differentiation in cardiac myocytes.

It is possible to envisage at least two models by which the interaction between E1A and p300 might result in this effect. The most likely model, in light of p300's recognized transcriptional coactivator function, is one in which E1A sequesters p300 (and/or a p300-related protein) making it unavailable to participate in the transcriptional activation of cell type-specific genes. Strong support for the first model is provided by the ability of p300 constructs to relieve E1A's repression of the β-MHC promoter and, in particular, by the ability of p300del33 to effect this rescue independently of binding E1A. Another non-mutually exclusive model is that complexes consisting of E1A and p300 protein(s) actively participate in transcriptional repression. Our functional data neither support nor refute this model; additional biochemical experiments would be necessary to address this possibility.

A corollary of the first model is that member(s) of the p300 family participate in transcriptional activation of the α- and β-MHC, and presumably other cell type-specific genes in cardiac myocytes under normal circumstances. In light of the functional redundancy that has been observed between p300 family members (21, 22), proof of this requires a cell in which the levels of all p300 family members are present in limiting quantities. Given E1A's ability to bind both p300 and CBP (21, 22), the two members of this family recognized thus far, it is likely that we have created such a cellular milieu by overexpressing E1A. In this setting, overexpression of p300 constructs augments the transcriptional activity of a β-MHC promoter. In the case of p300del33, β-MHC promoter activity increases to levels that exceed even those observed in the absence of E1A. These data are consistent with p300 family member(s) playing an important role in β-MHC transcription in normal cardiac myocytes. Other approaches to induce a deficiency of p300 family members will be useful in confirming this hypothesis.

Given the potential role played by p300 protein(s) as coactivator(s) of α- and β-MHC transcription, the question arises as to which cardiac transcription factor(s) interact with and transactivate through these proteins. Included among the motifs present in the promoter sequences used in this study are binding sites for TEF-1 (50–56), GATA-4/5/6 (57, 58), and MEF-2 (59, 60) proteins. It will be interesting to determine whether any of these and other transcription factors interact in a functionally significant manner with p300 family members in cardiac myocytes. Of note, p300 and MEF-2 have been shown to interact in skeletal myocytes (27, 28).

Recent work has demonstrated that, in some systems, p300 protein(s) are present in the nucleus in limiting concentrations (30) and, thus, may be well positioned to coordinate changes in the transcription of multiple genes in response to signals from the cytoplasm. Therefore, it will be important to determine which p300 family members, including possibly novel ones, are present in cardiac myocytes, what their levels are relative to each other and the proteins with which they interact, and how these interactions change in response to various developmental and physiological stimuli.

Our results differ somewhat from an earlier report (14) in which an E1A mutant defective in p300 binding alone retained the ability to repress the activity of a skeletal α-actin promoter in neonatal cardiac myocytes, while a second mutant unable to bind both p300 and pocket proteins lacked transcription repression. The reason for this discrepancy is unclear but might be due to differences in the E1A mutations in the adenoviruses used in the two studies, the promoter sequences studied or the developmental state of the cells. Although the ability of p300del33 to effect a more than complete rescue of E1A's repression of β-MHC promoter activity argues that additional pocket proteins are not needed for this effect, the possibility cannot be excluded that pocket proteins play some role in activation of the β-MHC promoter. Of note, pocket proteins are required for the transcriptional activity of the skeletal myogenic determination factors (61, 62), and the interaction between the coactivator hBrm and Rb enhances transcription in other systems (63). Despite this difference between the two studies, however, the results of both underscore the importance of the p300 family in the activation of cell type-specific genes in cardiac myocytes.

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