Transcriptional regulation of the human glycoprotein hormone common α subunit gene by cAMP-response-element-binding protein (CREB)-binding protein (CBP)/p300 and p53

Xian ZHANG*, Roger J. A. GRAND*, Christopher J. McCABE†, Jayne A. FRANKLYN†, Phillip H. GALLIMORE* and Andrew S. TURNELL*¹

*Cancer Research U.K. Institute for Cancer Studies, The Medical School, University of Birmingham, Edgbaston, Birmingham B15 2TT, U.K., and †Division of Medical Sciences, The Medical School, University of Birmingham, Edgbaston, Birmingham B15 2TT, U.K.

We have investigated the functional interactions between adenovirus early region 1A (AdE1A) protein, the co-activators cAMP-response-element-binding protein (CREB)-binding protein (CBP)/p300 and SUG1, and the transcriptional repressor retinoblastoma (Rb) in mediating T3-dependent repression. Utilizing the human glycoprotein hormone common α -subunit (α -subunit) promoter and AdE1A mutants with selective binding capacity to these molecules we have determined an essential role for CBP/p300. In normal circumstances, wild-type 12 S AdE1A inhibited α -subunit activity. In contrast, adenovirus mutants that retain both the SUG1- and Rb-binding sites, but lack the CBP/p300-binding site, were unable to repress promoter activity. We have also identified a role for the tumour-suppressor gene product p53

in regulation of the α -subunit promoter. Akin to 12 S AdE1A, exogenous p53 expression repressed α -subunit activity. This function resided in the ability of p53 to interact with CBP/p300; an N-terminal mutant incapable of interacting with CBP/p300 did not inhibit α -subunit activity. Stabilization of endogenous p53 by UV irradiation also correlated positively with reduced α -subunit activity. Intriguingly, T3 stimulated endogenous p53 transcriptional activity, implicating p53 in T3-dependent signalling pathways. These data indicate that CBP/p300 and p53 are key regulators of α -subunit activity.

Key words: adenovirus E1A, SUG1, thyroid receptor.

INTRODUCTION

Thyroid hormone receptors (TRs) mediate T3-dependent regulation of gene expression. Two TR genes, termed α and β , have been characterized, and several receptor isoforms, resulting from either differential splicing or the use of alternative promoters, have been identified [1,2]. T3 interacts with a C-terminal ligand-binding domain present in TR α_1 , TR β_1 and TR β_2 , but which is lacking in TR α_2 [3,4], to stimulate or inhibit transcription of specific thyroid hormone-responsive genes in a cell-type-specific manner. In the presence of T3, TRs bind specifically to positive thyroid-response elements (pTREs) to activate, or negative TREs (nTREs) to repress, the transcription of target genes, depending on the physiological context [5].

In the absence of T3, pTREs are repressed by the recruitment of co-repressors, such as N-CoR (nuclear co-repressor), SMRT (silencing mediator of retinoid and thyroid receptors) and SUN-CoR (small unique nuclear receptor co-repressor) to unliganded TR [6]. These repressor molecules also recruit histone deacetylases (HDACs) and Sin3 to unliganded TR, to produce a large multi-subunit repression complex [6]. In the presence of T3, conformational changes of the TR facilitate the dissociation of the repression complex and stimulate the recruitment of nuclear co-activators, cAMP-response-element-binding protein (CREB)-binding protein (CBP)/p300, steroid receptor co-activator (SRC-1), thyroid receptor-interacting proteins and TR-associated factors ('TRAFs'). These proteins form a large multi-subunit co-activator complex that possesses intrinsic histone-directed acetyl-

transferase activity that relaxes chromatin structure around the promoter and facilitates T3/TR-dependent activation of transcription.

The molecular mechanisms underlying TR-mediated, T3-dependent transcriptional repression have also been studied, albeit in less detail [7–10]. Recent studies investigating negative regulation of the human glycoprotein hormone common α -subunit (α -subunit) promoter have suggested that, in the absence of T3, TR can stimulate the basal transcriptional activity of negatively regulated genes [7,8] by recruiting co-repressors and sequestering them from the nTRE; mutant TR that is unable to bind co-repressors cannot stimulate transcriptional activity [8]. Exogenous expression of SMRT and N-CoR can also stimulate transcriptional activity through an nTRE [8]. In the presence of T3 it has been suggested that co-repressors dissociate from the TR, allowing for recruitment and sequestration of co-activators by the TR, facilitating repression of nTRE by co-repressor complexes [7,8]. In contrast to this, negative regulation of the β -subunit gene has been suggested to occur through T3-dependent recruitment of TR and HDAC2 to the negative regulatory element ('NRE') in the promoter [10], indicating that TR can also bind co-repressor in mediating T3-dependent repression.

DNA tumour virus oncoproteins are powerful deregulators of host cell transcription and have been used to probe the basic mechanisms of transcriptional control. Adenovirus early region 1A (AdE1A) protein, which is essential for both viral replication and adenovirus-mediated transformation, is expressed pre-

Abbreviations used: α -subunit, human glycoprotein hormone common α -subunit; Ad5, adenovirus serotype 5; AdE1A, adenovirus early region 1A; CAT, chloramphenicol acetyltransferase; CR, conserved region; CREB, cAMP-response-element-binding protein; CBP, CREB-binding protein; DAPI, 4,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; HDAC, histone deacetylase; N-CoR, nuclear co-repressor; nTRE, negative thyroid-response element; pTRE, positive thyroid-response element; PTTG, pituitary tumour-transforming gene; Rb, retinoblastoma; SMRT, silencing mediator of retinoid and thyroid receptors; SRC-1, steroid receptor co-activator; TR, thyroid hormone receptor; wt, wild type.

¹ To whom correspondence should be addressed (e-mail A.S.Turnell@bham.ac.uk).

dominantly as two differentially spliced mRNAs, 12 S and 13 S, that give rise to translated products of 243 and 289 amino acids respectively, in adenovirus type 5 [11]. The 12 S transcript encodes a protein identical to 13 S, except for a region of 46 amino acids that is unique to the larger molecule [conserved region (CR) 3]. CR3 possesses inherent transactivating potential, stimulating transcription from both viral early and cellular promoters. The transactivating potential attributable to CR3 depends upon its ability to interact with transcription factors such as activating transcription factor-2 [12], transcriptional mediator component hSur2 [13], and the basal transcriptional machinery TATA-box binding protein directly [14]. Other identifiable regions of conservation are also found between different serotypes. CR1 and CR2 are important in defining interactions with a number of cellular proteins, such as the retinoblastoma (Rb) tumour-suppressor gene family [15], serving to alleviate Rb-mediated repression of E2F-responsive elements. Rb also plays a general role in transcriptional repression by recruiting co-repressors such as HDACs to promoter complexes [16,17].

In contrast, AdE1A can also inhibit transcription through its N-terminus and elements in CR1 [18,19]. The N-terminus of AdE1A defines many interactions with cellular proteins including the co-activators CBP/p300 and SUG1 [20–23]; the binding sites for CBP/p300 and SUG1 are, however, distinct. A single Arg-to-Gly substitution at residue 2 of adenovirus serotype 5 (Ad5) E1A inhibits CBP/p300 binding, but not SUG1 binding [21,23]. CBP/p300 are structurally and functionally related co-activators, possessing intrinsic histone-directed acetyltransferase activity [24,25]. CBP/p300 have previously been implicated in stimulating transcription through pTREs, by direct interaction with SRC-1 [26]. SUG1, which is identical to the S8 component of the 19 S proteasome, possesses inherent DNA helicase activity [27] and stimulates transcription through direct binding to nuclear hormone receptors [28–30].

The tumour-suppressor gene product p53 plays a crucial role in the negative regulation of cell growth in response to DNA damage [31]. It regulates genes critical in controlling cell cycle progression, initiating DNA repair and inducing apoptosis. Recent evidence has suggested that there is an intimate functional relationship between CBP/p300 and p53. CBP/p300 can potentiate p53-dependent transcriptional activity through direct binding of its C-terminus to the N-terminal activation domain present in p53 [32,33]. Interestingly, p53 can, depending on the cellular promoter, potentiate [32] or inhibit [34] CBP/p300-dependent transcription. Consistent with these observations, AdE1A has been shown to inhibit CBP/p300-dependent activation of p53-responsive genes by direct binding to CBP/p300 [33,35].

Using the choriocarcinoma cell line JEG-3 we have previously shown that T3 can repress human α -subunit promoter activity in the presence either of TR α_1 or TR β_1 , TR β_1 being more potent than TR α_1 in mediating T3-dependent repression [36]. In the present study we have attempted to elucidate the contribution of the co-activator molecules CBP/p300 and SUG1 and the transcriptional repressor Rb in regulating T3/TR β_1 -dependent repression of α -subunit activity. Using AdE1A mutants that possess selective binding capacity for each of these molecules, we have determined that AdE1A represses α -subunit promoter activity by directly targeting CBP/p300, but not SUG1 or Rb. Given the functional relationship between CBP/p300 and p53, the effects of p53 on α -subunit activity were also investigated. Expression of wild-type (wt) p53 repressed α -subunit activity, whereas an N-terminal p53 mutant incapable of binding to CBP/p300 was unable to inhibit α -subunit activity. Similarly, activation of endogenous p53 by UV irradiation correlated positively with

inhibition of α -subunit activity. T3 also stimulated p53 transcriptional activity, suggesting a potential physiological role for p53 in regulation of the α -subunit promoter, and more generally in T3-mediated signalling pathways. Exogenous CBP/p300 expression served not only to relieve repression mediated by T3/TR β_1 , AdE1A and p53, but moreover served as a co-activator of α -subunit activity in the absence of other exogenous factors. Taken together these data suggest that signalling pathways that impinge on CBP/p300 are crucial in both the positive and negative regulation of α -subunit promoter activity.

EXPERIMENTAL

Plasmids

The reporter plasmid p846 α CAT comprised a 846 bp insert of 5'-flanking regulatory sequence from the human α -subunit gene inserted into a pBR322-based plasmid upstream of the chloramphenicol acetyltransferase (CAT) gene [37]. A putative nTRE is located between –22 and –7 of the 5' flanking sequences, at a region adjacent to a proximal TATA box and a cAMP-response element [38]. pCDM8-TR β_1 [39,40] was used for *in vivo*-regulated expression of TR β_1 . pRc/RSV-CBP [41] and pCMV β -p300 [20] were used for *in vivo*-regulated expression of CBP and p300 respectively. pcDNA3-p53 was used for *in vivo*-regulated expression of wt human p53. pcDNA3-mp53 and pcDNA3-mttmp53 were used to express wt mouse p53 and an N-terminal mouse p53 double point mutant [32], respectively. pAd5 *dl*520 was used for *in vivo*-regulated expression of Ad5 12 S E1A and pAd5 *dl*1101/520, similarly used for *in vivo* expression, contained a deletion of amino acids 4–25 of 12 S E1A [23]. PG13CAT consists of 13 direct repeats of the p53 binding oligonucleotide 5'-CCTGCCTGGACTTGCCTGG-3' upstream of the polyoma-virus early promoter tethered to a CAT reporter gene [42]. The reporter plasmid pCMV β -gal was used as an internal control to determine transfection efficiencies. All plasmids were purified by CsCl gradient ultracentrifugation.

Viruses

Ad5 viruses used in this study were propagated in permissive Ad5-transformed HEK-293 cells, and titred by plaque assay on permissive Ad5 911 cells. All viruses used in this study only express the smaller 12 S E1A protein and do not express 13 S E1A. Ad5 *dl*520 is essentially a wt virus except that 13 S E1A is not expressed. Ad5 *dl*1101/520 E1A has a deletion between amino acids 4 and 25, and consequently is unable to bind either CBP/p300 or SUG1 [23]. Ad5 RG2/520 expresses 12 S E1A that lacks only the CBP/p300-binding site because of an Arg-to-Gly substitution at residue 2 [21]. Ad5 *dl*1108/520 has a deletion of amino acids 124–127 in CR2, and fails to bind pRb, p107 or p130 [43].

Cell culture, transfection and UV irradiation

Human choriocarcinoma JEG-3 cells and colon carcinoma cell lines HCT116 and H1299 were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% foetal calf serum, penicillin (5000 units/ml), streptomycin (5 μ g/ml), Hepes (10 mM) and glutamine (2 mM). JEG-3 cells were used in this study as they lack functional TRs, whereas HCT116 cells possess two wt p53 alleles and H1299 cells are nullizygous for p53. Typically, 3×10^5 cells were plated on 6 cm dishes, using complete medium for overnight attachment to the plates. Cells were then cultured with DMEM containing 8% charcoal-stripped serum (First Link U.K., Brierley Hill, West Midlands, U.K.) for 24 h. Transfection was achieved by a

6 h incubation with the complex of plasmid DNA and DNA and LIPOFECTAMINE[®] according to the manufacturer's instructions (Invitrogen). Where appropriate, the total DNA per dish was kept constant by using pCDM8 plasmid alone. The amounts of reporter and expression plasmids used are indicated in the Figure legends. In order to calculate differences in transfection efficiency between different experimental groups, cells were co-transfected with pCMV β -gal. After transfection, cells were either infected with adenoviruses or maintained in medium containing charcoal-stripped serum. Cells were washed and incubated with fresh medium containing charcoal-stripped serum in the absence or presence of 10 nM T3 (Sigma), unless otherwise stated, for 24 h prior to cell harvesting. Upon harvesting CAT and β -galactosidase activities were determined by ELISA according to the manufacturer's instructions (Roche). To allow for variation in transfection efficiency, CAT reporter activities were normalized by comparison with corresponding β -galactosidase activities, and arbitrary relative activity quotients calculated. For UV irradiation, medium was removed from the plates, the cells exposed to UVC irradiation (254 nm) for the appropriate time and subsequently re-immersed in medium.

Viral infections

After transfection, cells were infected with Ad5 *d*/520, Ad5 *d*/1101/520, Ad5 RG2/520 or Ad5 *d*/1108/520 at a multiplicity of infection of 50 plaque-forming units/cell for 2 h, with intermittent rocking every 15 min. Cells were then washed and incubated in fresh medium containing charcoal-stripped serum, in the absence or presence of 10 nM T3, for an additional 24 h before harvesting.

Western blot analysis

Protein concentrations were determined by Bradford assay using the Bio-Rad protein assay kit. Protein samples of 50 μ g were separated on SDS/polyacrylamide gels, and electroblotted on to nitrocellulose filters (Gelman Sciences). The blots were blocked and then hybridized with the appropriate antibodies. AdE1A was detected using M58 monoclonal antibody (Pharmingen); p53 was detected using DO-1 monoclonal antibody or a sheep polyclonal antibody; Hdm2 (human homologue of Mdm2) was detected using 2A10 monoclonal antibody; and p21 was detected using a rabbit polyclonal antibody (Santa Cruz Biotechnology). Antigens were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences).

Immunofluorescence

H1299 cells were grown on glass multiwell slides and transfected with a pcDNA3 construct expressing either wt mouse p53 or the N-terminal p53 mutant. After transfection (24 h) cells were washed once in PBS and fixed in paraformaldehyde [4% (w/v) in PBS] for 8 min and subsequently permeabilized with acetone at -20°C for an additional 10 min. Non-specific binding sites were blocked using blocking buffer [20% (v/v) heat-inactivated goat serum, 0.2% BSA (w/v) and 0.1% sodium azide in PBS] for 1 h at room temperature. Cells were co-stained for p53 and p300, using CM-1 rabbit polyclonal antibody diluted 1:250 in blocking buffer and RW128 monoclonal antibody (Upstate Biotechnology) at a dilution of 1:250 in blocking buffer respectively. Cells were incubated in primary antibodies for 2 h at 37°C in a humidified atmosphere. Cells were washed twice in PBS for 15 min and then incubated with anti-rabbit Alexa 594 and anti-mouse IgG Alexa 488 secondary antibodies (Molecular Probes) at a dilution of 1:200 for an additional h at 37°C . Cells

were subsequently washed for 30 min in PBS. 4,6-Diamidino-2-phenylindole (DAPI), at a final concentration of 0.1 $\mu\text{g}/\text{ml}$, was included in the final PBS wash in order to visualize DNA. Slides were then mounted in 2% (w/v) DABCO (diazabicyclo-[2.2.2]octane) in 80% glycerol/PBS. Cells were viewed using a Nikon Eclipse E600 microscope. z-Layer images (0.2 μm horizontal sections) were recorded with a biovision software package (Openlab 3.0.9.; Improvision) and selected z-layers subsequently volume deconvolved. Single, equivalent z-layers were merged to show co-localization.

RESULTS

TR β_1 -mediated, T3-dependent repression of the α -subunit promoter in JEG-3 cells

Initially, we determined dose- and time-dependent effects of T3 on the TR β_1 -mediated repression of the α -subunit promoter in JEG-3 cells. In the presence of TR β_1 , T3 inhibited promoter activity in a dose- and time-dependent manner (Figure 1). The IC_{50} for T3-dependent repression was approx. 10 nM after 24 h

Figure 1 TR β_1 -mediated, T3-dependent repression of the human α -subunit promoter in JEG-3 cells

JEG-3 cells were transfected with 2 μg of TR β_1 -pCDM8, or pCDM8 vector alone, together with 4 μg of p846 α CAT reporter and 1 μg of pCMV β -gal. (A) Dose-dependent repression by T3 in the presence of TR β_1 . Unpaired Student's *t* tests: 10 nM T3 treatment, $P < 0.02$; 100 nM T3 treatment, $P < 0.01$; 1 μM T3 treatment, $P < 0.01$. (B) Time-dependent repression by T3 in the presence of TR β_1 . Cells were incubated in the absence or presence of 10 nM T3 for the times indicated: 6 h T3 treatment, $P < 0.025$; 24 h, $P < 0.02$; 48 h, $P < 0.005$; 72 h, $P < 0.01$; means $\pm$ S.D. ($n = 4$). $\circ$, Transfection of TR β_1 in the absence of T3 treatment; $\bullet$, T3 treatment in the presence of TR β_1 .

Figure 2 Schematic representation of AdE1A constructs used

Ad5 *dl520* expresses wt 12 S E1A, which is capable of binding CBP/p300, SUG1 and Rb. Ad5 *dl1101/520* expresses an AdE1A deletion mutant which retains the capacity to bind Rb. Ad5 *RG2/520* expresses an AdE1A mutant which retains the capacity to bind SUG1 and Rb. Ad5 *dl1108/520* expresses an AdE1A deletion mutant which retains the capacity to bind CBP/p300 and SUG1.

Figure 3 AdE1A inhibits the transcriptional regulation of α -subunit promoter through binding to endogenous CBP

(A) JEG-3 cells were first transfected with 4 μ g of p846 α CAT reporter, 1 μ g of pCMV β -gal and 2 μ g of TR β ₁-pCDM8 or pCDM8, then infected with either Ad5 *dl520*, Ad5 *RG2/520* or Ad5 *dl1101/520* viruses. Cells were cultured for 24 h in the absence or presence of 10 nM T3. #*P* < 0.005, significant difference from cells not treated with T3; **P* < 0.01, CAT activities from Ad5 *dl520*-infected cells were significantly different from mock-infected cells; ***P* < 0.05, CAT activities from T3/TR β ₁-treated, Ad5 *dl520*-infected cells were significantly different from cells treated with T3/TR β ₁ alone; means $\pm$ S.D. (*n* = 8). (B) Western blot analysis of AdE1A proteins, using M58 monoclonal antibody, from the experiment outlined in (A). (C) 1 μ g of pAd5 *dl520* or pAd5 *dl1101/520* was co-transfected with 2 μ g of pTR β ₁-pCDM8 or pCDM8, together with 4 μ g of p846TSH α CAT reporter vector. The cells were subsequently cultured for 24 h in the absence or presence of 10 nM T3. #*P* < 0.02, CAT activities from T3/TR β ₁-treated cells were significantly different from mock-treated cells; **P* < 0.02, CAT activities from Ad5*dl520*-transfected cells were significantly different from mock-transfected cells; ***P* < 0.05, CAT activities from T3/TR β ₁-treated, Ad5*dl520*-transfected cells are significantly different from cells treated with T3/TR β ₁ alone; means $\pm$ S.D. (*n* = 8). (D) Western blot analysis of AdE1A proteins, using M58 monoclonal antibody, from the experiment outlined in (C).

treatment. Thus in subsequent experiments designed to determine the roles of co-activators and co-repressors in T3/TR β ₁-mediated repression of the α -subunit promoter, cells were typically treated

with 10 nM T3 for 24 h, prior to harvesting. In addition, 12 S AdE1A-expressing plasmids and viruses lacking CR3 were used to discriminate from general CR3 effects (Figure 2).

Figure 4 Repression by AdE1A does not necessitate binding to Rb

(A) Repression of the α -subunit promoter by AdE1A does not require an intact Rb-binding site. JEG-3 cells were transfected with 4 μ g of p846 α CAT reporter vector, 1 μ g of pCMV β -gal and 2 μ g of pTR β -pCDM8 or pCDM8. Cells were then infected for 2 h with Ad5 *dl*520 or with the mutant virus Ad5 *dl*1108/520. Cells were then cultured for 24 h in the absence or presence of 10 nM T3. [#] $P < 0.005$, CAT activities from T3/TR β_1 -treated cells are significantly different from mock-treated cells; ^{*} $P < 0.01$, CAT activities from Ad5 *dl*520 infected cells are significantly different from mock-infected cells; [†] $P < 0.05$, CAT activities from T3/TR β_1 -treated, Ad5 *dl*520-infected cells are significantly different from cells treated with T3/TR β_1 alone; means $\pm$ S.D. ($n = 4$). (B) Western blot analysis of AdE1A species from the experiment outlined in (A).

AdE1A inhibits transcriptional activity of α -subunit by binding to CBP/p300

AdE1A mutants, with selective binding capacities for CBP/p300, SUG1 and Rb (Figure 2), were used to determine the contribution of these proteins in the regulation of α -subunit promoter activity. Wt 12 S Ad5E1A (Ad5 *dl*520) inhibited α -subunit promoter activity by approx. 40 %, whereas AdE1A mutants that do not bind CBP/p300 or SUG1 (Ad5 *dl*1101/520) or CBP/p300 alone (Ad5 RG2/520) failed to inhibit α -subunit activity (Figure 3A). Indeed, there was no discernible difference in α -subunit activity between mock-infected cells and cells infected with either Ad5 *dl*1101/520 or Ad5 RG2/520 (Figure 3). Since Ad5 *dl*1101/520 and Ad5 RG2/520 have different binding capacities for p300/CBP and SUG1, these data suggest that AdE1A inhibits transcriptional activity of the α -subunit promoter by exclusively binding to CBP/p300 but does not function through binding to SUG1. In the presence of AdE1A, T3/TR β_1 was further able to repress transcription, suggesting that T3/TR β_1 may also target molecules other than CBP/p300 in repressing α -subunit activity. These findings were substantiated by transient transfection assays. Expression of wt AdE1A (pAd5 *dl*520) inhibited α -subunit promoter activity, whereas a mutant incapable of binding to CBP/p300 (pAd5 *dl*1101/520) lost the ability to repress gene

Figure 5 Role of CBP in α -subunit activity

(A) p846 α CAT reporter (4 μ g) and 1 μ g of pCMV β -gal were transfected into JEG-3 cells with 0.5 μ g of pAd5 *dl*520 vector and an increasing amount of pRc/RSV-CBP, as indicated. [#] $P < 0.005$, CAT activities from Ad5 *dl*520-transfected cells were significantly different from mock-transfected cells; ^{*} $P < 0.05$, CAT activities from cells co-transfected with CBP and AdE1A were significantly different from cells transfected with AdE1A alone; means $\pm$ S.D. ($n = 4$). (B) TR β_1 -pCDM8 (2 μ g) was co-transfected into JEG-3 cells with an increasing amount of pRc/RSV-CBP, together with 4 μ g of p846 α CAT reporter and subsequently treated with 10 nM T3 for 24 h. [#] $P < 0.01$, CAT activities from T3/TR β_1 -treated cells were significantly different from mock-treated cells. ^{*} $P < 0.01$, CAT activities from cells co-transfected with CBP/TR β_1 and T3-treated cells were significantly different from cells treated with T3/TR β_1 alone. Means $\pm$ S.D. ($n = 4$). (C) JEG-3 cells were transfected with different amounts of pRc/RSV-CBP or pCMV β -p300 and 4 μ g of p846 α CAT reporter vector in the absence of TR β_1 . After transfection, cells were cultured for 24 h in the absence (open bars) or presence (hatched bars) of 10 nM T3. CAT activities from cells transfected with CBP or p300 were significantly different from mock-transfected cells; [#] $P < 0.005$, ^{*} $P < 0.005$ and [†] $P < 0.05$; means $\pm$ S.D. ($n = 4$).

transcription (Figure 3C). Western blot analysis of AdE1A expression revealed that the mutant viruses synthesized AdE1A protein in similar quantities and T3/TR β_1 did not affect their expression (Figure 3B). Similar results were obtained when AdE1A was expressed following transient transfection (Figure 3D).

Repression by AdE1A does not require interaction with Rb

To determine whether Rb plays a role in T3/TR β_1 -mediated repression of the α -subunit promoter, JEG-3 cells were infected with a virus that expresses an AdE1A protein lacking the capacity to bind Rb but which retains the ability to bind CBP/p300 and SUG1 (Ad5 *d*/1108/520; Figure 2). This AdE1A mutant retained the capacity to repress the transcriptional activity of the α -subunit promoter, similar to wt AdE1A (Figure 4A). This suggests that the repressive effect attributable to AdE1A does not necessitate sequestration of Rb from the promoter. Moreover, since there was no additional repression in the presence of this AdE1A mutant, when compared with the corresponding control (mock) value in each instance, it is unlikely that Rb plays a direct role in T3/TR β_1 -dependent repression of the α -subunit gene. The level of AdE1A expression was similar in each instance of viral infection, and was unaffected by the presence of T3/TR β_1 (Figure 4B).

CBP/p300 stimulates transcription of the α -subunit promoter and alleviates T3/TR β_1 - and AdE1A-mediated repression

Since AdE1A represses promoter activity by binding to endogenous CBP, we considered whether overexpression of CBP would overcome AdE1A-mediated repression. By co-transfecting increasing amounts of CBP with a constant level of wt AdE1A, we found that CBP could alleviate the repression mediated by AdE1A in a dose-dependent manner (Figure 5A). These findings are consistent with the results shown in Figure 3, strongly supporting the suggested mechanism that AdE1A represses the α -subunit gene by targeting CBP/p300. Similarly, co-transfection of increasing amounts of CBP also alleviated TR β_1 -mediated, T3-dependent repression in a dose-dependent manner (Figure 5B), implying that the transactivating capacity of CBP/p300 is normally inhibited during TR-mediated T3-dependent repression of the α -subunit promoter. In the absence of exogenous factors, CBP and p300 stimulated the basal activity of the α -subunit promoter (Figure 5C). These data indicate the pivotal role of CBP/p300 in the regulation of α -subunit activity.

p53 inhibits α -subunit promoter activity

Recent evidence suggests that there is an intimate functional relationship between CBP/p300 and p53. We were thus interested in examining the role of p53 in regulation of the α -subunit promoter, which lacks sequence-specific p53-response elements. However, the use of AdE1A as a tool to investigate the role of p53 in this system is inappropriate, since it has been determined that AdE1A may inhibit p53 transcriptional activity [35]. We therefore transfected increasing amounts of a plasmid expressing human wt p53 into H1299 cells to determine its effects on α -subunit promoter activity. p53 repressed α -subunit promoter activity, in direct contrast to the effects of CBP/p300 (Figure 6A). Indeed, CBP overexpression was able to alleviate the transcriptional repression mediated by p53 (Figure 6A), indicating that CBP/p300 and p53 exert functionally opposed effects in the regulation of the α -subunit promoter.

To further establish the relationship between p53 and α -subunit activity, H1299 cells were transfected with 846 α -CAT in

the presence of either a wt mouse p53 construct or an N-terminal mouse p53 mutant incapable of binding CBP/p300 [32]. Like human p53, wt mouse p53 similarly repressed α -subunit activity (Figure 6B, panel i). Interestingly, however, the N-terminal p53 mutant was unable to repress α -subunit activity (Figure 6B, panel i), suggesting that p53-CBP/p300 complex formation is required to repress α -subunit promoter activity. The N-terminal p53 mutant was expressed as well as wt p53 (Figure 6B, panel ii), indicating that the repressive effects attributable to wt p53, relative to mutant p53, did not merely reflect levels of protein expression. Similarly, wt p53 stimulated the p53-responsive genes Hdm2 and p21, whereas the N-terminal p53 mutant did not (Figure 6B, panel ii), indicating that functional p53-CBP/p300 complexes are required for transactivation.

In order to validate previous findings [32], and moreover establish the binding capacities of these p53 species for endogenous CBP/p300 in H1299 cells, transfected cells were stained for both p53 and p300 and visualized by immunofluorescence microscopy (see the Experimental section). Deconvolved images of a single z-layer show that a proportion of transfected wt p53 (Figure 6C, panel ii, red) co-localizes with endogenous p300 (Figure 6C, panel ii, green) indicated by regions of overlapping yellow staining (Figure 6C, panel ii, arrows indicate regions of co-localization), whereas the transfected N-terminal p53 mutant (Figure 6C, panel v, red) does not co-localize with p300 (Figure 6C, panel v, green), despite both p53 species having similar intra-nuclear staining patterns [Figure 6C, compare panels i (wt) and iv (mutant)]. Interestingly, p300 appeared to be excluded from mutant p53 foci; p300 foci were, however, often adjacent to p53 foci without obvious overlap (Figure 6C, panel v, indicated by arrows). These data confirm the ability of wt p53, but not the N-terminal p53 mutant, to form complexes with endogenous CBP/p300 in H1299 cells, and are supportive of the hypothesis that p53 complex formation with CBP/p300 is required to repress α -subunit activity.

T3 stimulates p53 transcriptional activity

To determine whether p53 might be regulated by T3, HCT116 cells expressing wt p53 were transfected with a p53 reporter construct (PG13CAT; see the Experimental section). After transfection, cells were washed and then maintained in DMEM containing charcoal-stripped serum for 24 h. Cells were subsequently challenged with 10 nM T3 for the appropriate time. Figure 7 indicates that T3 significantly enhanced p53 transcriptional activity (Figure 7A, panel i) in the absence of observable p53 stabilization (Figure 7A, panel ii). Post-translational modification of p53 often accompanies transcriptional activation. We were, however, unable to detect phosphorylation of serine residues implicated in transcriptional activation of p53 (e.g. Ser-15, Ser-33 and Ser-315; results not shown), upon treatment with T3.

To determine whether endogenous p53 can repress α -subunit activity *in vivo*, JEG-3 cells, which have previously been shown to express one wt p53 allele and one mutant allele (Gly to Thr, codon 167) [44], were treated with UV irradiation to specifically induce p53 expression and activity. p53 was induced in a dose- and time-dependent manner upon treatment with UV irradiation. Maximal p53 induction was observed at a dose of 30 J/m² 24 h after irradiation (Figure 7B, panel i). At this time, adherent cells stained with Acridine Orange showed no morphological evidence of apoptosis (results not shown). To determine whether the transactivating potential of p53 was stimulated at this dosage, JEG-3 cells were irradiated and adherent cells subsequently

Figure 6 p53-mediated repression of the α -subunit promoter in H1299 cells

(A) H1299 cells were co-transfected with 4 μ g of p846 α CAT reporter, 1 μ g of pCMV β -gal with increasing amounts of either pcDNA3-p53 or pRc/RSV-CBP, or co-transfected together with both pcDNA3-p53 and pRc/RSV-CBP. CAT activities were assayed 24 h after transfection. # P < 0.05, CAT activities from cells transfected with p53 were significantly different from mock-transfected cells; * P < 0.05, cells transfected with CBP were significantly different from mock-transfected cells; † P < 0.05, cells co-transfected with CBP and p53 were significantly different from cells transfected with p53 alone; means $\pm$ S.D. (n = 4). (B, panel i) H1299 cells were transfected with 4 μ g of p846 α CAT along with either 1 μ g of pRc/RSV-CBP, mouse p53 (mp53) or an N-terminal p53 mutant incapable of binding CBP (mtmp53). CAT activities were assayed 24 h after transfection. * P < 0.01, CAT activities from cells transfected with mouse p53 were significantly different from mock-transfected cells; †CAT activities from cells transfected with the N-terminal p53 mutant were not significantly different from mock-transfected cells. (B, panel ii) Western blots of p53, Hdm2 and p21^{KIP1} from the experiment described in (B) panel i. Lane 1, mock-transfected; lane 2, pRc/RSV-CBP; lane 3, wt mouse p53; lane 4, N-terminal p53 mutant. (C) Wt p53 co-localizes with p300, whereas the N-terminal p53 mutant does not. Panels i–iii, H1299 cells transfected with wt p53; panels iv–vi, H1299 cells transfected with the N-terminal p53 mutant. Panels i and iv, p53 (red) and DAPI (blue) staining; panels iii and vi, p300 (green) and DAPI (blue) staining; panels ii and v, merged images. Regions of p53/p300 dual staining appear yellow.

assayed for p53 transcriptional activity. The ability of p53 to bind DNA specifically was significantly enhanced upon UV treatment of JEG-3 cells, relative to mock-treated JEG-3 cells (Figure 7B, panel ii), suggesting that p53 is functionally active. Intriguingly, enhanced transcriptional activity of p53 in UV-treated cells correlated positively with the ability of UV irradiation to specifically repress α -subunit activity by approx. one-quarter (Figure 7B, panel iii, compare bars 1 and 2). Moreover, UV irradiation of the p53-null cell line H1299 did not significantly repress α -subunit activity (Figure 7B, panel iii, compare lanes 3 and 4), suggesting that functional p53 plays a direct role in repressing α -subunit activity in UV-irradiated cells;

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Figure 7 T3 stimulates p53 transcriptional activity

(A, panel i) HCT116 cells were transfected with a p53-responsive reporter (PG13CAT) construct and pCMV β -gal. Cells were subsequently cultured in the absence or presence of 10 nM T3 for 8 or 24 h in the continued presence of DMEM-containing charcoal-stripped serum. Lane 1, 8 h mock; lane 2, 24 h mock; lane 3, 8 h T3; lane 4, 24 h T3. p53 transcriptional activities from T3-treated cells were significantly different from mock-treated cells; * $P < 0.02$ and † $P < 0.001$. (A, panel ii) Western blots of p53 from the experiment outlined in panel i. (B, panel i) Induction of p53 in response to UV irradiation. JEG-3 cells were subjected to different doses of UV irradiation. Post-irradiation (24 h) cells were harvested, subjected to SDS/PAGE and Western blotted for p53. (B, panel ii) Effects of UV irradiation on transcriptional activity of p53. JEG-3 cells were transfected with PG13CAT, 24 h prior to UV irradiation. Post-irradiation (24 h) cells were harvested and CAT assays performed as described in the Experimental section. * $P < 0.003$, p53 transcriptional activity was significantly enhanced upon UV-irradiation. (B, panel iii) Effects of UV irradiation on transcriptional activity of α -subunit promoter in (lanes 1 and 2) JEG-3 cells expressing functional p53, and (lanes 3 and 4) H1299 cells nullizygous for p53. Cells were transfected with p846 α CAT 24 h prior to UV irradiation. Post-irradiation (24 h) cells were harvested and CAT assays performed as described in the Experimental section. p846 α CAT activity was significantly inhibited in JEG-3 cells upon UV irradiation (* $P < 0.001$), but not in H1299 cells (# $P > 0.2$), indicating the p53-dependency of α -subunit repression; means $\pm$ S.D. ($n = 4$).

p53-independent pathways do not contribute to the repression of α -subunit activity in UV-irradiated cells.

DISCUSSION

In the present study we have utilized AdE1A constructs that have differential binding affinities for a number of proteins that mediate TR-dependent responses, to investigate the contribution of co-activators and co-repressors in T3/TR β_1 -dependent negative regulation of the α -subunit promoter. We demonstrate that T3/TR β_1 represses the α -subunit promoter in a dose- and time-dependent manner (Figure 1). Utilizing the 846 α -CAT reporter we failed to observe the unliganded activation of the α -subunit promoter (Figure 1), in contrast to studies using the 846 α -luciferase reporter [7,8]. This apparent discrepancy may reflect the sensitivities of the different reporter constructs used, or reflect the observation that luciferase cDNA may itself contain an nTRE [45].

The AdE1A-binding proteins CBP/p300 and SUG1 have previously been shown to act as co-activators in T3/TR-dependent transcriptional activation: CBP/p300 stimulates T3/TR-dependent transcription by interacting with SRC-1 [26]; SUG1 stimulates T3-dependent transcription through its interaction with both TR α_1 and TR β_1 [29,30]. In contrast, Rb has been shown previously to repress T3/TR activation by binding to, and sequestering, a TR-interacting protein (TRIP230) normally involved in transactivation at a pTRE [46]. Moreover, AdE1A itself, has been shown to transactivate a pTRE directly, by binding to TR through CR1 and CR3, to synergistically enhance T3-stimulated activation [47]. In this study we have shown that in the absence of T3/TR β_1 , 12 S AdE1A represses α -subunit promoter activity by directly targeting CBP/p300 (summarized in Figure 8), since the Ad5 RG2/520 mutant, which does not bind CBP/p300, fails to repress promoter activity (Figure 3). However, AdE1A does not target the Rb family of proteins or SUG1 in mediating this response (Figure 3). As there is no additional repressive effect in the presence of an adenovirus that does not bind Rb (Ad5 dl1108/520), a role for Rb in general repression of the α -subunit promoter in the presence of T3/TR β_1 is unlikely. This is perhaps surprising given the role of Rb in transcriptional repression [16,17]. However, in the study by Chang et al. [46] a direct interaction between TR and Rb could not be found, suggesting that T3/TR β_1 recruits HDACs to the promoter independently of Rb.

A separate study has assessed the specific contribution of 12 S AdE1A-binding proteins in the negative regulation of a region comprising the first 132 bases of the α -subunit promoter [9]. These authors determined that targeting of CBP/p300 by AdE1A contributed to 40 % of the repressive effect, whereas targeting of Rb contributed approx. 20 %. This earlier study proposed that AdE1A needed to target both CBP/p300 and Rb for complete repression [9]. Using a region comprising the first 846 bases of the α -subunit promoter we propose, however, that the whole repressive effect attributable to AdE1A is through targeting CBP/p300 (Figure 3). Using an AdE1A mutant that does not bind Rb, p107 or p130 (Ad5 dl1108/520 [43]) our studies showed that the Rb family of proteins was not involved in mediating AdE1A repression in this instance (Figure 4). The differences between the two studies presumably reflect the different promoter regions used.

Other studies have attempted to determine the relative contribution of co-activators and co-repressors in T3/TR-dependent regulation of the α -subunit promoter [7–10]. A model has been proposed in which T3 promotes the recruitment of co-activators to TR that allows for dissociation of co-repressors to effect

A. -T3

B. + T3

Figure 8 Schematic illustration depicting the role of the TR, CBP/p300, p53 and E1A in T3-independent (A) and T3-dependent (B) regulation of the α -subunit promoter

The roles of other co-activators and co-repressors in this process are also shown. RXR, retinoid X receptor; CRE, cAMP-response element; P/CAF, p300/CBP-associated factor.

deacetylation and inhibition of the promoter. Many of these experiments, however, were performed in TSA-201 cells [7,8], a human embryonic kidney cell line that expresses not only adenovirus E1 proteins (including both AdE1A 12 S and 13 S), but simian virus 40 (SV40) large T-antigen as well. These transforming proteins bind and affect activities of both co-activators such as CBP/p300 and p300/CBP-associated factor (P/CAF), regulate proteins involved in co-repressor function, such as Rb, and moreover regulate transcription factors directly [11,48]. Thus the precise roles of co-activators and co-repressors in T3/TR β_1 -dependent repression of the α -subunit in cells not expressing AdE1A or SV40 large T-antigen awaits clarification.

Due to the intimate functional relationship between CBP/p300 and the tumour-suppressor protein p53, we analysed the effect of p53 on α -subunit promoter activity. We demonstrate here that p53 inhibits α -subunit promoter activity (Figures 6 and 8). The α -subunit promoter region utilized in this study does not possess any consensus binding motifs for p53, suggesting either that p53 represses α -subunit activity through non-specific DNA binding or by direct protein-protein interaction. The observation that the p53-mediated repression can be specifically reversed by p300 (Figure 6), and that a p53 mutant incapable of binding CBP/p300 ([32]; Figure 6C) does not inhibit α -subunit activity (Figure 6B, panel i), strongly suggests that p53 represses α -subunit activity by directly targeting CBP/p300. Indeed, this notion is consistent with previous studies showing that, in the absence of specific p53-response elements, p53 can inhibit CBP/p300-dependent transcription [34]. Intriguingly, T3

also enhanced p53 transcriptional activity, suggesting a potential physiological role for endogenous p53 in T3-mediated signalling pathways. However, it is not clear at present how T3 activates latent p53. Previous studies have suggested that phosphorylation of p53 upon any number of serine residues correlates with enhanced p53 transcriptional activity [49,50]. In this instance however there was no evidence for enhanced phosphorylation at residues previously implicated in controlling p53 activity (e.g. Ser-15, Ser-33 and Ser-315) upon T3 treatment. Interestingly, T4 (and to a lesser extent T3) have recently been shown to stimulate the phosphorylation of p53 in a mitogen-activated protein kinase-dependent manner [51]. Unfortunately, the critical phosphorylated residue(s) were not identified in that study. p53 is also a substrate for sumoylation and acetylation reactions that also affect transcriptional activity [52,53]. Identifying the critical p53 residues that are modified upon T3 treatment will facilitate an understanding of how T3 signalling pathways couple to p53 transcriptional activity. p53 has previously been shown to physically associate with TR β_1 , inhibiting its ability to dimerize, bind to hormone-response elements [54] and stimulate T3-mediated production of growth hormone [55]. Data presented in this report indicate that p53 can also regulate TR-responsive genes independently of binding to TR, suggesting that p53 can differentially regulate TR-mediated signalling.

Intriguingly, recent evidence has indicated that the protein product of the pituitary tumour-transforming gene (PTTG) induces apoptosis by both p53-dependent and -independent mechanisms [56]. The ability of PTTG to induce p53-dependent apoptosis suggests that p53 function may be ablated in pituitary tumours overexpressing PTTG. It will be of considerable importance to determine p53 functional status in tumours overexpressing PTTG and relate p53 status to expression of the α -subunit. The data presented in this report indicate the essential requirement for the tumour-suppressor proteins CBP/p300 and p53 in regulating the activity of the α -subunit promoter. The role of these tumour suppressors in the regulation of α -subunit may be particularly relevant and of physiological significance given that recent reports implicate altered TR-mediated repression in the inappropriate expression of glycoprotein hormone α -subunit gene observed in clinically non-functioning pituitary tumours in humans [57,58].

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