Differential role of p300 and CBP acetyltransferase during myogenesis: p300 acts upstream of MyoD and Myf5

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Studies in tissue culture cells have implicated p300 and CBP acetyltransferases in myogenic regulatory factor (MRF) mediated transcription and terminal differentiation of skeletal muscle cells. However, in vivo data placing p300 and CBP on myogenic differentiation pathways are not yet available. In this report we provide genetic evidence that p300 but not CBP acetyltransferase (AT) activity is required for myogenesis in the mouse and in embryonic stem (ES) cells. A fraction of embryos carrying a single p300 AT-deficient allele exhibit impaired MRF expression, delayed terminal differentiation and a reduced muscle mass. In mouse embryos lacking p300 protein, Myf5 induction is severely attenuated. Similarly, ES cells homozygous for a p300 AT or a p300 null mutation fail to activate Myf5 and MyoD transcription efficiently, while Pax3, acting genetically upstream of these MRFs, is expressed. In contrast, ES cells lacking CBP AT activity express MyoD and Myf5 and undergo myogenic differentiation. These data reveal a specific requirement for p300 and its AT activity in the induction of MRF gene expression and myogenic cell fate determination in vivo.

Keywords: cell fate determination/differentiation/ES cells/HAT/myogenin

Introduction

Most sequence-specific transcription factors regulate gene expression by interacting with transcriptional co-regulators, which influence chromatin structure. Acetyltransferases (ATs) and chromatin remodelling complexes facilitate chromatin opening, while deacetylases (HDACs) and lysine 9-specific methyltransferases frequently contribute to gene silencing (Jenuwein and Allis, 2001; Narlikar et al., 2002). The transcriptional co-regulators CBP and p300 possess histone acetyltransferase activity (Bannister and Kouzarides, 1996; Ogryzko et al., 1996) and are capable of interacting with a large variety of transcription factors playing central roles in a wide range of cellular processes including proliferation, differentiation and apoptosis (for reviews see Shikama et al., 1997; Goodman and Smolik, 2000; Chan and La Thangue, 2001). Moreover, CBP and p300 exhibit tumour-suppressing activity, especially in hematopoietic cells (Kung et al., 2000; Rebel et al., 2002). A growing body of evidence indicates that p300 and CBP are also able to acetylate proteins other than histones, such as transcription factors, components of the transcription machinery and tumour suppressors (Gu and Roeder, 1997; Imhof et al., 1997; Boyes et al., 1998; Hung et al., 1999; Soutoglou et al., 2000), adding yet another level of control to their transcriptional regulatory potential.

The p300/CBP family is specific to multicellular organisms and evolutionarily conserved from plants to humans (Bordoli et al., 2001b), suggesting that p300 and CBP are likely to play important roles in organ development and morphogenesis. Drosophila melanogaster and Caenorhabditis elegans only possess the cbp ortholog (Akimaru et al., 1997; Shi and Mello, 1998), while both p300 and CBP are present in humans and mice. The two proteins are highly related (63% identity) and their expression pattern during mouse development is almost identical (Partanen et al., 1999), suggesting that their respective functions overlap to some degree. However, there is evidence that they are not interchangeable, in particular from the analysis of p300 and cbp knockout mice which exhibit in part distinct phenotypes (Yao et al., 1998; Tanaka et al., 2000). For example, an increased incidence of haematological malignancies and abnormal skeletal patterning was reported in cbp but not in p300 heterozygous null mice (Tanaka et al., 1997; Kung et al., 2000). At the cellular level, it was shown that ribozyme-mediated ablation of p300, but not CBP, inhibits retinoic-acid-induced differentiation (Kawasaki et al., 1998). Similar studies in p300−/− fibroblasts implied that p300, but not CBP, is an essential retinoic acid receptor cofactor (Yao et al., 1998).

Previous in vitro studies have indicated that several ATs, including p300/CBP and PCAF, as well as HDACs are involved in the control of muscle differentiation (for review see McKinsey et al., 2001; Puri et al., 2001). During myogenesis, the primary myogenic regulatory factors (MRFs) MyoD and Myf5 are thought to cooperate with p300 and CBP (Eckner et al., 1996; Yuan et al., 1996; Puri et al., 1997a; Sartorelli et al., 1997) and with MEF2 transcription factors (Eckner et al., 1996; Sartorelli et al., 1997) to mediate activation of the secondary MRFs, myogenin and Mrf4. The primary MRFs specify myogenic identity of uncommitted somitic mesoderm cells. The
secondary MRFs, in conjunction with MEF2 proteins, allow myoblasts to exit the cell cycle and to differentiate into myocytes and mature myofibers (for reviews see Sabourin and Rudnicki, 2000; Buckingham, 2001). This process requires both repression of genes associated with proliferation and activation of muscle-specific genes.

CBP and the GCN5-related acetyltransferase PCAF were shown to acetylate MyoD, thereby facilitating heterodimer formation with ubiquitously expressed E-box proteins encoded by the E2A gene (Puri et al., 1997b; Sartorelli et al., 1999; Polesskaya et al., 2000). However, disruption of Pcaf in the mouse is not lethal and does not ostensibly impair myogenesis (Xu et al., 2000; Yamauchi et al., 2000), and chemical inhibition of CBP AT activity still allows MyoD to activate myogenin in C2C12 cells (Polesskaya et al., 2001). Therefore the significance of PCAF- and/or CBP-mediated MyoD acetylation in vivo remains unclear.

In this report, we provide the first in vivo evidence that p300 indeed plays a role in skeletal myogenesis. A fraction of mouse embryos bearing a monoallelic mutation inactivating p300 AT activity exhibit impaired myogenesis and MRF expression. In contrast, embryos with an equivalent mutation in cbp do not manifest overt muscle defects. Moreover, embryonic stem cells lacking p300 or its AT activity are strongly impaired in their ability to activate MyoD and Myf5 during skeletal myogenesis, while ES cells deficient in CBP or its AT activity are still capable of forming myotubes. These results suggest that the AT activity of p300 is distinct from that of CBP and reveal an essential function of p300 AT activity in myogenic cell fate specification.

Results

Generation of heterozygous mice and homozygous ES cells deficient in p300 or CBP AT activity

We have used a knock-in strategy in ES cells to introduce the previously described AT-inactivating point mutation in p300 [residues WY(1466–1467)AS] or CBP [residues WY(1503–1504)AS] (Bordoli et al., 2001b). Integration of the mutation by homologous recombination of the p300 and cbp targeting vectors (Figure 1A and Supplementary figure S1A available at The EMBO Journal Online, respectively) was detected by Southern blot (Figure 1B; Supplementary figure S1B). Heterozygous ES cells were injected into blastocysts that gave rise to chimeric mice transmitting the AT-mutant allele to their progeny.

The heterozygous ES cells were made homozygous for the p300 or CBP AT mutation by cultivation in medium containing a high G418 concentration (Figure 1B; Supplementary figure S1B and C). These homozygous cells still harbour the neo gene and p300 protein levels were undetectable in p300ASneoASneo cells (Figure 1C, lane 3), while in cbpASneoASneo cells the CBP protein levels were drastically diminished (reduced by more than 95%);

Fig. 1. Targeting strategy to generate p300 AT mutant ES cells and characterization of p300 or CBP AT-mutant ES cells. (A) Overall organization of the p300 protein, the wild-type p300 genomic locus, the targeting vector and the recombed loci is shown. The asterisks indicate the site of the mutation, which harbors a de novo Nhel restriction site. (B) Southern blot with probe 1 analysing genomic DNA isolated from wild-type, heterozygous (p300ASneoAS), and homozygous p300 KO (p300ASneoASneo) and p300 AT− (p300ASneoAS) ES cells. The probes and the size of the genomic DNA fragments following Nhel restriction digest are indicated in (A). (C and F) Western blot analysis showing p300 and CBP protein levels in (C) p300 KO and p300 AT− cells and (F) CBP KO (cbpASneoASneo) and CBP AT− (cbpKOASneoAS) cells. (E) E1A binding assay; western blot analysis following E1A immunoprecipitation from ES cells infected with adenovirus using, from top to bottom, E1A, p300 or CBP antibodies. (D and G) HAT assay with (D) p300 KO and three independent p300 AT− cell lines and (G) CBP KO and two independent CBP AT− cell lines.
Figure 1F, lane 2). Excision of the neo cassette (Figure 1B, lane 5, for p300 and Supplementary figure S1B, lanes 3 and 4, for CBP cells) restored p300 and CBP protein expression to levels comparable to those of wild-type cells, as illustrated in Figure 1C (lanes 4–6) for p300 and in Figure 1F (lanes 3 and 4) for CBP. Therefore excision of the neo cassette is required for full expression of AT-mutant p300 or CBP proteins. Furthermore, in agreement with previous in vitro studies (Bordoli et al., 2001a), CBP (Figure 1G) and p300 (Figure 1D) mutant proteins did not exhibit any AT activity. For simplicity, the AT-deficient cells will be referred to as p300 AT− and CBP AT−, and the p300KO/neo/AS− and the cbpKO/neo/AS− ES cells will be named p300 KO (knockout) and CBP KO, respectively.

In order to assess the conformational integrity of the mutant proteins, we have analysed their binding to the adenoviral E1A protein by immunoprecipitation. The AT-deficient proteins bound E1A as efficiently as wild-type p300 (Figure 1E, middle panel) or CBP (data not shown). These findings suggest that the AT mutation does not overtly perturb the overall structure of p300 and CBP and still allows binding of E1A to the C/H3 domain which is immediately adjacent to the AT domain (Figure 1A).

**Full dose of p300 AT activity is required for normal muscle development in mice**

The p300KO/AS− and cbpKO/AS− mice were viable and fertile. In vivo excision of the neo gene was performed by crossing these mouse lines with a CMV-Cre deleter strain whose Cre gene is active from the zygote stage onwards (Schwenk et al., 1995). Genotyping of several E8.5–E11.5 embryos derived from such crosses revealed efficient and complete in vivo excision of the neo gene (data not shown).

p300KO/AS embryos varied in size and did not survive embryogenesis. The majority of these embryos died between E12.5 and E16.5 due to heart failure or perinatally due to an inability to breathe (Shikama et al., 2003). In contrast, most of the cbpKO/AS animals reached term, but died within the first 1–2 days following birth, probably owing to respiratory failure. Since most mice heterozygous for a p300 or cbp null allele are viable (Tanaka et al., 1997, 2000; Yao et al., 1998), the lethality observed in the two AT-mutant mice indicates that the AT-deficient alleles are dominant negative. Mouse strains harbouring these AT-mutant alleles can only be propagated as long as expression of the mutant locus is suppressed by the neomycin gene transcribed in opposite direction relative to the p300 or cbp gene. In this article, we have focused on skeletal muscle development in the two AT-mutant mouse lines and in ES cells.

Histological analysis of E18.5 mouse embryos showed that in most of them the size of the muscle fibres was reduced and their interstitial space was increased, resulting in a loose and disorganized appearance (Figure 2, compare wt and p300KO/AS panels). This was clearly visible for the trapezius muscle (Figure 2C and D), the tongue (Figure 2E and F), the diaphragm (Figure 2G and H) and the intercostal muscles (Figure 2I and J). In contrast, no such disorganized muscle phenotype was ever seen in cbpKO/AS mice (data not shown). These observations indicate that hypaxial (tongue and limbs) as well as epaxial muscle formation is sensitive to a reduction in the dose of p300 but not CBP AT activity.

A reduction in the size of muscle groups was visible in all p300KO/AS embryos at E14.5 (Figure 3Ab and Ad), whereas cbpKO/AS mice of this age had a largely intact muscle compartment (Figure 3Bb and Bd). In addition, sarcomeric actin protein expression levels were diminished in the back muscles in a fraction of E14.5 p300KO/AS embryos (Figure 3Ac and Ad) but never in cbpKO/AS embryos (Figure 3Bc and Bd). All E14.5 p300KO/AS embryos also exhibited a peripheral edema (Figure 3Ab and Ad),
which is in part due to cardiovascular malformations (Shikama et al., 2003) and may contribute to the impaired myogenesis by exerting pressure on muscle tissue. However, a reduction in sarcomeric actin expression was also observed in muscle groups that are not directly exposed to the edema, such as the limbs (data not shown) and the tongue (Figure 3Ae and Af). Moreover, a proportion of p300+/AS embryos exhibited a diminished or delayed expression of myosin heavy chain (MHC) at E10.5 (Figure 3C, right panels), a time point that is well before the appearance of the edema around E12.5. Based on these observations, the edema is unlikely to account fully for the compromised myogenesis in p300+/AS embryos. Rather, the presence of smaller muscle groups in all embryos and the partially penetrant reduction in terminal differentiation suggest that muscle formation is directly affected by p300 AT mutation.

The reduction in muscle size was due to neither decreased proliferation nor increased apoptosis in p300+/AS embryos of the same size as wild-type littermates (Supplementary figure S2A and B, respectively). Migration of muscle precursor cells into the limb buds was also comparable between size-matched p300+/AS and wild-type littermates (Supplementary figure S2C). These findings suggest that the muscle defects of p300 AT mutant embryos are more likely to occur at the level of myogenic determination and differentiation, during maintenance of the differentiated state.

Since myoblast identity and terminal differentiation is determined by MRFs (Sabourin and Rudnicki, 2000; Buckingham, 2001), we investigated the expression levels of Myf-5, myogenin and MyoD in p300+/AS and wild-type embryos. Approximately 15% of E14.5 p300+/AS embryos exhibited a reduction in MyoD (Figure 4Aa and Ab) and myogenin (Figure 4Ac and Ad) protein expression. Similarly, RNA whole-mount in situ hybridization of embryos aged E9.5–E11.5 suggested that MRF expression is perturbed in a fraction of p300+/AS embryos relative to wild-type littermates. Thirty-seven embryos (16 wild type and 21 p300+/AS) were analysed. Eleven of the 21 p300+/AS embryos displayed an equivalent number of somites as wild-type littermates. Three of these mutant embryos showed diminished or delayed expression of Myf-5 or myogenin (Figure 4B, middle, and C, top and bottom). Among the 10 retarded p300+/AS embryos, two were of particular interest as they did not express myogenin or MyoD (Figure 4C, middle, and D) despite having reached a developmental stage (27 and 37 somites) where these transcripts are normally expressed (10–12 somite stage at E8.5 for myogenin and 35 somite stage at E10.5 for MyoD). In addition, another retarded p300+/AS embryo showed an irregular and disrupted Myf-5 expression pattern (Figure 4B, right). Taken together, these in vivo results show that monoallelic abrogation of p300 AT activity leads to a partial impairment of MRF expression, suggesting that the full complement of p300 AT activity is required to activate and/or sustain MRF gene transcription efficiently. Furthermore, the MRF activity itself is also likely to be affected by the p300 AT mutation since the impaired muscle formation was completely penetrant at E14.5 and E18.5 while the compromised MRF expression was not.
**CBP and p300 AT activities are not required for embryoid body formation and proliferation**

We have extended these in vivo analyses by investigating the myogenic differentiation potential of homozygous AT-deficient ES cells in which a requirement for AT activity should become more readily apparent than in the heterozygous mice. Pluripotent ES cells can form embryoid bodies (EBs) which are able to differentiate into a wide spectrum of cell types including neuronal, hematopoietic, skeletal muscle and bone cells (for a review see Desbaillets et al., 2000). In particular, skeletal myogenesis is well characterized in EBs and reflects the temporal and sequential MRF expression pattern seen during mouse development. (Braun and Arnold, 1994; Rohwedel et al., 1994; Weitze et al., 1995).

All five cell lines (E14 wild type, p300 KO, p300 AT−, CBP KO and CBP AT−) efficiently formed EBs within 2 days (D2) (Figure 5A), suggesting that neither protein nor their intrinsic AT activity is required for this process. On D4 and D6, wild-type, p300 AT−, CBP KO and CBP AT− EBs showed an increase in diameter due to cell proliferation (Figure 5B). However, EBs derived from p300 KO cells remained small and showed reduced cell proliferation, a defect that was maintained throughout differentiation until confluency was reached. A similar finding was recently reported for p300 nullizygous EBs (Rebel et al., 2002). Since p300 AT deficient EBs did not show such a defect, these results indicate that a function of p300 other than the AT activity plays a role in proliferation of ES cells upon induction of differentiation.

**Severe impairment of skeletal myogenesis in ES cells lacking p300 AT activity**

Having established EB formation of p300 and CBP AT-deficient cells, we assessed their myogenic differentiation potential by analysing MHC expression. After 44 days in culture (Figure 6A, left column), all cell lines were able to differentiate, albeit with strikingly different efficiencies. Wild-type and CBP mutant cell lines (KO and AT−) showed abundant striated myofibers, while both p300 mutant lines showed very few MHC-positive cells which exhibited little cell fusion and lacked striation as well as myofibrillar organization (Figure 6A). Quantification of the signal confirmed that wild-type and both CBP mutant
lines displayed comparable MHC expression (Figure 6B). In contrast, p300 KO and AT– cells showed a 6- to 18-fold and a 12- to 38-fold reduction in MHC expression, respectively (Figure 6B). In keeping with the above results, skeletal actin and embryonic MHC mRNA levels were also reduced in p300 KO and AT– cell lines at D44 (Figure 6C). Differentiation of both p300 mutant cells was not merely delayed, since even at D66 these cells failed to form myofibers, which were abundant in wild-type and CBP mutant cells (Figure 6A, right column). Confluency of p300 KO cells was reached well before D66, suggesting that the differentiation defect is unlikely to be due to the limited proliferative capacity of these cells. Taken together, these data demonstrate that cells deficient in p300 but not CBP AT activity are largely unable to undergo terminal myogenic differentiation. However, the presence of weak MHC staining in p300 KO and AT– cells suggests that differentiation was not completely blocked, implying the existence of a myogenic differentiation pathway independent of p300.
To exclude that p300 KO and AT− cells exhibit a general differentiation impediment, we subjected these cells to osteogenic differentiation (Phillips et al., 2001) and analysed the presence of mineralized bone nodules (Figure 7A). The percentage of EBs with calcium deposits was comparable for the five cell lines, ranging between 78% and 92% (Figure 7B). Similarly, the osteoblast-specific transcript osteocalcin was expressed at equivalent levels in all cell lines (Figure 7C). However, microscope examination revealed subtle differences in the fine structure of the bone nodules (J.-F. Roth et al., in preparation). Taken together, these results suggest that p300 KO and AT− cells are able to undergo osteogenesis as efficiently as wild-type and CBP cell lines, and thus do not manifest a general differentiation impairment.

These data clearly demonstrate that, while dispensable for osteoblast differentiation, the AT activity of p300 is required for myogenic differentiation including efficient cell fusion leading to myofibre formation. CBP AT activity does not appear to be required for this process, pointing to a striking functional difference between the two AT activities.

**Abrogation of p300 AT activity prevents Myf5 and MyoD gene expression**

The expression pattern of the MRF genes during EB differentiation was analysed in order to determine at which stage skeletal muscle differentiation was blocked in both p300 mutant cells (Figure 8). At D23 and D44 (Figure 6A, right column), all four MRF transcripts were present in wild-type cell lines in the expected temporal pattern (Figure 8, lanes 2 and 7). In contrast, in p300 mutant cell lines, MRF genes failed to be efficiently induced (Figure 8, lanes 5, 6, 10 and 11). Since Myf5 and MyoD define myoblast identity, these results suggest that p300 is required for myoblast specification and acts genetically upstream of these two genes.

Unexpectedly, CBP KO and CBP AT− cells, which were both able to undergo muscle cell differentiation as assessed by MHC expression (Figure 6), differed in their MRF gene expression pattern. Unlike CBP AT− cells, which showed a pattern similar to wild-type cells (Figure 8, cf. lanes 4 and 2), Myf5 was not induced in CBP KO cells at D23. In addition, MyoD and myogenin induction was reduced (Figure 8, lane 3). At D44, CBP KO cells still exhibited lower levels of all MRF transcripts, except MyoD, when compared with wild-type and CBP AT− cells, (Figure 8, cf. lanes 7, 9 and 8, respectively). Interestingly, the depressed MRF levels correlated with a small but notable reduction in myotube formation observed in CBP KO relative to wild-type and AT− cells (Figure 6A). Given the previously suggested concept of two separate myogenic subpopulations defined by either MyoD or Myf5 expression (Braun and Arnold, 1996; for a review see Tajbakhsh and Buckingham, 2000), our data may reflect a deficiency in determination of the Myf5 subpopulation in cells lacking CBP. The fact that the CBP AT mutation does not interfere with Myf5 gene activation suggests that one or several domains of CBP, distinct from the AT domain, are required for this process. Importantly, this result also provides evidence that the mutation introduced in the AT domain leaves other CBP functions intact.

Finally, we have analysed Pax3 gene expression, since Pax3 and Myf5 have been identified as upstream regulators of MyoD (Maroto et al., 1997; Tajbakhsh et al., 1997). Pax3 has also been shown to be essential for migration of somitic muscle precursor cells to the limb buds (Bober et al., 1994). Pax3 mRNA levels were comparable in all
cell lines at both D23 and D44 (Figure 8), suggesting that neither p300 nor CBP are essential for induction of Pax3 expression during ES cell differentiation. Nevertheless, since Pax3 is expressed in mesodermal and neuroectodermal tissues which are both generated upon in vitro differentiation, we cannot rule out the possibility that in this experiment a mesodermal defect in Pax3 expression could be partially masked by neuroectodermal Pax3 expression.

**Impaired Myf 5 induction in p300 KO embryos**

In order to complement the data obtained with the p300 KO ES cells, we have analysed Myf5 gene expression in p300−/− embryos. On a mixed 129SVJ × C57BL/6 background, these embryos die between E8.5 and E11 (Yao et al., 1998). Therefore, Myf5 was the only MRF whose expression could be examined since it is induced around E7.75 in presomitic mesoderm (Ott et al., 1991). At E8.5, Myf5 expression was readily detectable in the first four somites of a p300−/− embryo, while it was much weaker in two p300−/− littersmates (Figure 9). This result indicates that the absence of p300 leads to a compromised expression of Myf5 in vivo, thus confirming the findings with the p300 KO cells.

**Discussion**

We have taken a genetic approach in mouse and ES cells to investigate the role of p300 and CBP AT activity during skeletal myogenesis. Unexpectedly, the results of both lines of enquiry indicate that p300 AT but not CBP AT activity is required for the induction and/or maintenance of MRF gene expression and muscle differentiation. Hence, p300 plays a critical role in myoblast cell fate determination by acting genetically upstream of Myf5 and MyoD genes. Moreover, while myogenesis is inhibited to a comparable degree in ES cells homozygous for either a p300 null or an AT mutation, this is not the case for equivalent CBP mutations. A CBP null mutation prevents efficient induction of Myf5, in contrast with the CBP AT mutation which has no effect. These observations suggest that during myogenesis, the AT activity plays a central role in the overall function of p300 whereas it appears to be largely dispensable in CBP.

All previous studies investigating the role of p300 and CBP in skeletal myogenesis were carried out in immortalized tissue culture cell lines such as C3H10T1/2 fibroblasts and C2C12 myoblasts, the latter already being committed to a myogenic fate and recapitulating aspects of terminal differentiation. Transient transfection and microinjection experiments with these cells have suggested that p300 and CBP cooperate with MyoD in the activation of the Cdk inhibitor p21 and of the myogenin gene, allowing terminal differentiation and myotube formation to take place (Eckner et al., 1996; Yuan et al., 1996; Puri et al., 1997a; Sartorelli et al., 1997). More recently, using the synthetic inhibitor LysCoA to block p300 and CBP AT activity, induction of the terminal differentiation markers MHC and muscle creatine kinase (MCK) was suggested to depend on p300/CBP AT activity (Pollesson et al., 2001). This defect could in part be rescued by the overexpression of a wild type but not by a HAT mutant CBP protein lacking 138 amino acids. However, the above experiments could not address a possible role of p300 and CBP in the specification of the myogenic cell fate nor functionally distinguish between the two proteins.

Our analysis of skeletal myogenesis in AT-mutant and knockout mice provides the first evidence that p300 and its AT activity indeed play a role in this process in animals. The strength of our approach lies in the specificity of our point mutations and in our ability to assay myogenesis in its natural context without overexpression of genes. Taking advantage of ES cells recapitulating myogenesis more faithfully than myoblastic cell lines, we were able to distinguish p300 from CBP function and to show that p300 is required well before terminal differentiation, in particular at the level of myoblast specification. Moreover, we also demonstrate that CBP AT-deficient cells are capable of undergoing both myoblast specification and terminal differentiation.

It is important to point out that myogenesis is not completely inhibited in the absence of p300 or p300 AT activity since MRFs were expressed, albeit at very low levels, during skeletal muscle differentiation of p300 KO and AT− ES cells, and MHC could be detected by immunofluorescence. Similarly, p300−/− mice showed weak but detectable Myf5 gene expression at E8.5. Thus our results suggest the existence of p300 and p300 AT-independent pathways enabling myogenic specification and differentiation to occur. This observation points to a certain degree of redundancy among AT enzymes.

The defects observed in Myf5, myogenin and MyoD expression in a fraction of p300+/− and in Myf5 expression in p300−/− embryos are indicative of a requirement of p300 and its AT activity at the level of MRF gene induction. This process is known to depend on the local balance between myogenesis-inducing (Wnt1, Wnt7 and Shh) and myogenesis-repressing (BMP/TGFβ family members) morphogens (for reviews see Bailey et al., 2001; Brent and Tabin, 2002). Accordingly, p300 and its AT activity may be required to convert these opposing morphogen signals into an appropriate transcriptional response leading to MRF activation. Transcription factors responding to Shh are the Gli family members which contribute to the
activation of Myf5 (Tajbakhsh et al., 1998; Gustafsson et al., 2002). In turn, Myf5 induces myogenin (Kaul et al., 2000) and, together with Pax3, can contribute to MyoD activation (Maroto et al., 1997; Tajbakhsh et al., 1997). Thus we propose that p300 AT may cooperate with one or several of the transcription factors participating in Myf5 and MyoD gene induction (Figure 10). However, it is important to note that no Pax3 binding site is present in the MyoD core enhancer element, and thus its effect on MyoD activation may be indirect (for reviews see Borycki and Emerson, 1997; Arnold and Winter, 1998).

In addition to playing a role at the level of MRF gene expression, our data are consistent with a requirement for p300 AT activity at the level of MRF transactivation potential. The defect in myofiber formation present in most E18.5 p300+/−/AS embryos does not fully correlate with impaired MRF expression that is only detectable in approximately 15% of the embryos. Because of this partial correlation, it is likely that the p300 AT mutation also affects the transcriptional activity of MRFs themselves, resulting in the generation of fewer myoblasts and less efficient myofiber formation. However, further analyses are required to address this issue in greater detail.

Previous in vitro studies have provided some evidence for a differential role of p300 and CBP during certain differentiation processes. Retinoic-acid-induced differentiation of F9 embryonal carcinoma cells was inhibited when p300, but not CBP, protein levels were reduced (Kawasaki et al., 1998). During hematopoiesis, p300 is required for differentiation of specific precursor cells (Kasper et al., 2002; Rebel et al., 2002), while CBP contributes to maintenance of the hematopoietic stem cell pool (Rebel et al., 2002). Enhancing the link between p300 and differentiation, our work demonstrates that it is mainly p300, but not CBP, which permits myogenic cell specification and differentiation in vivo. In addition, it is specifically the AT activity of p300 that is essential for this process, implying that the two ATs may modify distinct substrates. Indeed, a previous mutagenesis study has revealed several structural differences between p300 and CBP AT domains (Bordoli et al., 2001a). Since p300 and CBP modify histones with equal efficiency, at least in vitro, our observations raise the possibility that histones may not represent the most critical acetylation substrates of p300 during differentiation. Among the MRFs, MyoD has been demonstrated to be acetylated in vivo, and can be modified in vitro by PCAF, p300 and CBP on several lysine residues (Sartorelli et al., 1999; Polesskaya et al., 2000). Therefore it will be interesting to determine whether MyoD acetylation is diminished in p300 or CBP AT-deficient cells and whether altered acetylation of MyoD changes its transcriptional activity. Moreover, the availability of the AT-deficient ES cells will facilitate the identification of physiologically relevant acetylation substrates of p300 and CBP.

Materials and methods

Generation of homozygous p300 or CBP AT-deficient ES cells and mice

p300 and CBP AT mutations were introduced into E14, subclone KPA, cells using a knock-in strategy. The targeting vectors contained a floxed pgk-neo cassette in an intron flanking the mutated exon encoding a segment of the AT domain. The use of a weak neo allele allowed rapid subsequent generation of homozygous cells by raising G418 concentration to 5 mg/ml. The neo cassette was excised in vitro by electroporating ES cells with pPGK/CREbPA plasmid (kindly provided by K. Rajewsky), and clones sensitive to low G418 were isolated and analysed by Southern blot and PCR (primer sequences are available upon request).

To generate AT-mutant mice, p300+/−neo or cbp+/−neo ES cells were injected into blastocysts, which gave rise to chimeric mice transmitting the mutant allele to offspring. To remove the neo cassette, heterozygous mice were mated with CMV-Cre deleter mice (Schwenk et al., 1995). The efficiency of the neo cassette deletion was monitored by isolating DNA from the embryo proper and yolk sac. Neo cassette deletion was complete in most embryos, and the results from embryo and yolk sac DNA were matching. Subsequently, the genotype of the embryos was inferred from yolk sac DNA analysis.

Western blot analysis, immunoprecipitation and HAT assays

Total cell extracts were prepared in IPH buffer [50 mM Tris–HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.1 mM phenyl-methylsulfonyl fluoride (PMSF)]. Western blot and immunoprecipitation experiments were performed with the p300 antibody RW128 and the CBP antibody AC26 (Upstate Biotechnology). For EIA binding experiments, ES cells were infected with adenovirus Ads5 at a multiplicity of infection of 10. Following cell lysis in IPH buffer, the EIA protein was immunoprecipitated with M73 antibody and co-purified CBP and p300 proteins were detected by western blotting. HAT activity was determined by assaying the enzymatic activity of p300 or CBP proteins immunoprecipitated from 2 mg of total cell extract incubated with purified histones and acetyl-CoA (Sigma) as described previously (Bordoli et al., 2001b).

ES cell growth and differentiation

Wild-type E14 ES cells and their derivatives were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 15% defined fetal calf serum (FCS) (Hyclone), 2 mM l-glutamine, 100 IU penicillin, 100 μg/ml streptomycin, 20 mM HEPES buffer, 0.085 mM β-mercaptoethanol and 500 IU/ml leukaemia inhibitory factor (LIF) on dishes coated with 0.1% gelatine.

For skeletal muscle differentiation experiments, ES cells were maintained in Iscove’s modified Eagle’s medium (IMDM) supplemented with 15% FCS, 2 mM l-glutamine, 100 IU penicillin, 100 μg/ml streptomycin, 450 μM mornithyglycol and 1/2 non-essential amino acids (Invitrogen). EBs were generated as described (Wobus et al., 2001).

At day 0 (D0), 600 cells were allowed to aggregate into EB in 20 μl hanging drops. At D2, the EBs were transferred to bacterial dishes. At D5, the EBs were plated out. The medium was exchanged every 3 days.
Osteogenesis was induced as described previously (Gollner et al., 2001; Phillips et al., 2001).

**Immunofluorescence**

Differentiated EBs, attached to coverslips, were fixed in 3:1 methanol–acetic acid at −20°C for 20 min prior to staining with the MHC antibody MF 20 overnight at 37°C in a humidified chamber, following standard procedures. Quantification was performed using the Adobe quantification toolpack.

**RT–PCR**

RNA was extracted from 100–150 differentiated EBs using Trizol® reagent (Life Technologies) according to the manufacturer’s instructions. The RNA was treated with RNase-free DNase and 1 µg of total RNA was used per reaction. The reaction was performed using the Qiagen OneStep RT–PCR kit. The PCR primers are designed within different exons so that they amplify exon as well as intron sequences, allowing PCR products derived from mature or precursor transcripts to be distinguished. Primer sequences are available upon request.

**Mouse histology**

Haematoyxlin–eosin (HE) staining was performed according to standard procedures. Immunohistochemistry on paraffin sections was performed using the histomouse Kit (Zymed), including a quenching step and using the Dako solution® for unmasking. The antibodies used were anti-reticulin (Sigma A2172), anti-MyOD (Santa Cruz Biotechnology M318) and anti-amyogin (Santa Cruz Biotechnology M225).

**Whole-mount hybridization**

Whole-mount in situ hybridization was performed as described (Wilkinson 1992). The antisense probes were labelled with digoxigenin and generated using the following plasmids: mouse MyoD from pEMC11s (a gift from A.Lassar), mouse Myf5 from pBS-Myf5 (kindly provided by T.Braun) and myogenin from pSP-myogenin, which contains a 1.4 kb EcoRI fragment of the rat myogenin gene. Whole-mount immunohistochemistry was performed according to standard procedures with MF20 antibody.

**Supplementary data**

Supplementary data are available at The EMBO Journal Online.

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**References**


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