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Molecular Mechanisms Underlying the Valproic Acid-Induced Degradation of Transcriptional Coactivators p300 and CBP

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Abstract

p300 and CBP are closely related transcriptional coactivators involved in a wide range of cellular processes. They interact with a large array of transcription factors and possess intrinsic acetyltransferase activity which allows them to acetylate many nuclear proteins including nucleosomal histones. Although much is known about the functions of p300 and CBP, what in turn regulates these coactivators is less understood. The present study reveals that the stability of both proteins is affected by the histone deacetylase inhibitor valproic acid and elucidates the molecular mechanisms involved in this pathway. These results show that valproic acid induces degradation of p300 and CBP through the 26S proteasome, and suggest that this occurs in an ubiquitin-dependent manner and that this effect is mediated by the B56γ3 regulatory subunit of protein phosphatase 2A. Considering the involvement of valproic acid, p300 and CBP in such processes as embryonic development and tumorigenicity, these findings potentially entail important biological significance.

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List of Abbreviations

ACTR	Activator of retinoid receptor
AML	Acute myeloid leukemia
AP-1	Activating protein-1
APS	Ammonium persulfate
AR	Androgen receptor
Az	Antizyme
Aza-dC	5-aza-2'-deoxycytidine
BSA	Bovine serum albumine
C-terminal	Carboxy terminal
CBP	CREB-binding protein
ChIP	Chromatin immunoprecipitation
CITED2	CBP/p300-interacting transactivator with ED-rich tail 2
CREB	cAMP response element binding protein
DNMT	DNA methyltransferase
EGR-1	Early growth response-1
EKLF	Erythroid Krüppel-like factor
eNOS	Endothelial nitric oxide synthase
ER	Estrogen receptor
ERK	Extracellular regulated kinase
GABA	γ-aminobutyric acid
GAD	Glutamate dehydrogenase
GNAT	Gcn5-related N-acetyltransferase
GR	Glucocorticoid receptor
HAT	Histone acetyltransferase
HBO1	HAT bound to the origin of replication complex
HDAC	Histone deacetylase
HEK	Human embryonic kidney
HIF-1	Hypoxic inducible factor-1
HPV	Human papilloma virus
HRP	Horseradish peroxidase
IP	Immunoprecipitation
KLF2	Krüppel-like factor 2
MAPK	Mitogen-activated protein kinase
MEF	Mouse embryonic fibroblast
MEF2	Myocyte enhancer factor 2
MEK	Mitogen activated ERK kinase
MLL	Mixed lineage leukemia
MORF	MOZ related factor
MOZ	Monocytic leukemia zinc finger protein
MPF	M-phase promoting factor
N-terminal	Amino terminal
NaB	Sodium butyrate
NF-κB	Nuclear factor-κB
ODC	Ornithine decarboxylase

PBS	Phosphate buffered saline
PCAF	p300/CBP-associated factor
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PMSF	Phenylmethylsulphonylfluoride
PPAR	Peroxisome proliferator activated receptor
PP2A	Protein phosphatase 2A
PSA	Prostate-specific antigen
RAR	Retinoic acid receptor
RT-PCR	Reverse transcription-polymerase chain reaction
RTS	Rubinstein-Taybi syndrome
RXR	Retinoid X receptor
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SRC-1	Steroid receptor coactivator-1
SUMO	Small ubiquitin-like modifier
TBP	TATA-binding protein
TCA	Tricarboxylic acid
TEMED	Tetramethylethylenediamine
TFAP2	Transcription factor activating protein 2
VEGF	Vascular endothelial growth factor
VPA	Valproic acid
WCE	Whole cell extract

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Introduction

Post-translational modifications of histones include acetylation, ubiquitination and phosphorylation. Together with methylation and ADP-ribosylation, these modifications compose the "histone code", where different combinations of histone modifications act as epigenetic markers to regulate such cellular activities as transcription, DNA replication, repair, recombination and chromosomal segregation (Strahl and Allis, 2000; Turner, 2000). Acetylation has been the most extensively studied histone post-translational modification. It occurs in the N-terminal tail of core histones, where an acetyl group is covalently added to the ε-amino group of conserved lysine residues. Such a modification is thought to neutralize the positive charge of these lysines and reduce the interaction between the histone tails and the negatively charged DNA. This results in DNA being more accessible for transcription factors and the basal transcriptional machinery. In general, chromatin regions where histones are highly acetylated are associated with increased transcriptional activity, while hypoacetylation correlates with less active chromatin. The acetylation state of histones is governed by the opposing actions of two types of enzymes. Histone acetyltranferase (HAT) enzymes catalyze the transfer of acetyl groups from acetyl-CoA to the lysines of target substrates, while histone deacetylase (HDAC) enzymes remove the acetyl groups from these lysines.

Based on sequence homology, HATs are divided into five major groups (reviewed in Timmermann et al., 2001). The Gcn5-related N-acetyltransferases (GNATs) contain the Gcn5 and the p300/CBP-associated factor (PCAF) human enzymes. Human members of the MYST family include the monocytic leukemia zinc finger protein (MOZ), the

MOZ-related factor (MORF), Tip60 and the histone acetyltransferase bound to the origin of replication complex (HBO1). Some nuclear receptor coactivators such as the steroid receptor coactivator 1 (SRC-1) and the activator of retinoid receptor (ACTR) also possess acetyltransferase activity and represent another family of HATs. The $TAF_{II}250$ subunit of the general transcription factor TFIID complex also possesses HAT activity, although weaker than most members of the other families. Finally, the last family of HATs is composed of two proteins involved in a large array of cellular activities: p300 and the CREB-binding protein (CBP).

Transcriptional coactivators p300 and CBP

The discovery of p300 was made when an unidentified 300 kDa protein immunoprecipitated with the viral oncoprotein encoded by the early region of the adenovirus E1A (Yee et al., 1985; Harlow et al., 1986; Egan et al., 1988). Early on, this 300 kDa protein was described as a nuclear protein that could be phosphorylated, and exhibited modified migrating patterns throughout the cell cycle (Yaciuk et al., 1991). The protein was shown to be involved in DNA synthesis and also as being a part of the TATA-binding protein (TBP) complex, thus hinting toward an involvement of the protein in transcriptional activation (Howe et al., 1990; Abraham et al., 1993). Molecular cloning of p300 was done by Eckner and colleagues (1994), who characterized the three cysteine/histidine-rich regions along with the bromodomain of p300. Bromodomains are present in many transcriptional coactivators and histone acetyltransferases and are involved in the binding of acetylated lysines (Dhalluin et al., 1999; Jacobson et al, 2000). The study by Eckner et al. (1994) demonstrated that p300 could increase the activity of

an SV40 enhancer, thus adding further evidence to the involvement of p300 as a transcriptional coactivator. The coactivating function of p300 was confirmed when the protein was shown to be structurally and functionally related to the CREB-binding protein (CBP, Lundblad et al., 1995). CBP was initially identified as a coactivator due to its interaction with the PKA-phosphorylated form of CREB, a nuclear transcription factor involved in cAMP-responsive gene expression (Chrivia et al., 1993). It was found that CBP, like p300, could interact with the 12S E1A early region protein, which disrupted its coactivating function. This study also revealed that p300, like CBP, could interact with CREB and increase its transcriptional activity (Lundblad et al., 1995). Therefore, the overlapping functions of p300 and CBP revealed the closely related nature of the two transcriptional coactivators. As such, in many contexts these proteins are referred to as p300/CBP.

The intrinsic HAT activity of p300 and CBP was reported by two independent groups (Bannister and Kouzarides, 1996; Ogryzko et al., 1996). The HAT domains of p300 and CBP were shown to be located between the bromodomain and the second cysteine/histidine region, and have 91 % of sequence homology. Unlike other HATs such as the p300/CPB-associated factor (PCAF), which acetylates only histones H3 and H4, p300 and CBP exhibit acetyltransferase activity toward all nucleosomal histones (H2A, H2B, H3 and H4). This acetylation occurs at the conserved lysine residues in the N-terminal histone tails.

Since their characterization as transcriptional coactivators possessing acetyltransferase activity, p300 and CBP have been established as essential factors in many biological processes through their interactions with a wide range of transcription

factors (reviewed in Goodman and Smolik, 2000). Expression of p300 mRNA was shown to be widely distributed early during mouse embryonic development, which initially suggested a role for p300 in this process (Yao et al., 1998). This was confirmed when it was observed that p300-null embryos were not viable past embryonic day 11.5 (E11.5). In fact, 50 % of the null embryos were dead as early as E10.5. Some lethality was also observed in $p300^{+/-}$ embryos at E10.5. The null animals were significantly smaller then the wild-type mice and exhibited developmental defects of the neural tube. A major observable phenotype in the null embryos consisted of complete or partial opening of the neural tube in the mutant embryos. However, it was proposed that lethality resulted from abnormal heart development. These developmental malformations were suggested to be caused by lower cell proliferation since mesenchymal and neural crest cells from null embryos, as well as $p300^{-/-}$ mouse embryonic fibroblasts (MEFs), showed proliferation defects. Embryos null for CBP displayed similar developmental abnormalities, as well as embryonic lethality. Interestingly, the same embryonic lethality and developmental malformations were observed in p300 and cbp double heterozygous knock-out animals, suggesting that total gene dosage of p300 and CBP was critical during embryogenesis. In a more recent study, the acetyltransferase activity of p300 was found to be required for embryonic development (Shikama et al., 2003). Inactivation of the HAT domain of one allele of p300 resulted in embryonic or neonatal lethality in mice. This was also observed, albeit to a lesser extent, with inactivation of CBP HAT activity. The HAT inactivation of p300 caused malformations of the heart, including reduced cardiac muscle mass and closure defects of the atrio-ventricular septum. Severe defects in lung and small intestine formation were also observed with p300 HAT inhibition.

The requirement for CBP function in development has been highlighted in the context of the Rubinstein-Taybi syndrome (RTS). This disorder is characterized by abnormal facio-cranial development, unusually broad thumbs and big toes, as well as mental retardation (reviewed in Berry, 1987). It is quite common among mentally challenged patients, with an incidence of 1 in 300 among institutionalized cases. Interestingly, there seems to be a higher risk of tumor development in RTS patients (Miller and Rubinstein, 1995). RTS has been associated with germline single allele mutations (deletions, translocations or point mutations) of the CBP gene (Petrij et al., 1995). The resulting developmental defects are thought to be due to haploinsufficiency of CBP. Support for this claim comes from a study by Tanaka et al. (1997), which shows that CBP heterozygous mutant mice exhibit skeletal development reminiscent of RTS. A more recent study revealed that mutations occurring in the HAT domain of the CBP gene were present in patients with RTS and that this loss of acetyltransferase activity was sufficient to cause the disorder (Kalkhoven et al., 2003). Although most of the studies have associated RTS to disruption of the CBP gene, recent evidence suggests that p300 could also play a role in the disease. Indeed, mutations at the p300 gene locus were identified in RTS patients, suggesting that a loss of either p300 or CBP acetyltransferase activity is responsible for causing the disease (Roelfsema et al., 2005).

Interaction of p300 and CBP with many transcription factors involved in hematopoiesis has also highlighted the requirement for the coactivators in this system (reviewed in Blobel, 2000). The hematopoietic transcription factor c-Myb is involved in maintaining the proliferation of precursors of myeloid, erythroid and lymphoid lineages. Its levels of expression decrease along the differentiation process of mature cells.

Transcriptional activity of c-Myb is increased upon interaction with CBP and disruption of this interaction by E1A was shown to impede c-Myb activity (Blobel, 2000). GATA-1 is another important transcription factor involved in both survival of erythroid and megakaryocytic precursors and differentiation of mature cells. Both CBP and p300 were shown to enhance the transcriptional activity of GATA-1. Acetylation of GATA-1 by p300 and CBP was also demonstrated, although it is not yet clear how this affects its DNA binding affinity or transcriptional activity (Blobel, 2000). The erythroid Krüppel-like factor (EKLF) activates β-globin genes during differentiation of erythroid cells and is another substrate for CBP and p300 acetylation (Blobel, 2000). Again, although p300 and CBP increase the activity of this transcription factor, if and how acetylation affects this process is unclear. The transcriptional activities of many other important hematopoietic transcription factors such as NF-E2, C/EBPβ, members of the Ets family, and AML1 are regulated by p300 and CBP (Blobel, 2000). Although both p300 and CBP participate in the development of the hematopoietic system by binding to these numerous transcription factors, recent evidence suggests that the two proteins might also play distinct roles in hematopoiesis. Indeed, Rebel and colleagues (2002) engineered chimeric $CBP^{-/-}$;WT/WT or $p300^{-/-}$;WT/WT mice in order to study the role of CBP and p300 in hematopoietic stem cells. Their study revealed that renewal and maintenance of a pool of hematopoietic stem cells was dependent on CBP but not on p300. Conversely, p300, but not CBP, was required for the differentiation of these stem cells into different cell lineages. Hence, this suggests that rather then overlapping and redundant, the functions of p300 and CBP are complementary and act in coordination during the development of the hematopoietic system.

Development of skeletal muscle is another process in which p300, and possibly CBP, are involved. Indeed, important myogenic factors including MyoD, Myf5 and the myocyte enhancer factor 2 (MEF2) have been shown to interact with p300 (reviewed in Goodman and Smolik, 2000). It was revealed that E1A prevented activation of MyoD-responsive promoters, and that this could be countered by over-expression of p300 (Yuan et al., 1996). Experiments conducted with microinjected antibodies against p300 also revealed inhibition of MyoD transcriptional activity, along with prevention of cell differentiation (Eckner et al., 1996; Puri et al., 1997). These experiments revealed that PCAF was also involved in MyoD activity and that acetylation of MyoD by PCAF but not by p300 enhanced its DNA binding affinity and transcriptional activity. However, recent studies have reported that myogenesis was compromised when p300 lacked its acetyltransferase ability, and that activation of MyoD and Myf5 was impaired in this context (Roth et al., 2003). Also, acetylation of MEF2 by p300 increased its binding to DNA as well as its activation (Ma et al., 2005). Therefore, further investigation will be needed to clarify the molecular mechanisms of p300-regulated myogenic gene expression. Current evidence also suggests that p300 seems to have a more central role than CBP in myogenesis. Again however, more data will be required to confirm the distinct roles of each coactivator in this process.

A role for p300 and CBP in tumorigenesis was initially suggested due to targeting of the coactivators by viral oncoproteins. Interaction of the 12S early region product of the adenovirus E1A with p300 and CBP was shown to be required for E1A-induced cell transformation (reviewed in Goodman and Smolik, 2000). Although the molecular mechanisms underlying this process are not completely understood, E1A was

shown to compete with PCAF for a common binding site in the CH3 domain of p300, thus preventing the association between PCAF and p300 required for transcriptional activation of certain genes (Yang et al., 1996). Direct inhibition of the acetyltransferase activity of p300 by E1A was also demonstrated (Chakravarti et al., 1999). The large T antigen of SV40 is another oncoprotein that was shown to interact with p300 and CBP, inhibit their transcriptional activity and induce cell transformation (Yaciuk et al., 1991; Eckner et al., 1996; Avantaggiati et al., 1996). As E1A and SV40, the human papillomavirus (HPV)-encoded E6 protein interacts with p300 and CBP and disrupts binding of p53 to p300/CBP, thus impeding p53 activity (Zimmerman et al., 1999; Patel 1999). Targeting of p300 and CBP by E6 has also been demonstrated to block activation of NF-κB Patel et al., 1999).

Chromosomal translocations targeting the CBP and p300 genes have been associated with development of different types of leukemia (reviewed in Goodman and Smolik, 2000). In some cases of acute myeloid leukemia (AML), the gene encoding the MOZ protein was found fused to the N-terminal region of the CBP gene (Borrow et al., 1996). This translocation is rare but still more frequent than a similar fusion between MOZ and p300, although some cases have been reported (Kitabayashi et al., 2001; Chaffanet et al., 2000). CBP and p300 are also involved in translocations resulting in fusion of these genes with the mixed lineage leukemia (MLL) gene (Rowley et al., 1997; Sobulo et al., 1997; Ida et al., 1997). Translocations involving MLL are highly frequent in children treated for other malignancies with drugs such as etoposides (Goodman and Smolik, 2000). The mechanisms leading to CBP and p300 translocation-induced leukemia remain obscure. However, both a gain and a loss of function seem possible.

Indeed, the HAT domains of MOZ and both p300 and CBP are present in the fusion proteins. Therefore, it is conceivable that aberrant acetyltranferase activity could account for the development of the disease. The impact of acetylation on cancer development is highlighted in the context of androgen receptor (AR) activity in prostate cancer. Indeed, p300 has been shown to acetylate the AR, resulting in increased activity of the hormone receptor, which is thought to be involved in cell proliferation and progression of prostate cancer (reviewed in Fu et al., 2004). Of course, the fusion of the two proteins along with the loss, although minor, of some regions of MOZ, p300 and CBP most likely results in the inability of these proteins to interact with their respective factors and exert their normal functions.

Further evidence in support of a role for p300 and CBP in cell cycle control is the ability of the coactivator to interact with tumor suppressor proteins such as p53 or the TGF-β pathway effectors Smad and RUNX (reviewed in Iyer et al., 2004). The regulation of p53 stability and activity has been shown to involve p300 through various mechanisms. The coactivating function of p300 and CBP is required by p53 to induce the expression of genes such as p21, mdm2, cyclin G and Bax following DNA damage (reviewed in Goodman and Smolik, 2000). Acetylation of p53 by p300 was also shown to increase its DNA binding affinity and activity (Gu and Roeder, 1997). In contrast however, some data show that p300 is involved in the degradation of p53. Indeed, although it was demonstrated that E1A products inhibit p53 activity, the stability of the tumor suppressor was enhanced in cells where E1A was expressed (reviewed in Goodman and Smolik, 2000). Furthermore, it was demonstrated that the ubiquitin-dependent degradation of p53, involving the ubiquitin ligase MDM2, required binding of

p300 to the p53-MDM2 complex (Zhu et al., 2001; Grossman et al., 1998). Moreover, Grossman and colleagues reported that p300 also performed an ubiquitin ligase activity toward p53, resulting in polyubiquitination of the transcription factor (Grossman et al., 2003). Together, these observations indicate that p300, and possibly CBP, carry the dual responsibility of coactivating p53 transcriptional activity while ensuring turnover of the tumor suppressor.

Smads and RUNX are transcription factors involved in the TGF-β tumor-suppressing pathway and recruit p300 to express TGF-β-induced genes (reviewed in Iyer et al., 2004). In an oral carcinoma cell line expressing a truncated form of p300, reintroduction of the full-length protein resulted in decreased proliferation of the cells (Suganuma et al., 2002). This study showed that the observed decrease in cell proliferation was due to activation of genes responsive to the TGF-β pathway. Of note, overexpression of CBP, which is wild-type in this cell line, failed to reduce cell growth, thus indicating that CBP and p300 perform distinct functions in this context.

Although infrequent, truncations of p300 and CBP have been identified in a variety of human cancer cells and primary tumors. Indeed, mutations causing truncation of p300 proteins have been identified in ovarian, breast, colorectal, lung, cervical, pancreatic and oral squamous cancers (Muraoka et al., 1996; Gayther et al., 2000; Ozdag et al., 2002; Ohshima et al., 2001). In some of these tumors or cell lines, mutations were accompanied by loss of the wild-type allele. Because loss of heterozygosity is considered a hallmark of tumor suppressors, this evidence supports such a role for p300. Although very few CBP mutations were identified in cancer cell lines or solid tumors, the loss of heterozygosity observed in hematological malignancies in RTS patients or CBP

heterozygous mice offers additional support for a similar tumor suppressor role of CBP (Kung et al., 2000).

The versatility of p300 and CBP functions results from the ability of these coactivators to acetylate and interact with a large array of cellular factors. The numerous studies assessing the functions of p300 and CBP reveal that it can participate in cell differentiation as well as in cell proliferation. The effect of p300 and CBP seems to be highly context-dependent. Because p300 and CBP are tightly regulated and rate-limiting under physiological conditions, the abundance of p300 and CBP-dependent transcription factors might determine the effect produced by these coactivators. Although their functions have been extensively studied, the mechanisms involved in the regulation of p300 and CBP have been much less described. Hence, considering the important role of these factors in development and tumorigenesis, it seems essential to further elucidate the molecular mechanisms regulating p300 and CBP activity and stability.

Histone deacetylases and the inhibitor valproic acid

Classical HDACs are divided into two groups. Class I HDACs include HDAC 1, 2, 3 and 8, are widely expressed and have a nuclear localization, while class II is composed of HDACs 4, 5, 6, 7, 9 and 10 and are localized in both the nucleus and cytoplasm (reviewed in de Ruijter et al., 2003). HDAC activity has been shown to be essential for important biological processes, notably embryonic development. Indeed, HDAC 1, 2 and 3 were found to be highly expressed and widely distributed in the developing mouse embryo (Lagger et al., 2002). This study also showed that embryos null for HDAC1 were not viable past embryonic day 9.5. Embryonic lethality was

demonstrated to be due to a deficiency in cell proliferation resulting from increased levels of cyclin-dependent kinase inhibitors such as p21 and p27, of which gene expression have been shown to be increased upon treatment with HDAC inhibitors (Archer et al., 1998; Park et al, 2003). Hence, these results reveal that in early embryonic development, HDAC activity is required to repress gene expression of cell-cycle inhibitors. This illustrates how deacetylation of histones causes higher DNA-histone interaction resulting in decreased transcriptional activity.

Valproic acid (VPA; 2-propylpentanoic acid) is a short-chained fatty acid that has been used for over 30 years in the treatment of epilepsy to control a variety of seizures. The discovery of its anti-epileptic properties was quite serendipitous. Initially, valproic acid was used as a solvent in which other compounds were dissolved. This led to the finding by Eymard and colleagues (1962) that compounds dissolved in valproic acid produced anti-seizure effects (reviewed in Blaheta and Cinatl, 2002). Valproic acid was shown to be responsible for this action. Soon after, valproic acid was being marketed and prescribed as an anti-epileptic drug. Although it has been widely prescribed for many years, the mechanisms of the anti-seizure actions of this drug remain unclear. However, some evidence shows that valproic acid increases the levels of the inhibitory neurotransmitter GABA (γ-aminobutyric acid; reviewed in Johannessen, 2000). Release of GABA inhibits the onset of seizures, while blockage of GABAergic transmission induces convulsions (Lösher, 1989). The increase in GABA levels induced by valproic acid occurs rapidly and is accompanied by a concomitant decrease of aspartate, an excitatory amino acid (reviewed in Johannessen, 2000). The production of aspartate in GABAergic neurons occurs through the tricarboxylic acid (TCA) cycle, which

metabolizes succinic acid, a product of GABA degradation. The mechanisms by which valproic acid increases GABA levels are thought to relate to enzymes involved in its metabolic pathway (reviewed in Johannessen, 2000). Indeed, valproic acid was shown to inhibit enzymes involved in the degradation of GABA, such as GABA transaminase which converts GABA to succinate semialdehyde, a precursor of succinic acid (Lösher, 1993). Also, valproic acid increases the activity of glutamate dehydrogenase (GAD), which converts glutamate to GABA (Lösher, 1989). The effect of valproic acid on these enzymes collaterally results in decreased production of succinic acid. However, direct inhibition by valproic acid of enzymes involved in succinic acid production has also been reported in one study (Luder et al., 1990). Hence, increased GABA accompanied with decreased aspartate levels could explain how valproic acid produces its anti-convulsive effects.

Another major explanation for the anti-epileptic properties of valproic acid is its ability to inhibit voltage-gated ion channels. Studies report that valproic acid increases hyperpolarization of neuronal cell membranes due to its ability to inhibit voltage-gated sodium channels and increase potassium conductance (reviewed in Johannessen, 2000). Such inhibition results in reducing the high frequency firing of neurons observed in epilepsy. Other therapeutic purposes of valproic acid include migraine prophylaxis and treatment of bipolar disorder (Tunnicliff, 1999; Sörensen, 1988) but the mechanisms by which valproic acid produces these therapeutic effects are poorly understood. However, it has been postulated that valproic acid might mimic the effects of lithium, a drug used in the treatment of bipolar disorder. In a study by Dixon and Hokin (1997), valproic acid was shown, as lithium, to increase the release of glutamate and inositol 1, 4, 5-

triphosphate. Unfortunately, solid evidence of the mechanisms involved in the actions of valproic acid in epilepsy, bipolar disorder and migraine prevention remains unavailable.

Although valproic acid possesses valuable therapeutic properties, it is also associated with important side-effects. Generally, valproic acid produces few adverse effects but women treated with the drug in early stages of pregnancy can, in 1-2% of cases, give birth to children with spina bifida, which is a disorder characterized by incomplete closure of the neural tube (DiLiberti et al., 1984; Robert, 1988). Other defects associated with valproic acid-induced teratogenicity include heart and facial skull malformations. Such valproic acid effects have been observed in both mice and humans (Nau et al., 1991). In mice, treatment with valproic acid at E8.25 and E9.5 respectively caused incomplete closure of the anterior and posterior parts of the neural tube (Nau et al., 1991). Using valproic acid derivatives, the teratogenicity of the drug was shown to be independent of its anti-epileptic property. Indeed, no correlation was established between teratogenicity and anti-epileptic activity since teratogenic as well as non-teratogenic derivative compounds exhibited anti-epileptic properties (Nau et al., 1991). However, the teratogenicity of the valproic acid derivatives did correlate with inhibition of HDAC activity (Göttlicher et al., 2001; Phiel et al., 2001). Krämer et al. (2003) revealed that valproic acid not only inhibited the catalytic activity of HDACs, but also caused degradation of class I HDAC2 by the proteasome. Teratogenicity was also associated with the ability of these compounds to differentiate F9 mouse teratocarcinoma cells and activate the peroxisome proliferator-activated receptor δ (PPARδ; Werling et al., 2001; Lampen et al., 1999, 2001). HDAC inhibition by valproic acid was shown to result in differentiation of mouse teratocarcinoma cells as well as human colon and breast

carcinoma cells (Göttlicher et al., 2001). This was further confirmed recently by Gurvich and colleagues (2004), who demonstrated that class I and II HDACs were inhibited by valproic acid, which resulted in differentiation of hematopoietic cells. In many studies, the potential of valproic acid in reducing growth and proliferation of cells from a variety of tumor types by increasing differentiation and apoptosis was shown to be promising (reviewed in Blaheta et al., 2002, 2005). Indeed, the fate of cells from neuroblastoma, colon, breast, prostate, lymphoid, myeloid, endometrial and glioblastoma origins were shown to be affected upon exposure to valproic acid.

Some cellular pathways and factors were shown to be affected by valproic acid (reviewed in Blaheta et al., 2002, 2005). As mentioned previously, PPARδ, which is a steroid receptor that acts to regulate gene expression by forming a heterodimer with retinoid X receptors (RXR), is activated by valproic acid. Also, the phosphorylation of the extracellular-regulated kinase (ERK) is increased by valproic acid, and the DNA binding affinity and transcriptional activity of the AP-1 transcription factor, a downstream target of ERK, is enhanced by the drug. Valproic acid was also shown to inhibit other kinases, such as protein kinase C (PKC) and GSK-3β, which in the latter case results in transclocation to the nucleus of β-catenin and expression of β-catenin/Tcf/Lef-responsive genes. However, the mechanisms underlying these effects of valproic acid are not well understood, and their relevance in biological processes such as cell growth, proliferation, differentiation and apoptosis remains unclear.

Since the discovery of its deacetylase inhibition activity, a number of studies have assessed the effect of valproic acid on gene expression in the context of transformed and undifferentiated cells. Results from such studies indicate that indeed, modulation of gene

expression through valproic acid-induced HDAC inhibition is responsible for the induction of apoptosis or differentiation of cancer cells. In the Gurvich study, alteration of gene expression through inhibition of HDAC by valproic acid was shown to cause differentiation of the hematopoietic cell line U937. Indeed, using valproic acid and its analogs, it was shown that the potency of HDAC inhibition of the compounds correlated with their ability to induce cell differentiation. Upon valproic acid treatment, p21 gene expression was increased, accompanied by an increase in the differentiation marker CD11b. In cells expressing p21 antisense mRNA however, no such increase in CD11b levels was observed following exposure to valproic acid. This ability to differentiate cells was therefore associated with increased expression of the cell-cycle inhibitor p21 due to HDAC inhibition. Reduction of proliferation and induction of differentiation of human neuroblastoma cells was also observed by Rocchi and colleagues (2005) upon valproic acid treatment. This effect was accompanied by increased gene expression of p21 and another cyclin-dependent kinase inhibitor, p27. Hence, these results support a tumor-suppressor function for such cell-cycle inhibitors as targets of valproic acid. Clear evidence of the relief of transcriptional repression by valproic acid-induced HDAC inhibition was also demonstrated in MCF-7 breast cancer cells (Mongan and Gudas, 2005). Chromatin immunoprecipitation (ChIP) analysis revealed that valproic acid, in concert with retinoic acid and 5-aza-2'-deoxycytidine (Aza-dC), an inhibitor of DNA methyltransferase (DNMT), was able to increase acetylation of histone H3 at the retinoic acid receptor β2 (RARβ2) promoter. This resulted in increased expression of RARβ2 mRNA, which is a tumor suppressor known to be epigenetically down-regulated in breast cancer (reviewed in Widschwendter, 2001). In this context, valproic acid inhibited

growth and proliferation of the breast cancer cells. Clinically, a combination treatment with valproic acid and retinoic acid was recently shown to have only minor beneficial effects in patients with acute myeloid leukemia (AML; Kuendgen et al., 2005). Depending on the criteria grid utilized, the rate of positive response ranged between 5 % and 16 %. Thus, as suggested by the authors and consistent with results observed in the Widschwendter study, relief of RARβ2 repression seems to require demethylation of its promoter along with HDAC inhibition and the presence of retinoic acid.

Although valproic acid increases the expression of many genes, it was found that decreased gene expression could also occur in certain contexts after treatment with valproic acid. In MCF-7 cells, valproic acid was demonstrated to inhibit gene expression of the estrogen receptor α (ERα; Reid et al., 2005). In this study, valproic acid increased the mRNA expression of members of a distinct class of deacetylase enzymes, the NAD-dependent SIRT 2, 4 and 7, which were suggested to act as suppressors of gene expression at the ERα promoter. Another mechanism by which valproic acid can down-regulate gene expression was recently proposed by Qiao et al. (2006). In their study, valproic acid was found to decrease gene expression of adiponectin in mature adipocytes. In part, decreased DNA binding affinity of the transcription factor C/EBPα was shown to be responsible for this decrease in adiponectin mRNA levels. The mechanism by which valproic acid decreases the activity of C/EBPα is not assessed in the study, but it is suggested by the authors that by inhibiting deacetylase activity, valproic acid could accumulate C/EBPα in a hyperacetylated state and thus cause its loss of function. This claim is supported by the findings of Duan et al. (2005), which show that HDAC inhibitors can indeed inhibit C/EBPα activity by augmenting its acetylation level.

Although the effect of valproic acid on non-histone protein acetylation has not been extensively reported, the involvement of this aspect of its mode of action should always be considered. Indeed, acetylation is thought to parallel other post-translational modifications, such as phosphorylation, in the regulation of many cellular factors. Alteration of acetylation levels of transcription factors by inhibition of deacetylase activity therefore represents another mean by which valproic acid can affect gene expression. Hence, regulation of gene expression by valproic acid is proving to be much more complex than the simple model of hyperacetylation of histones resulting in increased transcriptionally active chromatin. The ability of valproic acid to reduce growth and proliferation of cancer cells through alteration of the expression of certain genes allows the extrapolation that it exerts its teratogenic properties through similar mechanisms. Some studies have revealed altered expression of genes involved in neurulation of mouse embryos upon exposure to valproic acid. These studies showed that many genes, such as p53, bcl-2, c-fos, c-jun, creb, Emx-1, Emx-2, gadd45b, ier5, per1, phfl3, pou3f1, and sox4 were up-regulated, while decreased expression of the ccne2, ccnl, gas5, egr2, sirt1, and zfp105 genes was observed (Okada et al., 2005; Wlodarczyk et al., 1996). These results, however, are correlative; whether and how these genes are involved in the neural tube defects observed upon valproic acid treatment is still unknown. Many of the molecular events involved in the teratogenic and anti-cancer effects of valproic acid remain to be elucidated. Considering available data, alteration of gene expression through HDAC inhibition by valproic acid offers a mechanism by which this drug might exerts its teratogenic and differentiating activities.

Protein phosphatase 2A and regulatory subunits

As mentioned previously, post-translational modification by phosphorylation is an important regulatory mechanism for many proteins. Phosphorylation is involved in controlling the function and stability of many cellular factors, from cytoplasmic signal transduction messengers to nuclear transcription factors. As with acetylases and deacetylases in the case of acetylation, the level of phosphorylation of a given protein is the result of the opposing actions of two types of enzymes: kinases and phosphatases. While kinases catalyze the transfer of a phosphate group to a substrate, phosphatases hydrolyze a phosphate group from a given site, resulting in dephosphorylarion of the substrate. Depending on the substrate being phosphorylated and the site of phosphorylation, this post-translational modification exerts different effects. Indeed, some proteins are activated and stabilized by phosphorylation, while others are inactivated and targeted for degradation when phosphorylated. On proteins, reversible phosphorylation (dynamic action of phosphorylation/dephosphorylation) can occur at tyrosine, serine and threonine residues. In general, a given kinase or phosphatase is specific for tyrosine or serine/threonine residues.

One of the major serine/threonine phosphatase is protein phosphatase 2A (PP2A). PP2A is a heterotrimeric enzyme composed of a 65 kD structural subunit, a 36 kD catalytic subunit, and a variable B regulatory subunit (for extensive review see Janssens and Goris, 2001). The structural (also known as A or PR65) and catalytic subunits each have two isoforms encoded by distinct genes. Both isoforms of the structural subunit, PR65α and PR65β, have 86 % sequence identity and are widely distributed. Sequence homology of the catalytic subunit isoforms α and β is even greater with 97 %, and is also

highly conserved across organisms from yeast to humans. The variable regulatory B subunit is responsible for conferring substrate specificity and subcellular localization to the PP2A holoenzyme. A number of B subunit families (B55/B, B56/B', B'' and B''') among which exists no homology have been described (Janssens and Goris, 2001). The B subunits are very diverse and each family is comprised of different isoforms, which in turn can be alternatively spliced into different variants. The family of the B55/B regulatory subunits is composed of the B55α, β, γ and δ isoforms. While B55α and δ are widely distributed in different tissues, B55β and γ are highly expressed in neurons. B55α is present in both the nucleus and cytoplasm, B55β is restricted to the cytoplasm and B55γ was demonstrated to be associated with the cytoskeleton. The B56 subunit family contains five distinct isoforms (α, β, γ, δ and ε). There exists two different B56β isoforms, B56β1 and B56β2, and the gene encoding the B56γ isoform is alternatively spliced, giving rise to three different products, B56γ1, B56γ2 and B56γ3 (McCright et al., 1995). The B56γ1 isoform exhibits both nuclear and cytoplasmic staining, while B56γ2 and B56γ3 isoforms have nuclear localization, with the B56γ3 isoform containing a nuclear localization signal in its carboxyl-terminal region (Tehrani et al., 1996; McCright et al., 1996). The sequences of the B56γ2 and B56γ3 isoforms are identical, with the exception of a 39-amino acid exon present in B56γ3 but absent in B56γ2 (Tehrani et al., 1996). B56α and all three B56γ isoforms are widely distributed but highly expressed in the heart and skeletal muscles, whereas B56β and B56δ are abundant in the brain (Tehrani et al., 1996; Janssens and Goris, 2001). The B'' family of regulatory subunits contains the PR72, PR130, PR59 and PR48 members (Janssens and Goris, 2001). It is thought that the PR72 and PR130 subunits result from alternative splicing of a single

gene. PR72 is exclusively expressed in heart and skeletal muscle while PR130 is widely distributed, although highest expression was detected in heart and skeletal muscle. PR59 is expressed in testis, kidneys, liver, brain, heart and lungs, but excluded from skeletal muscle. Both PR59 and PR48 have been shown to be involved in arrest of cell cycle in G1. Although the diversity of serine/threonine protein phosphatases is much more restricted than the abundance of different kinases, it is thought that different combinations of structural, catalytic and regulatory subunits allow specificity for substrate recognition. Indeed, considering the variety among the isoforms of the PP2A subunits, over 70 different PP2A heterotrimers can theoretically be formed.

PP2A is involved in a large number of biological activities including metabolism, transcription, translation, RNA splicing, DNA replication, development, morphogenesis, transformation and regulation of cell cycle (reviewed in Janssens and Goris, 2001; Schönthal, 2001). Attribution to PP2A of such functions was often observed following inhibition of its catalytic activity by okadaic acid. This PP2A inhibitor has proven helpful in establishing the functions of PP2A. Strong evidence supports an important role for PP2A in controlling cell cycle progression. The M-phase-promoting factor (MPF) is involved in allowing G2/M transition of the cell cycle. It is a complex composed of the cyclin-dependent kinase Cdc2 and cyclin B. In its inactive state, MPF is phosphorylated at threonine residues 14, 15 and 161 on Cdc2. Activation of the complex and progression to M phase occurs when MPF is activated by dephosphorylation of threonine 14 and 15 by the phosphatase Cdc25 (reviewed in Dunphy, 1994). PP2A acts at different steps of this pathway to block activation of MPF. First, the activating phosphorylation of threonine 161 on Cdc2 is prevented by dephosphorylation by PP2A of the kinase

responsible for phosphorylating this site. Also, PP2A is thought to dephoshorylate, and in this case activate, the kinase responsible for phosphorylation of threonine 15 on Cdc2, therefore maintaining MPF in its inactive state. Furthermore, dephosphorylation by PP2A of the phosphatase Cdc25 prevents dephosphorylation of threonine 14 and 15 on Cdc2 which again maintains MPF in its inactive form (reviewed in Janssens and Goris, 2001).

PP2A was also suggested to inhibit expression of growth-factor-induced genes such as c-jun and c-fos. Dephosphorylation of kinases in the mitogen-activated protein (MAP) kinase pathway is thought to be the mechanism by which PP2A produces this effect (Schöntal, 1995; Millward et al., 1999). Further evidence supporting this function of PP2A came with the finding that the enzyme was a target of viral oncoproteins such as the SV40 small t antigen (reviewed in Janssens and Goris, 2001). This was shown to displace the regulatory B subunit from the core enzyme and thus reduce PP2A activity. Consequently, kinases such as PKC, PKA, ERK and MEK are activated, which results in phosphorylation and activation of transcription factor such as CREB, NF-κB and AP-1 (reviewed in Janssens and Goris, 2001). Taken together, these observations suggest that PP2A is involved in inhibiting cell cycle progression, which could possibly render PP2A important in tumor suppression. Consistently, mutations of the structural Aβ subunit were identified in many types of human cancers. Indeed, Wang and colleagues (1998) found mutations in this gene in primary lung tumors (15 %), lung cancer cell lines (6 %) and colorectal cancers (15 %). These findings strongly supported a tumor-suppressor function for PP2A.

However, contrasting results suggest that PP2A is also required for cell growth and proliferation. Indeed, mice lacking the α catalytic subunit of PP2A are unable to develop a mesodermal germ layer and die during embryonic development (Götz et al., 1998). Also, PP2A was demonstrated to be involved in DNA replication. Its activity was shown by Lin and colleagues (1998) to be required in the initiation of elongation at replication origins. The B regulatory subunit PR48 was also shown to be involved in the pre-replication complex through an interaction with the cyclin-dependent kinase Cdc6 (Yan et al., 2000). Interestingly, and contrasting with the previously mentioned role of PP2A in blocking cell cycle progression at the G2/M transition, the phosphatase was demonstrated to be required for passage of cells through the G1 phase of the cell cycle (Yan and Mumby, 1999; Schönthal and Feramisco, 1993). These studies showed that without PP2A, expression of cyclins such as cyclin D2, cyclin E and cyclin A was prevented and cell cycle was arrested at G1. Hence, there is existing evidence both for progression of cell proliferation and inhibition of cell cycle as functions of PP2A. It is suggested that different B regulatory subunits composing the PP2A heterotrimer might account for the observed opposing effects of the phosphatase.

The B56γ subunit of PP2A has been suggested to play a role in preventing cell transformation and metastasis. B56γ1 was shown by Ito and colleagues (2000) to be involved in preventing metastasis in mouse melanoma tumor cells. This study revealed that by interacting with and dephosphorylating the focal adhesion regulatory protein paxillin, the B56γ1-containing PP2A reduced the metastatic potential of these cells. The expression of a truncated form of B56γ1 protein, unable to allow dephosphorylation of paxillin by PP2A, resulted in increased metastasis of cells. Furthermore, the B56γ3

subunit of PP2A was shown to be involved in transformation of human embryonic kidney (HEK) cells (Chen et al., 2004; Moreno et al., 2004). In these studies, B56γ knock-down using B56γ3 anti-sense RNA favored cell transformation, while over-expression of B56γ3 resulted in reduced cell growth and proliferation.

Ubiquitin and the 26S proteasome pathway

The 26S proteasome is a massive 2.5 MDa protein complex composed of a core proteolytic 20S particle and two 19S regulatory particles capping the 20S complex (reviewed in Voges et al., 1999). While the 19S regulatory particle is involved in substrate recognition and unfolding in an ATP-dependent manner, the 20S core particle is responsible for hydrolysis of the peptide bonds of the substrate protein through an ATP-independent mechanism. Prior to degradation by the proteasome, target proteins are covalently conjugated to chains of small 76-amino acid ubiquitin moieties following a series of enzymatic reactions (Hershko et al., 1980; reviewed in Ciechanover, 1994). The first step in this process involves the activation of ubiquitin in an ATP-dependent manner by an ubiquitin-activating enzyme (E1). Subsequently, ubiquitin is transferred to an ubiquitin-conjugating enzyme (E2), generally followed by an ubiquitin ligating-enzyme (E3) which covalently attaches ubiquitin to a lysine residue on a target substrate. Following ligation of this ubiquitin monomer to the substrate, ubiquitin moieties are added to form a chain of polyubiquitin on the protein, targeting it for degradation through the proteasome. It is suggested that an additional enzyme, E4, is involved in the polyubiquitination step (Koegl et al., 1999). These ubiquitinated proteins are then recognized by the 19S complex of the proteasome, which unfolds the proteins and

removes their ubiquitin chains, followed by their subsequent breakdown in the 20S core particle (reviewed in Voges et al., 1999). Although the subunits of the 19S regulatory particle recognize polyubiquitinated proteins, other non-proteasomal chaperone and shuttle proteins have been shown to be involved in proteasomal recognition of polyubiquitinated proteins. Some of these proteins contain UBA and UBL domains, the former being responsible for interaction with polyubiquitin chains, and the latter delivering the tagged substrate to the proteasome by interacting with the 19S particle (reviewed in Miller and Gordon, 2005).

Although degradation through the 26S proteasome generally involves prior ubiquitination of the substrate, proteasomal degradation of proteins in an ubiquitin-independent manner has also been reported. Initial evidence of such a process came from studies on ornithine decarboxylase (ODC), an important enzyme in the biosynthesis of polyamines. The turn-over rate of ODC is very rapid, is increased in response to elevated polyamine levels, and the enzyme is degraded through the proteasome independently of ubiquitin (Murakami et al., 1992). Instead, its proteasomal degradation is dependent on its interaction with the protein antizyme (Az), of which the levels are increased by polyamines. This induces a conformational change of the carboxyl terminal region of ODC which allows proteasomal recognition of the ODC-Az complex (reviewed in Pickart, 1997). Other proteins have also been demonstrated to be degraded by the proteasome through ubiquitin-independent mechanisms (reviewed in Pickart, 1997 and in Orlowski and Wilk, 2003). Although the signal-induced proteasomal degradation of IκBα was shown to require ubiquitination, its basal turn-over however, was revealed to occur without prior ubiquitination (Krappman et al., 1996). The transcription factor c-

Jun can also be degraded through the proteasome in an ubiquitin-independent fashion, as purified c-Jun was shown to be a substrate of purified 26S proteasome (Jariel-Encontre et al., 1995; reviewed in Pickart, 1997 and in Orlowski and Wilk, 2003). Similarly, p21 is subject to proteasomal degradation both through ubiquitin-dependent and independent pathways. Indeed, ubiquitination of p21 was observed prior to its proteasomal degradation, but mutation of all lysine residues in p21 did not prevent its degradation, which was in turn blocked by proteasome inhibitors (reviewed in Pickart, 1997 and in Orlowski and Wilk, 2003). Ubiquitin-independent degradation by the 26S proteasome has also been reported for calmodulin, troponin and p53 (reviewed in Pickart, 1997 and in Orlowski and Wilk, 2003). Therefore, although degradation through the 26S proteasome pathway generally depends on prior ubiquitination of the target protein, proteasomal degradation must not be systematically associated with ubiquitination. Conversely, polyubiquitinated substrates are not necessarily targeted to the proteasome, since polyubiquitination can also target proteins for lysosomal or autophagic degradation (Klionsky et al., 2000; Bjorkoy et al., 2005).

Initially thought to be a major degradation pathway only for misfolded and non-functional proteins, the 26S proteasome is now also known to play an important role in many cellular processes by ensuring turnover of functional proteins. Tightly controlled activity of the proteasome is essential for proper cellular function, as aberrant degradation of proteins involved in cell cycle regulation could result in tumorigenesis. As such, the proteasome has been shown to be highly expressed in leukemia cells. These findings are not surprising, considering the numerous proteins of which the stability is regulated by degradation through the 26S proteasome. Proteins involved in cell cycle control,

transformation and apoptosis such as p53, Rb, p21, MDM2, Bax, IκB and E2F have all been demonstrated to be regulated by proteasome function (reviewed in Zavrski et al., 2005). IκB inhibits the nuclear translocation of the transcription factor NF-κB, which is involved in transcriptional activation of genes involved in oncogenesis. This nuclear translocation of NF-κB occurs after ubiquitination and degradation of IκB by the proteasome (reviewed in Zavrski et al., 2005). Therefore, this represents a mechanism where aberrant proteasome activity can contribute to tumorigenesis. Consistent with this idea is the effect of proteasome inhibitors on cancer cells and solid tumors. Indeed, regression of many types of cancer cell lines and tumor-derived cells has been observed following inhibition of the proteasome. Furthermore, the therapeutic value of proteasome inhibitors has been, and continues to be evaluated in phase I, II and III clinical trials (reviewed in Zavrski et al., 2005).

The present study has examined the effect of valproic acid on the stability of the transcriptional coactivators p300 and CBP. Currently, little is known about the mechanisms involved in the regulation of these proteins. However, some evidence suggests that the stability of these coactivators is affected by HDAC inhibition. The objective of this present study was to verify if the HDAC inhibitor valproic acid increased p300 and CBP degradation and if so, to elucidate the molecular mechanisms underlying this valproic acid effect. Because of the clinical use of valproic acid, accompanied by its teratogenic properties, the effect of this drug on p300 and CBP stability could possibly encompass relevant biological implications. Our results show that valproic acid increases the degradation of p300 and CBP through the 26S proteasome

pathway and suggest that this degradation is ubiquitin-dependent. These experiments also propose that this valproic-acid induced proteasomal degradation is mediated by the B56γ3 regulatory subunit of protein phosphatase 2A.

Material and Methods

Cell culture and reagents

Human cervical carcinoma cells HeLa were obtained from American Type Cell Culture (ATCC). Human squamous oral carcinoma cells HOC313 were a gift from Dr. Masa-Aki Ikeda. Human colorectal carcinoma cells HCT116 were a gift from Dr. Bruce McKay. HeLa and HOC313 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Invitrogen) containing L-glutamine, supplemented with non-essential amino acids, penicillin/streptomycin and 10% fetal bovine serum. HCT116 cells were maintained in McCoy's 5A modified medium (GIBCO, Invitrogen) also containing L-glutamine and supplemented with non-essential amino acids, penicillin/streptomycin and 10 % fetal bovine serum. Cells were incubated in 5 % CO_2 at 37 °C. Valproic acid and MG132 were obtained from Sigma-Aldrich. Anti-p300 N15 antibody (used for Western and IP) is a rabbit polyclonal antibody raised against the amino-terminal region of p300. Anti-p300 H-272 antibody (used for immunofluorescence microscopy) is a rabbit polyclonal antibody raised against amino acids 774-1045 of p300. Anti-CBP A-22 antibody (used for Western, IP and immunofluorescence microscopy) is a rabbit polyclonal antibody raised against the amino-terminal region of CBP. Anti-ubiquitin P4D1 antibody is a mouse monoclonal antibody (used for Western and immunofluorescence microscopy). N15, H-272, A-22 and P4D1 were obtained from Santa Cruz Biotechnology. Anti-B56 M-880 antibody is a non-commercial polyclonal antibody (used for Western and immunofluorescence microscopy) given by Dr. Craig Kamibayashi. Antibodies against EGR-1, SRC and

RARβ were obtained from Santa Cruz Biotechnology. Hoechst-33258, goat anti-rabbit Alexa Fluor® 488 and goat anti-mouse Alexa Fluor® 555 fluorescent secondary antibodies for microscopy were obtained from Molecular Probes. HRP-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies for Western blotting were also obtained from Molecular probes.

Western and Immunoprecipitation

Cells were grown in 6 cm tissue culture plates. Following treatment, cells were harvested in PBS. Whole cell protein extraction was done at 4 °C for 30-40 minutes in whole cell extract (WCE) buffer (10 % glycerol, 50 mM Tris-HCl pH 7.6, 400 mM NaCl, 5 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 % NP-40). Protein concentration was quantified with the Bradford method. 1 µl of WCE was added to 1 ml of protein dye reagent (Bio-Rad). As a standard, different quantities of bovine serum albumin (BSA) were added to 1 ml of Bradford dye. Optical density (OD) of samples was measured at 595 nm with the Multiskan Spectrum spectrophotometer (Thermo) and protein concentration was determined according to the OD from the standard curve. Equal amounts of proteins were added to Laemmli reducing buffer (25 % glycerol, 125 mM Tris-HCl pH 6.8, 4 % SDS, 700 mM β-mercaptoethanol, bromophenol blue) and migrated by electrophoresis on an SDS-polyacrylamide gel (375 mM Tris-HCl pH 8.8, 0.1 % SDS, 0.05 % APS, 0.05 % TEMED) containing 6 or 8 % acrylamide/bis-acrylamide, depending on the protein of interest. The proteins were transferred overnight to an Immobilon-P membrane (Bio-Rad) and incubated overnight at 4 °C in the proper antibody diluted in PBS containing 1% of non-fat powdered milk. The membrane was

incubated at room temperature for one hour in the proper secondary antibody diluted in PBS containing 1% of non-fat powdered milk. The Western Lightning™ chemiluminescence reagents kit (Perkin Elmer) was used for signal detection. For loading control, membranes were stripped 30 minutes at 60 °C in stripping buffer (2 % SDS, 62.5 mM Tris-HCl pH 6.7, 100 mM β-mercaptoethanol) and reprobed with the appropriate antibody. Quantification of Western blots was performed using Scion Image software.

For immunoprecipitation (IP), cells were grown in 10 cm tissue culture plates. Cell harvest, whole cell protein extraction, and protein concentration were performed as previously mentioned. IP samples were diluted with dilution buffer (10 % glycerol, 20 mM Tris-HCl pH 8.0, 0.5 EDTA, 1 mM DTT, 1 mM PMSF, and 10 μg/ml BSA) to reduce NaCl concentration to 150 mM. Samples were precleared for one hour at 4 °C with protein A sepharose beads (Sigma-Aldrich) without antibody. IP was conducted for 2.5 hours at 4 °C with protein A sepharose beads and the proper antibody. Beads were washed (10 % glycerol, 100 mM NaCl, 20 mM Tris-HCl pH 8.0, 0.5 mM EDTA pH 8.0, 1 mM DTT, 1 mM PMSF, 0.1 % NP-40) prior to addition of Laemmli reducing buffer. Samples were heated 5 minutes at 90 °C, vortexed and heated another 5 minutes at 90 °C. Samples were migrated by SDS-PAGE on an 8 % gel. Protein transfer, membrane incubation, signal detection, quantification, stripping and reprobing were performed as described previously.

Reverse Transcription and Polymerase Chain Reaction

Cells were grown in 6-well tissue culture vessels. Following treatment, total RNA was isolated with the RNeasy Mini kit (Qiagen) according to the manufacturer's protocol. RNA concentration and quality was measured using the Multiskan Spectrum spectrophotometer (Thermo). For quantitative RT-PCR, 1 μg of RNA was reverse transcribed with 200 units of SuperScript™ II reverse transcriptase according to the manufacturer's protocol (Invitrogen) in a 20 μl final volume. 1 μl of cDNA was used for the PCR reaction performed with 2.5 units of Taq DNA polymerase according to manufacturer's protocol (Invitrogen). The number of PCR cycles to obtain optimal detection within a linear range was found to be 30 following testing of 20, 25, 28 and 30 cycles. One pair of primers (F-GCTATTTGATGACTG TACACAAC, R-TCCTTCTTCGGATCTTTCTGTG, Qiagen) was designed to amplify both B56γ2 and B56γ3, giving rise to amplicons of 140 and 257 bp respectively. The p300 primer pair (F-GATCTTCGAAATCATCTTGTTCACA, R-CATACATGTCCCCT TCAACTTTCC, Qiagen) produced an amplicon of 129 bp. PCR reactions were multiplexed with primers for the 18S ribosomal RNA housekeeping gene as an internal standard (Ambion), resulting in a 489 bp PCR product. PCR products were migrated by electrophoresis on a 1.5 % agarose gel in TAE buffer stained with Vistra Green™ DNA dye (1: 10 000, Amersham Biosciences). Detection was done with the Typhoon 8600 Variable Mode Imager instrument (Molecular Dynamics) and quantification was performed with Scion Image software (Scion).

For Real-Time PCR, RNA isolation was performed as described previously. 5 μg of RNA was reverse transcribed with 200 units of Multiscribe reverse transcriptase

(Applied Biosystems) in a 100 µl total volume reaction according to the manufacturer's protocol. Gene expression assay kits with Taqman® probes were used for p300, CBP and 18S single-primer pair reactions (Applied Biosystems) with 5 µl of cDNA input. The cDNA for the 18S rRNA endogenous control PCR reactions was diluted 1/10 000 to reach similar amplification range as the target genes. 40-cycle PCR reactions were conducted with the 7500 Real-Time PCR System (Applied Biosystems) and results were analyzed with the Sequence Detection System software (Applied Biosystems).

Immunofluorescence and Apotome Microscopy

Cells were grown on coverslips in 6-well tissue culture vessels. Following treatment, cells were fixed 15 minutes on ice with 4 % paraformaldehyde (in PBS) and rendered permeable with 0.2 % NP-40 (in water) for 15 minutes at room temperature. Coverslips were incubated with appropriate antibodies in PBS with 10 % of non-fat powdered milk and 0.2 % Tween-20 overnight at 4 °C. Incubation of coverslips with the appropriate fluorescent secondary antibodies in PBS with 10 % of non-fat powdered milk and 0.2 % Tween-20 was conducted for 45 minutes at room temperature. When indicated, coverslips were incubated for 10 minutes in PBS with 0.5 µg/ml of Hoechst DNA stain. Coverslips were mounted on slides with 90% glycerol for microscopy. Microscopy was performed with the Zeiss Axiovert 200M instrument with Apotome technology. Optical sectioning of cells was performed with increments of 0.2 µm. Cells were observed through a Zeiss 63X oil objective and image acquisition was done with the AxioCam HRm monochrome camera. Images captured through different fluorescence

filters (488001, 488010 and 488015) were processed and merged by the Zeiss AxioVision 4.3 software.

Results

Valproic acid increases p300 degradation via the 26S proteasome pathway

The human cervical carcinoma cell line HeLa has been extensively used in the study of p300. This cell line has proven useful in the initial characterization of some properties of p300, such as its altered migrating pattern during cell cycle due to its phosphorylation state, its nuclear localization and its close relationship to DNA (Yaciuk and Moran, 1991; Rikitake and Moran, 1992). The use of HeLa cells was also involved in other major findings describing the functions of p300 such as its presence in the TATA-binding protein complex and in the RNA polymerase II holoenzyme (Abraham et al., 1993; Cho et al., 1998). HeLa cells have been shown to express a full-length p300 (Ohshima et al., 2001; Suganuma et al., 2002). In studying the impact of histone acetylation on glucocorticoid receptor (GR) activity in HeLa cells, it was revealed that the HDAC inhibitor sodium butyrate (NaB) inhibited GR-mediated transcriptional activation by inducing degradation of p300 through the 26S proteasome pathway (Li et al., 2002). The present study initially set out to examine the effect of the HDAC inhibitor valproic acid on p300 stability. HeLa cells were treated with 2 mM of valproic acid, concentration at which valproic acid was shown to inhibit HDAC activity (Gottlicher et al., 2001; Kramer et al., 2003; Phiel et al., 2001). Western analysis, using a polyclonal p300 antibody, revealed that p300 levels remained unchanged following 8 hours of treatment with valproic acid (Figure 1A and 1B). However, p300 levels were decreased by 68 % following 16 hours of valproic acid treatment (Figure 1A and 1B). In order to verify if this observed decrease in protein levels was due to degradation through the 26S

proteasome, cells were treated with the 26S proteasome inhibitor MG132. Also known as Cbz-leu-leu-leucinal, MG132 is a peptide aldehyde which reversibly inhibits the 26S proteasome function by acting as a substrate analogue of the proteasome and inhibiting the transition state of the chymotrypsin-like activity of the 20S particle (reviewed in Lee and Goldberg, 1998). It is a highly potent inhibitor of proteolysis in cultured cells, with an IC_{50} in the micromolar range (reviewed in Lee and Goldberg, 1998). Results of a 16-hour treatment with 5 μM of MG132 show that p300 protein levels were accumulated to 72 % above control levels in HeLa cells (Figure 1A and 1B). Moreover, p300 protein levels were also increased by 63 % following 16 hours of co-treatment with valproic acid and MG132 (Figure 1A and 1B). Therefore, with inhibition of the 26S proteasome, valproic acid was unable to cause a decrease in p300 protein levels, suggesting that the observed decrease in p300 levels caused by valproic acid is dependent on intact proteasome function. Following the analysis of protein levels upon valproic acid and MG132 treatment, investigation into the levels of gene expression of p300 was done to confirm that the observed decrease of p300 protein was due to degradation rather then a decrease in transcript production. Quantitative RT-PCR was therefore performed on HeLa cells after treatment with valproic acid and MG132. Total RNA was extracted from the cells, was reverse transcribed and subjected to PCR for amplification of a 129-base pair sequence specific to the junction of exons 8 and 9 of p300 mRNA. The 18S ribosomal RNA housekeeping gene was used as an endogenous control. Results from this assay show that neither valproic acid nor MG132 significantly affected p300 transcription after 16 hours of treatment (Figure 1C and 1D). Taken together, these

results show that the decrease in p300 protein levels upon valproic acid treatment is due to increased degradation of the protein through the 26S proteasome pathway.

Figure 1. Degradation of p300 through the 26S proteasome pathway. (A) Western analysis of p300 levels in HeLa cells following 8 and 16 hours of treatment with the histone deacetylase inhibitor valproic acid (2 mM) and/or the proteasome inhibitor MG132 (5 μM). 50 μg of total protein extracts were migrated on 6 % SDS-PAGE. An anti-SRC-1 antibody was used as loading control. **(B)** Quantification of Western results from (A) using Scion Image software. Results represent p300 fold variation of treated samples compared to control. Experiment was repeated three times in duplicates. Error bars represent standard deviations. Unpaired T-tests were performed for statistical analysis. For 16-hour treatments, $p \leq 0.05$ for VPA-treated samples compared to control, $p \leq 0.005$ for MG132-treated samples compared to control, $p \leq 0.01$ for VPA-treated samples compared to MG132+VPA-treated samples. **(C)** PCR products on agarose gel showing p300 gene expression in HeLa cells following 16 hours of treatment with valproic acid (2 mM) or MG132 (5 μM). Total RNA was isolated, reverse transcribed and amplified by PCR in multiplexed reactions with p300 and endogenous control 18S rRNA housekeeping gene primers. **(D)** Quantification of RT-PCR results of (C). PCR products were quantified using the ImageQuant software. Results show average fold variation of p300 mRNA levels from treated cells compared to control samples. Error bars represent standard deviations from five experiments performed with duplicates.

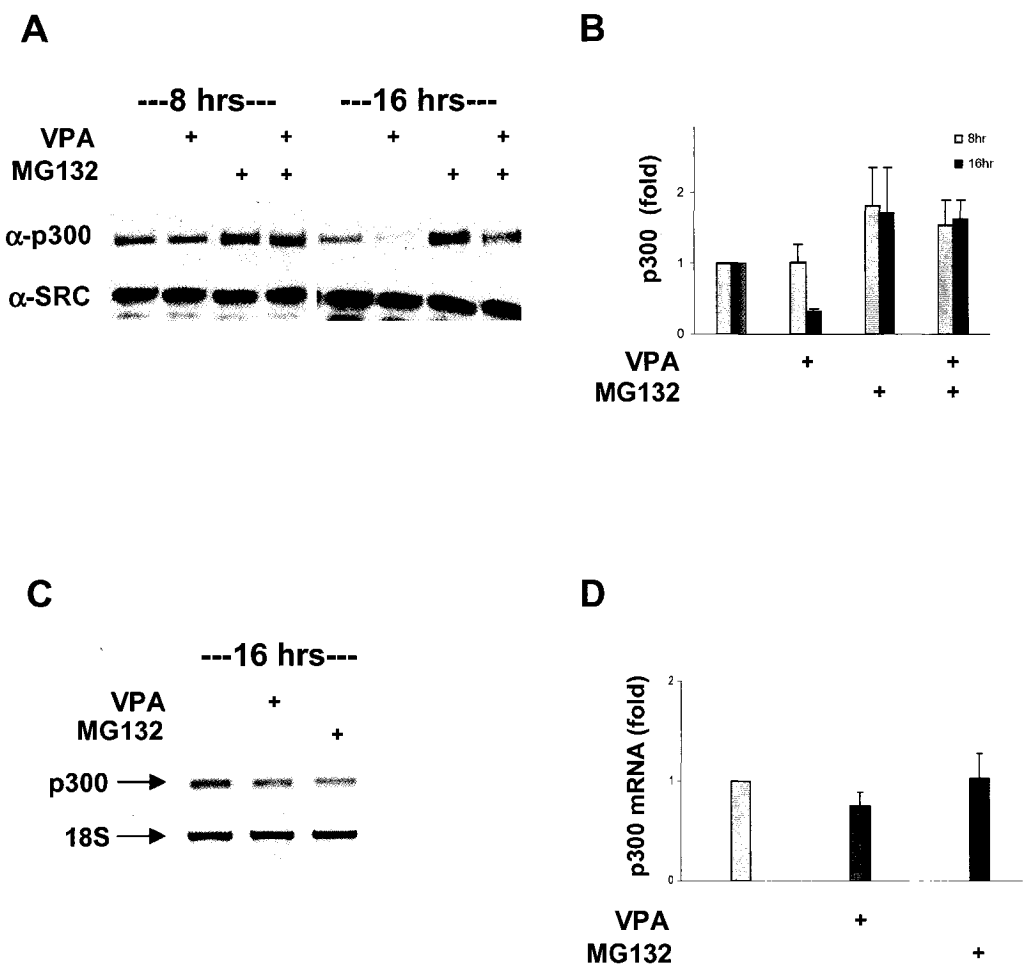

A
---8 hrs---
---16 hrs---
VPA
MG132
α-p300
α-SRC
B
p300 (fold)
8hr
16hr
VPA
MG132
C
---16 hrs---
VPA
MG132
p300
18S
D
p300 mRNA (fold)
VPA
MG132

Figure 1.

Increased expression of the B56γ regulatory subunit of PP2A by valproic acid

As previously mentioned the current model describing the impact of HDAC inhibition on gene transcription states that inhibitors of these enzymes accumulate histones in a hyperacetylated form. This results in a decreased interaction between DNA and nucleosomal histones, which renders chromatin more accessible to transcription factors and the basic transcriptional machinery (Wolffe et al., 2000). Thus, the transcription of any gene whose promoter region is located around nucleosomes could potentially be affected by HDAC inhibitors such as valproic acid. However, this model does not seem to apply to the effect of valproic acid on p300. Indeed, rather than increasing p300 protein levels through increased gene expression, valproic acid decreased p300 protein levels through degradation, without affecting the expression pattern of its gene. Also, the effect of valproic acid was only observed after 16 hours of treatment. Considering these facts, it was possible that valproic acid produced its effect through an indirect mechanism. This hypothesis would imply that valproic acid could increase p300 degradation by increasing the levels of one of its negative regulators. In order to identify p300-relevant potential target genes of valproic acid, microarray analysis was performed with HeLa cDNA (Chen et al., 2005). This analysis revealed that 2 isoforms of the B56γ regulatory subunit of PP2A, B56γ2 and B56γ3 were up-regulated by valproic acid (Chen et al., 2005). To confirm these findings, mRNA levels of both B56γ2 and B56γ3 isoforms were measured by quantitative RT-PCR performed on HeLa cells treated with valproic acid or MG132. As described, the sequences of B56γ2 and B56γ3 are practically identical, with the important exception of a sequence of 117 bp corresponding to exon 13 of the B56γ3 isoform which, through alternative splicing, is absent in the B56γ2 isoform.

Therefore, by designing primers which border exon 13 of the B56γ3 mRNA, both isoforms were amplified in a single PCR reaction with a single primer pair. This PCR reaction produced amplicons of respectively 140 and 257 base pairs for B56γ2 and B56γ3. In this manner and due to different migrating patterns of both amplicons, distinction between B56γ2 and B56γ3 mRNA levels was made possible. Quantification of both isoforms revealed that indeed, the mRNA of B56γ2 and B56γ3 subunits of PP2A were up-regulated respectively by 90 and 60 % in HeLa cells following valproic acid treatment (Figure 2A and 2B). This increase was observed after 8 hours of treatment and was maintained following 16 hours of exposure to valproic acid. Western analysis using a polyclonal antibody against B56γ2 and B56γ3 isoforms was performed to verify that the increase in gene expression was translated to increased protein levels. Migration of whole cell protein extracts on an 8 % SDS-polyacrylamide gel allowed an appropriate resolution of the two distinct isoforms (Figure 2C). An increase in protein levels of B56γ3 subunit was detected after 16 hours of valproic acid treatment (Figure 2C). However, an increase in B56γ2 was not detected, which is consistent with previous results which suggest that the levels of this subunit are tightly regulated in HeLa cells (Figure 2C; Chen et al., 2005).

Data from our laboratory have shown that over-expression of the B56γ3 subunit decreased p300 protein levels in a dose-dependent manner and that knock-down of this subunit using siRNA resulted in increased p300 levels (Chen et al., 2005). Also, B56γ3 was shown to interact with the C-terminal region of p300. Taken together, these results suggest that the effect of valproic acid is mediated through the B56γ3 regulatory subunit

of PP2A. Moreover, these findings suggest that the C-terminal end of p300 is involved in the valproic acid-induced p300 degradation.

Figure 2. Valproic acid up-regulates the B56γ regulatory subunit of protein phosphatase 2A. (A) Agarose gel of PCR products representing gene expression of the B56γ2 and B56γ3 subunits. HeLa cells were treated for indicated time periods with valproic acid (2 mM). Total RNA was isolated, reverse transcribed and amplified by PCR in a multiplex reaction with primers for the B56γ2/B56γ3 and 18S rRNA transcripts. **(B)** Quantification of results from (A) was done using ImageQuant software. Results represent fold variation of B56γ2 and B56γ3 mRNA from treated cells compared to untreated cells. Average values from at least three independent experiments in duplicates are shown. Error bars show standard deviations of average values. Unpaired T-tests were performed for statistical analysis. At 8 hours of treatment, $p \leq 0.01$ for VPA-treated samples compared to control for B56γ2, and $p \leq 0.05$ for VPA-treated samples compared to control for B56γ3. **(C)** Western analysis shows levels of B56γ2 and B56γ3 in Hela cells following treatment with valproic acid (2 mM). 50 μg of whole cell proteins were migrated by 8 % SDS-PAGE. Anti-RARβ antibody was used for loading control.

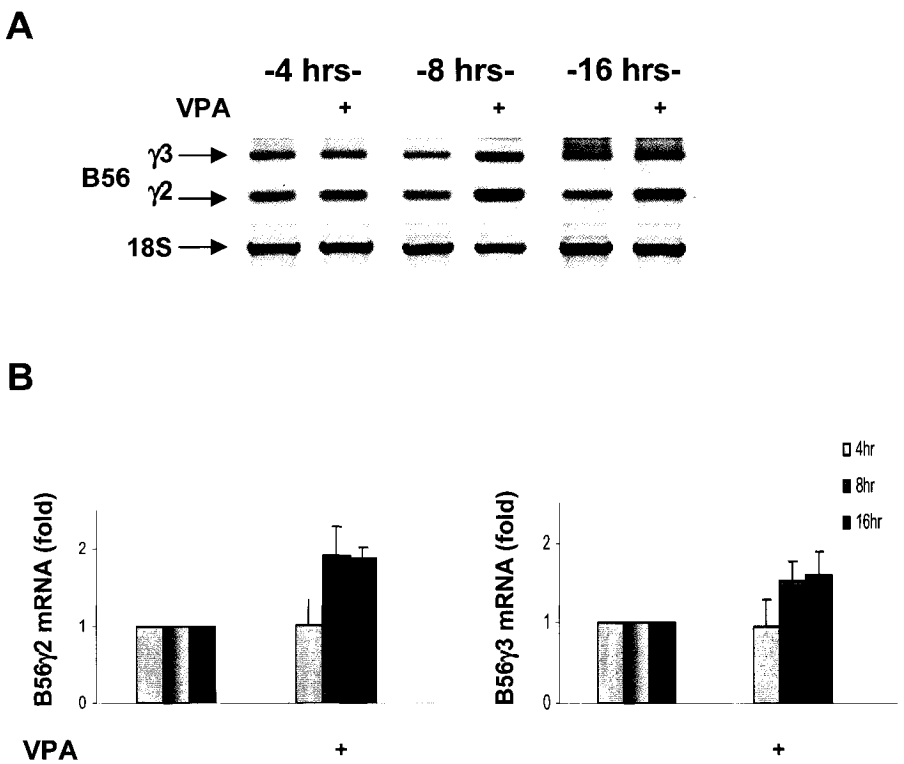

A
-4 hrs-
-8 hrs-
-16 hrs-
VPA
+
+
+
B56
γ3
γ2
18S
B
B56γ2 mRNA (fold)
B56γ3 mRNA (fold)
4hr
8hr
16hr
0
1
2
VPA
+
+
C
-8 hrs-
-16 hrs-
VPA
+
+
α-B56
γ3
γ2
α-RAR

Figure 2.

Valproic acid increases degradation of CBP but not of C-terminal-truncated p300 in HOC313 cells

Following the finding that B56γ3 interacts with the C-terminal region of p300, further investigation was conducted to examine the role of this region of p300 in its valproic acid-induced degradation. To do so, cell lines expressing a truncated p300 protein in its C-terminal region were used as experimental systems. The first to be investigated was the HOC313 cell line from human oral squamous carcinoma origin (Tadokoro et al., 1989). HOC313 cells have been used for over 15 years as an experimental system for the study of oral cancer. They have been a model system used in the study of metastasis due to the fact that they do not express E-cadherin, which correlates with increased invasiveness of many cancer cells (Hoteiya et al., 1999; Yokoyama et al., 2001). These cells were of use for the present study since they express a p300 protein with a truncation in its carboxy-terminal region (Suganuma et al., 2002; Figure 3A and 3B). Indeed, it was revealed that a nonsense mutation caused the truncation of the protein at amino acid 1874, representing a loss of the last 541 amino acids of p300 (Suganuma et al., 2002). To examine the impact of the loss of this region of p300 on its stability, Western analysis was performed following treatment of HOC313 cells with valproic acid (2 mM). Consistent with the observed results in HeLa cells, exposure of HOC313 to 8 hours of valproic acid treatment did not affect the levels of p300 protein (Figure 4A and 4B). However, contrary to what was observed in HeLa cells, 16 hours of valproic acid treatment failed to decrease p300 protein levels (Figure 4A and 4B). The HOC313 cells express a full-length CBP protein (Suganuma et al., 2002; Figure 3A). Therefore this coactivator, which is closely related to p300, was used

as an internal control to support the claim that the lack of effect we observed on p300 was due to the truncation of the protein rather than a cell-specific phenomenon. HeLa cells however, express a near-undetectable full-length CBP, although a faster migrating band is observed with the CBP antibody on Western (Figure 3A and data not shown). Western analysis revealed that levels of the full length CBP in HOC313 cells were decreased by 53 % after 16 hours of valproic acid treatment (Figure 4A and 4B), which is consistent with the results seen with full-length p300 in HeLa cells. Moreover, this decrease was blocked by co-treatment with MG132, resulting in a 30 % increase in CBP protein levels (Figure 4A and 4B). To verify if high rates of transcription could account for the undetected decrease in protein levels, real-time PCR using Taqman® probe technology was performed to compare gene expression of CBP to p300. Indeed, high basal levels of p300 gene expression could possibly have masked increased degradation of p300 in HOC313 cells. However, results from the real-time PCR analysis showed that CBP had higher mRNA levels (Figure 4C). This indicated that the inability of valproic acid to decrease p300 protein levels is due to higher stability of the protein - possibly because of the loss of its C-terminus - rather than over-abundant production of its transcript. Taken together, these results show that valproic acid increases CBP degradation through the 26S proteasome pathway but fails to increase the degradation of a C-terminal truncated form of p300, suggesting an involvement of this region in the regulation of the stability of the protein.

Another cell line used in this study was the colorectal carcinoma cell line HCT116. It was reported in this cell line that a single nucleotide mutation in the p300 gene causing a frame shift results in a protein of 1708 amino acids, of which only the first

1699 amino acids correspond to the p300 wild-type sequence (Gayther et al., 2000; Ionov et al., 2004; Figure 3A). In HCT116 cells, therefore, p300 lacks its last C-terminal 715 amino acids. This truncation is distal to the HAT domain of p300, which allows p300 to retain its acetyltransferase activity, as shown by its ability to acetylate p53 in HCT116 cells (Sakaguchi et al., 1998). This cell line also expresses wild-type CBP (Ozdag et al., 2002; Figure 3A). As with HOC313 cells, Western analysis was performed to assess p300 and CBP protein levels in HCT116 cells following valproic acid treatment. This revealed that p300, which lacks in HCT116 a larger region of its C-terminus than in HOC313 cells, was in turn affected by valproic acid, as shown by decreased protein levels of 47 % after 16 hours of treatment (Figure 5A and 5B). Consistent with HOC313 cells, wild-type CBP protein levels in HCT116 were decreased to 64 % by valproic acid treatment (Figure 5A and 5B).

Following the phenotype observed in HOC313 cells, the results obtained with HCT116 cells in regards to p300 are somewhat contrasting. However, some available evidence, discussed below, can offer a potential explanation for this phenomenon. Because of the interesting phenotype observed in HOC313 cells, further investigation was done in order to elucidate the molecular mechanisms involved in the regulation of p300 within this cell line. The HCT116 results, although intriguing, were put aside for future investigation.

Figure 3. Western analysis of p300 and CBP proteins in different cell lines. (A) Western analysis showing endogenous p300 and CBP proteins in HeLa, HOC313 and HCT116 cells. 50 μg of whole cell protein were migrated by 6 % SDS-PAGE. RARβ antibody was used for loading control. **(B)** Schematic representation of full-length p300 and CBP proteins, as well as different truncated forms of p300 (Bromo = Bromodomain; CH1, 2, 3 = Cystein/Histidine-rich region1, 2, 3; HAT = Histone acetyltransferase domain; Q-rich = Glutamine-rich region). Numbers indicate length of p300 or CBP in amino acids. Broken line on protein from HCT116 cells indicates an amino acid sequence non-specific to wild-type p300.

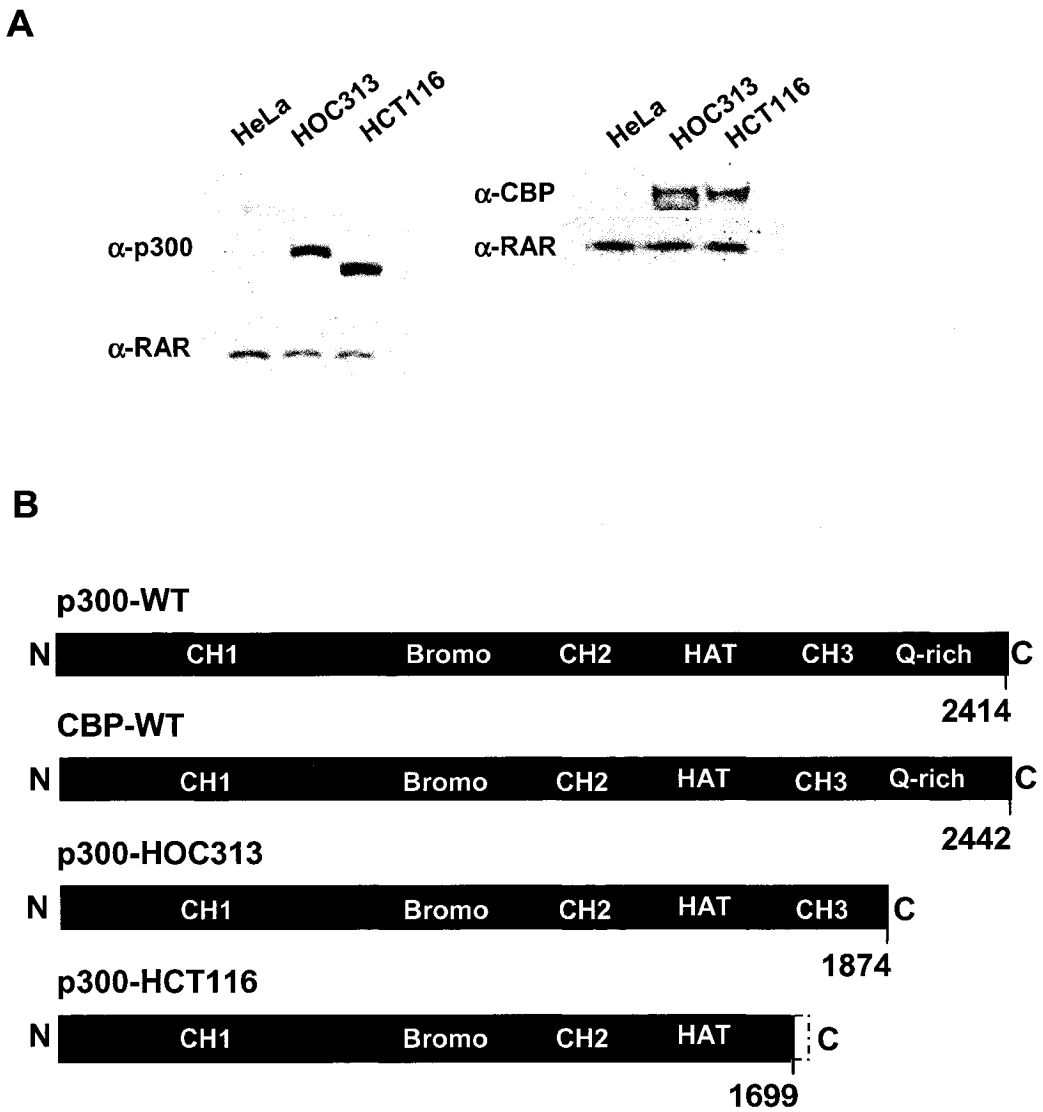
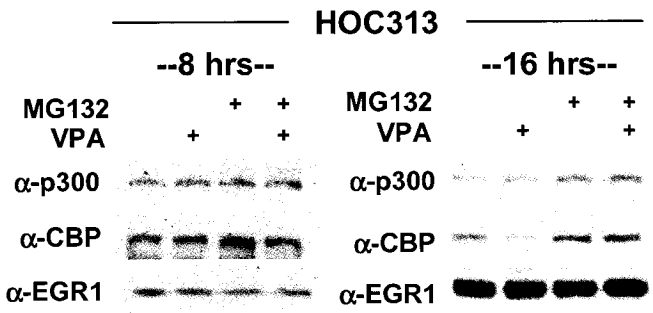

A
HeLa
HOC313
HCT116
α-p300
α-RAR
HeLa
HOC313
HCT116
α-CBP
α-RAR
B
p300-WT
N
CH1
Bromo
CH2
HAT
CH3
Q-rich
C
2414
CBP-WT
N
CH1
Bromo
CH2
HAT
CH3
Q-rich
C
2442
p300-HOC313
N
CH1
Bromo
CH2
HAT
CH3
C
1874
p300-HCT116
N
CH1
Bromo
CH2
HAT
C
1699

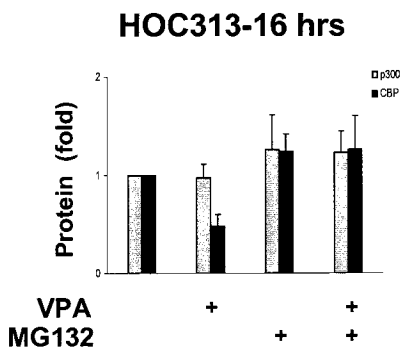
Figure 3.

Figure 4. Valproic acid reduces CBP but not truncated-p300 protein levels in HOC313 cells. **(A)** Western blot of p300 and CBP levels following treatment of HOC313 cells with valproic acid and/or MG132 for indicated time periods. 50 μg of whole cell extract were migrated by 6 % SDS-PAGE. EGR-1 antibody was used as loading control. **(B)** Quantification of results from (A) with Scion Image software. Results show fold variation of p300 and CBP from treated cells compared to control samples. Values are averages from three experiments performed in duplicates. Error bars represent standard deviations. Unpaired Student T-tests were performed for statistical analysis. For CBP, $p \leq 0.005$ for VPA-treated samples compared to control, $p \leq 0.01$ for VPA-treated samples compared to MG132+VPA-treated samples. **(C)** Real-time PCR of endogenous p300 and CBP mRNA levels. Total RNA isolated from HOC313 cells and was reverse transcribed and amplified by real-time PCR using primers and fluorescent Taqman® probes specific to p300, CBP or 18S ribosomal RNA genes. Values show averages of three real-time PCR reactions and represent fold variation of CBP mRNA levels compared to p300 mRNA levels. Error bar represents standard deviation. Unpaired T-test was performed for statistical analysis. $p \leq 0.05$ for CBP mRNA levels compared to p300 mRNA levels.

A


HOC313
--8 hrs--
--16 hrs--
MG132
VPA
α-p300
α-CBP
α-EGR1

B


HOC313-16 hrs
p300
CBP
Protein (fold)
VPA
MG132

C

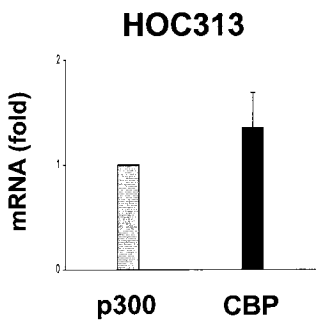

HOC313
mRNA (fold)
p300
CBP

Figure 4.

Figure 5. Valproic acid decreases protein levels of truncated-p300 and CBP in HCT116 cells. (A) Western results show levels of p300 and CBP following a 16-hour treatment of HCT116 cells with valproic acid or MG132. 50 μg of whole cell protein extracts were migrated by 6 % SDS-PAGE. Anti-EGR-1 antibody was used for loading control. **(B)** Quantification of Western results of **(A)** using Scion Image software. Values show fold variation of p300 and CBP protein levels from treated HCT116 cells compared to untreated control cells. Error bars represent standard deviations of averages of three experiments performed in duplicates. Unpaired T-tests were performed for statistical analysis. For p300 and CBP, $p \leq 0.05$ for VPA-treated samples compared to control.

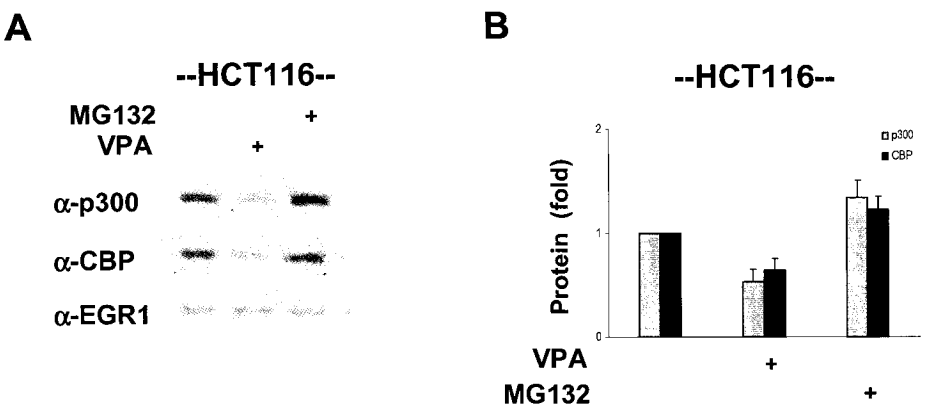

A
--HCT116--
MG132
VPA
+
+
α-p300
α-CBP
α-EGR1
B
--HCT116--
Protein (fold)
2
1
0
p300
CBP
VPA
MG132
+
+

Figure 5.

Valproic acid increases ubiquitination of CBP but not of p300 in HOC313 cells

Having shown that both p300 and CBP degradation occurred through the 26S proteasome pathway upon valproic acid treatment, and having also demonstrated that this increased degradation caused by valproic acid was prevented in the context of a C-terminal truncation of p300 in HOC313 cells, further investigation into the molecular mechanisms involved in the proteasomal degradation of these two proteins was conducted. As mentioned previously, protein degradation through the 26S proteasome pathway generally involves prior tagging of the target protein with ubiquitin, which not only sends misfolded proteins for degradation, but is also a regulatory mechanism for turnover of functional proteins. In order to determine if increased degradation of p300/CBP through the 26S proteasome pathway is associated with increased ubiquitination of these proteins, ubiquitination experiments were performed with HOC313 cells. The tagging of a protein with ubiquitin moieties involves covalent isopeptide bonds between ubiquitin and the target protein. Hence, the ubiquitin groups remain linked to the target protein even upon exposure to denaturing agents such as sodium dodecylsulfate (SDS), which allows the use of immunoprecipitation (IP) followed by SDS-PAGE to observe the ubiquitination pattern of a chosen protein by Western blotting. The goal of this experiment being to compare the basal ubiquitination state of p300/CBP to that upon valproic acid treatment, HOC313 cells were treated for 16 hours with MG132 alone or in cotreatment with valproic acid, prior to IP of p300 and CBP. As a negative control, an antibody directed against the E1A adenoviral oncoprotein was used since this viral product is absent from HOC313 cells. Inhibition of the proteasome is essential in such experiments since accumulation of the target proteins is required for

detection of the ubiquitin signal on a protein blot. The resulting immunoprecipitates were migrated by SDS-PAGE for Western analysis. The protein blot was probed with a monoclonal anti-ubiquitin antibody prior to its stripping and reprobing with the antibodies against p300 and CBP. Results from the IP experiments revealed no changes in the levels of ubiquitin signal of the p300-immunoprecipitate following valproic acid treatment (Figure 6A and 6B). In turn, ubiquitin signal was increased by 92 % in the CBP-immunoprecipitated sample treated with valproic acid (Figure 6A and 6B). This suggests that ubiquitination of CBP is increased by valproic acid, while ubiquitination of the truncated p300 is not.

Figure 6. Valproic acid increases ubiquitination of CBP but not of truncated-p300 in HOC313 cells. (A) Western results show ubiquitination levels in HOC313 cells following IP of p300 or CBP. HOC313 cells were treated for 16 hours with MG132 (5 μM) alone or in combination with valproic acid (2 mM). 500 μg were used for IP with p300 or CBP antibodies and protein A sepharose beads. The resulting immunoprecipitates were migrated on 8 % SDS-PAGE. Following Western blotting with an anti-ubiquitin antibody, the membrane was cut to separate the p300-immunoprecipitate lanes from the CBP-immunoprecipitate lanes. The resulting membranes were stripped and reprobed respectively with anti-p300 and anti-CBP antibodies. **(B)** Quantification of results in (A) with Scion Image software. Values represent the fold variations of ubiquitin levels in samples treated with both MG132 and valproic acid compared to samples treated only with MG132. Error bars represent standard deviations of averages from three different experiments performed in duplicates.

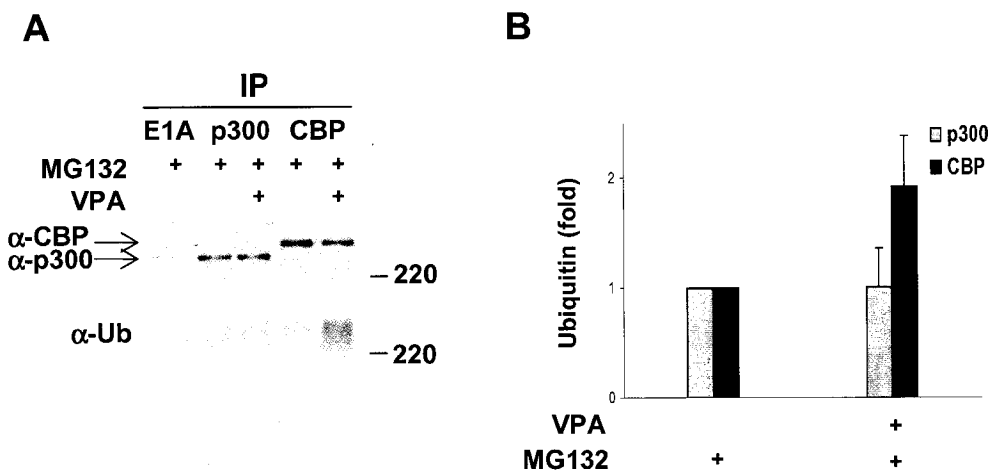

A
IP
E1A
p300
CBP
MG132
VPA
α-CBP
α-p300
α-Ub
220
220
B
p300
CBP
Ubiquitin (fold)
2
1
0
VPA
MG132

Figure 6.

Valproic acid increases co-localization of ubiquitin with CBP but not with p300 in HOC313 cells

To examine the cellular distribution of ubiquitin, p300 and CBP, immunofluorescence microscopy was performed with HOC13 cells. The cells grown on cover slips were treated with valproic acid (2 mM) and stained with ubiquitin and p300 or CBP antibodies. Using Apotome® technology, cells were optically sectioned in slices of 0.2 μm to increase resolution and co-localization specificity. It has been established that p300 and CBP are restricted to the nucleus (Chrivia et al., 1993; Yaciuk and Moran, 1991; Chen et al., 2004). In agreement with these previous findings, DNA staining of HOC313 cells with Hoechst confirmed that both p300 and CBP were restricted to the nucleus in this cell line (Figure 7). However, results showed different distribution patterns between CBP and p300. Indeed, while p300 was evenly distributed in the nucleus, intense small speckles were observed in cells stained with the CBP antibody (Figure 8A and 8B). Ubiquitin was more widely distributed throughout the cell, although it seemed to be predominantly concentrated in the nucleus (Figure 8A and 8B). Following 16 hours of treatment with valproic acid, ubiquitin was recruited to the small CBP aggregates and co-localized with CBP. As shown by the merged images of CBP and ubiquitin, yellow nuclear speckles represent sites of co-localization of CBP and ubiquitin (Figure 8B). Ubiquitin speckles in the cytoplasm, however, retain their original red color, demonstrating that the co-localization is specific to CBP in the nucleus. Conversely, p300 did not co-localize with ubiquitin speckles, even though such ubiquitin aggregates were present in the nucleus after valproic acid treatment (Figure 8A). In this case, neither nuclear nor cytoplasmic ubiquitin displayed yellow signal. Hence, these co-

localization results are consistent with the IP experiment and suggest that ubiquitination of CBP is increased upon valproic acid treatment, unlike the C-terminal truncated p300 which is not affected by the drug.

Figure 7. p300 and CBP exhibit a strictly nuclear distribution in HOC313 cells. Immunofluorescence microscopy shows nuclear distribution of p300 and CBP in HOC313 cells. Cells were subjected to 4% para-formaldehyde and 0.2% NP-40 prior to staining with anti-p300 or anti-CBP antibodies followed by staining with fluorescent secondary antibodies. Coverslips were then incubated in Hoechst/PBS (0.5 mg/ml) for DNA staining. Fluorescence microscopy was done using optical sectioning and Apotome technology (Zeiss). Sections of 0.2 μm are shown.

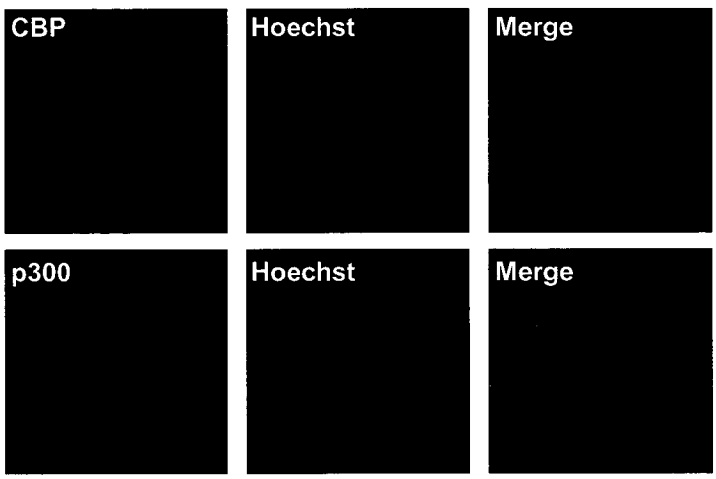


Figure 7.

Figure 8. Valproic acid increases co-localization of ubiquitin with CBP but not with truncated-p300 in HOC313 cells. (A) Immunofluorescence microscopy shows cellular distribution of ubiquitin and p300 in HOC313 cells treated with or without valproic acid (2 mM) for indicated time periods. After treatment, cells were subjected to 4% para-formaldehyde and 0.2% NP-40 prior to staining with anti-p300 and anti-ubiquitin antibodies followed by staining with fluorescent secondary antibodies. Fluorescence microscopy was done using optical sectioning and Apotome® technology. Sections of 0.2 μm are shown. **(B)** As in (A) with the exception that an anti-CBP antibody was used instead of an anti-p300 antibody. Results represent the consensus from 8 experiments.

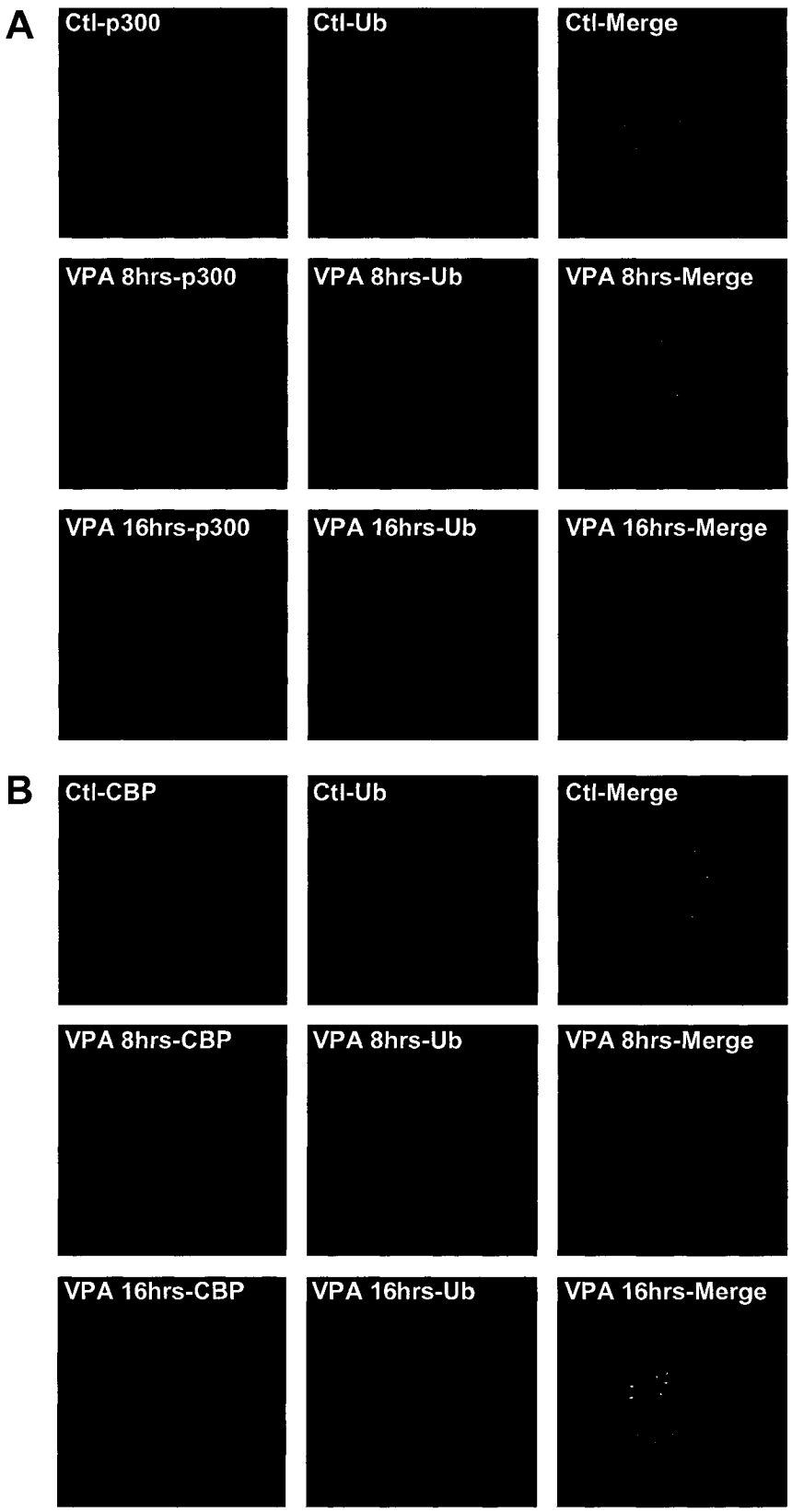

A
Ctl-p300
Ctl-Ub
Ctl-Merge
VPA 8hrs-p300
VPA 8hrs-Ub
VPA 8hrs-Merge
VPA 16hrs-p300
VPA 16hrs-Ub
VPA 16hrs-Merge
B
Ctl-CBP
Ctl-Ub
Ctl-Merge
VPA 8hrs-CBP
VPA 8hrs-Ub
VPA 8hrs-Merge
VPA 16hrs-CBP
VPA 16hrs-Ub
VPA 16hrs-Merge

Figure 8.

Discussion

This study reveals that valproic acid increases degradation of p300 and CBP through the ubiquitin-proteasome pathway and suggests that this effect is mediated by the B56γ3 regulatory subunit of PP2A. Indeed, these results show that the protein levels of the closely related transcriptional coactivators p300 and CBP are decreased in response to treatment with the HDAC inhibitor valproic acid. This decrease is prevented by a combination treatment of valproic acid and the 26S proteasome inhibitor MG132, demonstrating that the decrease in protein levels is due to degradation through the proteasome. Moreover, this proteasomal degradation is associated with ubiquitination of CBP, as shown by IP and co-localization assays, suggesting that the degradation is ubiquitin-dependent. Also, valproic acid up-regulates the B56γ regulatory subunits of PP2A, of which the B56γ3 isoform was shown to interact with the C-terminal region of p300, and cause its degradation through the 26S proteasome pathway (Chen et al., 2005). This suggests an important signaling role for the C-terminal region in the valproic acid-induced, B56γ3-mediated degradation through the ubiquitin-proteasome pathway. Consistently, neither ubiquitination nor degradation of p300 is increased by valproic acid in HOC313 cells, in which the p300 gene harbors a nonsense mutation leading to a truncation of its C-terminal region.

Effect of Akt on p300 stability

Although the present study reveals that increased degradation of p300 by valproic acid does not occur when the protein lacks its last 541 C-terminal amino acids, it also

shows that a deletion of the 715 C-terminal amino acids of p300 restores the ability of valproic acid to increase the degradation of the coactivator. Results from previous studies, including data from our laboratory (Chen et al., 2004; Huang and Chen, 2005) can offer a potential explanation for this phenomenon. In these studies, the stability and activity of p300 were shown to be regulated by the pro-survival factor Akt, also known as protein kinase B (PKB). Akt/PKB exerts its effect through phosphorylation of substrates harboring the consensus sequence RXRXXS/T (Alessi et al., 1996; Obata et al., 2000). Both p300 and CBP contain this amino acid sequence in their C-terminal region. Akt/PKB interacts with the C-terminal region of p300 and phosphorylates it at serine-1834, thus resulting in an increased stability of the coactivator. In HOC313 cells, p300 contains this Akt phosphorylation site since the protein is truncated downstream of amino acid 1874. Therefore, a potential explanation for the obtained results is that the stability of p300 in HOC313 cells is due to the inability of B56γ3 to exert its effect, while Akt/PKB remains as a positive regulator of p300 stability. In HCT116 cells, however, p300 lacks the amino acids required for Akt/PKB phosphorylation and so although the protein is missing the site potentially responsible for the B56γ3 effect, it is also deprived of its stabilizing Akt/PKB site, which could explain why decreased protein levels are observed in this cell line upon valproic acid treatment. However, this model implies that valproic acid acts to decrease p300 protein levels by a different mechanism, one which is either B56γ3-independent, or where B56γ3 plays a role in a different p300 region.

B56γ3 and the carboxyl-terminal region of p300

Inhibition of PP2A by okadaic acid has been shown to increase the stability of wild-type p300 and counteract the valproic acid-induced degradation of the protein (Chen et al., 2005). This suggests that the role of B56γ3 could be to direct PP2A to p300 for dephosphorylation at the C-terminus of the protein. Whether this possible dephosphorylation site would correspond to the Akt/PKB consensus site remains to be demonstrated. It is conceivable however that p300 stability would be decreased by the removal of a stabilizing phosphate group by PP2A, to which p300 would be exposed by interaction of the B56γ3 subunit with the C-terminal region of p300, thus allowing the catalytic subunit of the enzyme to have access to p300. This hypothesis is consistent with the observed results from this study in HOC313 cells, where valproic acid fails to induce a decrease in p300 degradation. Indeed, although serine 1834 of p300 is present in HOC313, the potential dephosphorylation of this site could be prevented by the incapacity of PP2A to anchor onto p300, possibly because of the inability of B56γ3 to interact with p300. This model suggests a direct effect of B56γ3 as part of the PP2A holoenzyme, is supported by the finding that p300 immunoprecipitates with the catalytic C subunit of PP2A under physiological conditions, and that p300 and B56γ3 interact *in vitro* as demonstrated by GST pull-down experiments (Chen et al., 2005). These results therefore suggest that PP2A forms a complex with p300 through an interaction between the C-terminus of p300 and the B56γ3 regulatory subunit of the phosphatase. However, a direct interaction between p300 and B56γ3 under physiological conditions has not been shown, which leaves the possibility that the effect of B56γ3 could also be indirect. As

such, B56γ3 could possibly negatively regulate p300 levels without directly interacting with the coactivator, but rather by inhibiting one of its positive regulators.

Dephosphorylation and inhibition of activity of Akt/PKB by PP2A has been clearly established (Andjelkovic et al., 1996). Furthermore, PP2A has been shown to be a major negative regulator of Akt/PKB and the main inhibitor of this kinase in certain model systems (Mora et al., 2002; Ugi et al., 2004; Resjo et al., 2002). Hence, the evidence showing the negative regulation of a positive regulator of p300 offers an interesting view on how PP2A could act to reduce p300 stability. By dephosphorylating Akt/PKB, PP2A would prevent it from phosphorylating p300 thus reducing the stability of the coactivator. The regulatory B subunit of PP2A involved in the dephosphorylation of Akt/PKB has not yet been identified so although such a role for B56γ3 in the regulation of Akt/PKB cannot be confirmed, neither can it be excluded at this point and time. This mechanism alone however, can not explain the lack of effect of valproic acid on p300 in HOC313 cells. Indeed, because the Akt site is present, preventing its phosphorylation should affect its stability, contrary to what is observed.

Regulation of p300 and CBP by phosphorylation

The observed increase of B56γ3 subunit induced by valproic acid, together with the concomitant decrease in p300 protein levels implies an important role for phosphorylation as a post-translational modification in the regulation of p300 function. Early on in their characterization, p300 and CBP were identified as being heavily phosphorylated (Yaciuk and Moran, 1991). Since then, studies have identified some of the kinases involved in the phoshorylation of p300 and CBP and have revealed how this

post-translational modification affects these coactivators. Phosphorylation of CBP at serine 436 through a growth factor-dependent pathway was shown to be essential for some of its transcriptional functions (Zanger et al., 2001). The cell cycle-dependent kinase complex cyclin E-CDK2 increases the HAT activity of CBP and interacts with the coactivator in its C-terminal region (Ait-Si-Ali et al., 1998). The C-terminus of CBP is also phosphorylated by MAP kinases such as the p44 MAP kinase/ERK1, which also increases its HAT activity (Ait-Si-Ali et al., 1999; Janknecht and Nordheim, 1996). As discussed above, stability of p300 is increased upon its phosphorylation at serine 1834 by Akt/PKB (Chen et al., 2004; Huang and Chen, 2005). PKA also increases phosphorylation of p300 during F9 cell differentiation, causing an increase in the acetyltransferase activity of the coactivator (Brouillard and Cremisi, 2003). Phosphorylation of p300 can also decrease its stability and inhibit its function. Indeed, serine 89 of p300 is a target for the AMP-kinase and PKC, resulting in its decreased interaction with nuclear receptors, thus inhibiting its function (Yuan and Gambbe, 2000; Yang et al., 2001). Also, degradation of p300 was shown following phosphorylation by the p38 MAP kinase (Poizat et al., 2005). Taken together, this demonstrates that the effect of phosphorylation on the stability and function of p300 and CBP is dependent on which kinase is involved and of the region where the phosphorylation occurs. Considering the available evidence, it seems that phosphorylation of the C-terminal region of these proteins, in general, results in increased stability and gain of function. This could explain how the inability of PP2A to interact with the C-terminal region of these proteins, thus preventing the dephosphorylation of the stabilizing phosphorylation site (s) of this region, would increase their stability. This offers additional support to the

results obtained in HOC313 and HCT116 cells. However, although strong evidence supports a role for PP2A in the stability of p300 and CBP, dephosphorylation of specific sites by this phosphatase, or any other, remains to be shown.

Ubiquitination of p300 and CBP

The B56γ regulatory subunit of PP2A has been shown to form a complex with cyclin G1 and the E3 ubiquitin ligase MDM2 to promote degradation of p53 in the later stages of response to DNA damage (Kimura and Nojima, 2002). It has been demonstrated that MDM2 is the ligase involved in the ubiquitination and subsequent degradation of PCAF (Jin et al., 2004). In early stages of the DNA damage response where p53 is stabilized and accumulated, cyclin G1 and MDM2 are complexed with ARF which sequesters MDM2 in the nucleolus and protects p53 from ubiquitination by the ligase activity of MDM2 and prevents its degradation through the proteasome (Weber et al., 1999; Honda and Yasuda, 1999). In the later response however, ARF is replaced in the complex by B56γ, which recruits protein phosphatase 2A to MDM2. This results in dephosphorylation and activation of MDM2, which causes ubiquitination and degradation of p53. Considering that the present study suggests an ubiquitin-dependent degradation of CBP and p300, activation of a ubiquitin ligase or conjugase by PP2A offers yet another possible mean by which B56γ3 could cause the degradation of these proteins.

To date, no such ubiquitinating enzyme has been identified for p300 or CBP. Valproic acid has, however, been shown to increase expression of the E2 conjugase Ubc8 (Krämer et al., 2003). It was shown that proteasomal degradation of HDAC2 induced by valproic acid was a result of increased expression of Ubc8, which in concert with the E3

ligase RLIM, increased ubiquitination of HDAC2, thus targeting it for degradation. A similar mechanism could also be involved in the valproic acid-induced ubiquitination and degradation of p300 and CBP. Valproic acid, by increasing a yet-to-be identified p300 and CBP ubiquitin ligase or conjugase, could enhance the ubiquitination state of the coactivators and target them in this manner to the 26S proteasome. This implies that multiple pathways of valproic acid are involved in the degradation of p300 and CBP, pathways which, far from being exclusive, could be complementary. Also, a role for the C-terminal region in this ligase/conjugase hypothesis could potentially be excluded, thus offering a possible explanation for the valproic acid-induced p300 degradation in HCT116 cells.

Few studies have examined the regulation of p300 and CBP by ubiquitin or ubiquitin-like modification. Yet in recent years, both p300 and CBP have been shown to be modified by ubiquitination. In F9 mouse embryonic carcinoma cells, p300 was found to be degraded through the proteasome during differentiation induced by retinoic acid (Brouillard and Cremisi, 2003). This degradation was shown to be associated with ubiquitination of p300. Likewise, CBP was ubiquitinated and degraded through the proteasome during HT22 neuronal cell death (Jiang et al., 2003). Also, modification of p300 by the ubiquitin-like protein SUMO was demonstrated in HeLa cells (Girdwood et al., 2003). This study demonstrated that lysines 1020 and 1024 of the consensus sequences ψKXE (where ψ is a large hydrophobic residue) in the CRD1 domain (1004-1044) of human p300 were the target sites of sumoylation. The CRD1 domain is known to be responsible for the transcriptional repression activity of p300 and is also highly conserved between p300 and CBP. Sumoylation of these lysine residues results in the

recruitment of HDAC6, which was shown to be the mechanism by which the CRD1 domain of p300 represses transcription this context. Recently, Kuo et al. (2005) revealed that murine CBP was also a target of SUMO modification in the region corresponding to the CRD1 domain. Interestingly, the resulting transcriptional repression activity of CBP was also shown to be the result of recruitment of HDAC although in this case, HDAC2 and not HDAC6, was recruited. Furthermore, the recruitment of HDAC2 by CBP sumoylation was shown to be mediated by the transcriptional corepressor Daxx. In both of these studies, the HDAC inhibitor Trichostatin A countered the effect of the sumoylation of p300 and CBP, resulting in relieved transcriptional repression.

Similarly to what is observed in this present study with valproic acid, Trichostatin A has been shown to increase ubiquitination of certain proteins. Upon Trichostatin A treatment, ubiquitin-dependent degradation of cyclin D1 was demonstrated in the MCF-7 breast cancer cell line (Alao et al., 2004). Interestingly, however, this study showed that Trichostatin A also reduced the levels of cyclin D1 transcription. Transcription levels of p300 were also affected by the Trichostatin A treatment of M12 prostate cancer cells (Yu et al., 2004). In this context, Trichostatin A increases acetylation of the transcription factor EGR-1, which reduces its ability to activate transcription at the p300 promoter, resulting in down-regulation of p300 expression. Thus, this represents an effect of Trichostatin A on the activity of the p300 promoter through an indirect mechanism mediated by acetylation of Egr-1. This contrasts with what has been observed in the present study in HeLa cells with valproic acid treatment, where p300 transcription was unaffected (Figure 1C and 1D). This could be due to a possible differential effect of valproic acid on the acetylation levels of EGR-1. Although valproic acid can reduce the

DNA-binding ability of EGR-1, this was only observed following a chronic 7-day treatment of human neuroblastoma cells (Grimes and Jope, 1999). Also, this study did not assess the acetylated levels of EGR-1. A possible explanation for the differential effects produced by valproic acid and Trichostatin A is their different HDAC inhibition properties. While the class II HDAC 6 and HDAC10 are inhibited by Trichostatin A, valproic acid fails to do so (Gurvich et al., 2004). Therefore, deacetylation of any protein by HDAC6 or HDAC10 would be prevented by Trichostatin A but not by valproic acid.

Regulation of p300 and CBP: differences and similarities

This study has examined the effect of valproic acid on the stability of both p300 and CBP. It suggests a role for the C-terminal region of these proteins in their increased degradation following valproic acid treatment. In this pathway, it is suggested that these proteins are targeted by the B56γ3 regulatory subunit of PP2A for ubiquitination and degradation by the 26S proteasome complex. However, the degradation of CBP by B56γ3 has not been demonstrated, nor has any interaction between both proteins been shown. Also, increased ubiquitination of full-length p300 in response to valproic acid treatment remains to be confirmed. However, both wild-type proteins are degraded through the proteasome following 16 hours of valproic acid treatment. Together with the consideration of the high degree of homology between these coactivators and the presence of consensus sequences such as the RXRXXS/T Akt/PKB site in the C-terminal region, this allows to hypothesize that this pathway applies to both proteins. Some studies show p300 and CBP to be differentially regulated. Such is the case during the above-mentioned differentiation of mouse F9 embryonic carcinoma cells. In this context,

only p300 is degraded through the ubiquitin-proteasome pathway, whereas retinoic acid-induced differentiation of F9 cells does not affect CBP levels (Brouillard and Cremisi, 2003). However, many studies assessing both p300 and CBP reveal common regulatory mechanisms for both proteins. Both p300 and CBP, in QT6 quail fibroblasts and HEK 293T cells respectively, are phosphorylated within their C-terminal region upon recruitment by the transcription factor C/EBPβ, although no kinase was identified in this process (Schwartz et al., 2003; Kovacs et al., 2003). At the transcriptional level, both CBP and p300 genes have been shown to be regulated by the transcription factor EGR-1 (Yu et al., 2004). Viral oncoproteins also target common regions of the two coactivators. Indeed, the large T antigen of the SV40 virus decreases phosphorylation of both proteins and thus impairs their transcriptional activity (Eckner et al., 1996). Also, results obtained in rat pheochromocytoma PC12 cells suggest that p300 and CBP are both accumulated in a hyperphosphorylated state in hypoxic conditions (Zakrzewska et al., 2005). Of note in this latter study, Zakrzewska and colleagues demonstrate that the electrophoretic mobility of both p300 and CBP is decreased under hypoxic conditions. However, they subsequently demonstrate that this is due to hyperphosphorylation and establish the molecular basis of this phenomenon based on experiments in regards to p300 only. Hence, from an initial observation with p300 and CBP, the authors conclude that this increased phosphorylation level is applicable to both p300 and CBP.

Valproic acid, p300 and CBP: functional allies?

The present study reveals, for the first time, a relationship between the HDAC inhibitor valproic acid and the acetyltransferases p300 and CBP. However, valproic acid

and both p300 and CBP have been shown to affect common proteins, pathways and biological processes much before a direct link between them was demonstrated. In some cases, the effect of valproic acid correlates with a loss of p300 and CBP, and is consistent with the observed increased degradation of these proteins in response to valproic acid. In others, valproic acid treatment and p300 or CBP activity produce similar effects. One of the initial findings of the HDAC inhibition property of valproic acid came from the observation that treatment of Neuro2A neuroblastoma cells with 2 mM of valproic acid increased the mRNA levels of the β-catenin protein (Phiel et al., 2001). This up-regulation of β-catenin, which was suggested to be caused by the concomitant inhibition of HDAC by valproic acid, resulted in activation of the Tcf/Lef-dependent promoters. Accumulation of β-catenin leads to its translocation to the nucleus where it associates with the Tcf/Lef transcription factors to induce transcriptional activation (reviewed in Eastman and Grosschedl, 1999 and Novak and Dedhar, 1999). It was demonstrated that β-catenin interacts with p300 and CBP, and requires its acetyltransferase function for transcriptional activation (Hecht et al., 2000). β-catenin has also been involved in cell transformation, and these transformation properties in some colorectal cancers were shown to be dependent of p300-associated transcriptional activation (Sun et al., 2000). Competition for p300 between β-catenin and the tumor supressor p53 was suggested as being the mechanism involved in conferring such a transforming property to p300 (Miyagishi et al., 2000). Thus, in regards to β-catenin, both valproic acid treatment and p300/CBP activity can produce similar effects, albeit through distinct mechanisms.

A comparable scenario, where the effects of valproic acid and both p300 and CBP result in related processes, involves PPARs. The teratogenicity of valproic acid

correlates with the activation of these receptors in F9 teratocarcinoma cells, chinese hamster ovary (CHO) cells and human embryo kidney (HEK293) cells (Werling et al., 2001; Lampen et al., 1999). Although PPARα, γ and δ were all activated by derivatives of valproic acid, only PPARδ activation was specific to the teratogenic derivatives. F9 cell-differentiation was also specific to valproic acid and its teratogenic derivatives, thus correlating both PPARδ activation and F9 cell differentiation with the teratogenic property of valproic acid. As with β-catenin, p300 and CBP are involved in binding PPARs and participating in PPAR-activity as a coactivator for the receptors (Chen et al., 2000; Dowell et al., 1997). Binding of p300 to PPARδ was shown to be strong in rat Caco-2 intestinal cells (Mochizuki et al., 2002).

Valproic acid, p300 and CBP are also involved in the activity of the AP-1 transcription factor complexes. The DNA-binding activity of AP-1 was increased up to 20 hours following treatment of C6 glioma and SK-SY5Y cells with valproic acid and gene expression involving AP-1 and its components Jun and Fos was also increased by the drug (Chen et al., 1997; Chen et al., 1999, Asghari et al., 1998). Studies have also revealed that both p300 and CBP are coactivators for the AP-1 complex through interactions with the Jun and Fos transcription factors (Albanese et al., 1999; Bannister and Kouzarides, 1995). Hence, β-catenin, PPARs and AP-1 are examples of proteins which are at least affected by the actions of valproic, p300 and CBP, if not dependent on them. Whether the activities of valproic acid, p300 and CBP regulate β-catenin, PPARs and AP-1 in common contexts remains to be seen. In fact, whether valproic acid, in such contexts, affects p300 and CBP is also unknown. However, it could be of interest, considering the relationship revealed in the present study between valproic acid and these

coactivators, to assess the effects of valproic acid on β-catenin, PPARs and AP-1 in a system where p300 and/or CBP are degraded by this HDAC inhibitor.

Biological significance of p300 and CBP degradation: teratogenicity

Considering the effect of valproic acid and the importance of correct p300 and CBP levels in early embryonic development, the finding that the coactivators are degraded upon valproic acid treatment appears very interesting. Indeed, for the first time, there is evidence to support that the side-effects associated with exposure to valproic acid in early embryonic stages and the malformations observed in mice embryos deficient in p300 and/or CBP may be more than correlative. The malformations and embryonic lethality reported in mice embryos lacking correct p300 and/or CBP levels highlight the importance of these coactivators in cell proliferation during embryonic development and confirms how their presence and function are essential at this fetal stage (Yao et al., 1998; Shikama et al., 2003). The defects in the neural tube closure of the fetus after administration of valproic acid during early pregnancy have been observed in both humans and mice (Nau et al., 1991). The same embryonic defects in neural tube formation have been observed in mice deficient in p300 or CBP (Yao et al., 1998). For obvious reasons, no such study has examined the levels of these coactivators in human cases of embryonic malformations. Moreover, similar embryonic malformations of the heart following valproic acid treatment have been shown in p300 or CBP deficient mice.

Although valproic acid produces phenotypes similar to p300 or CBP deficiency, no molecular mechanisms underlying this phenomenon have been elucidated. However, the finding that degradation of both p300 and CBP occurs after valproic acid treatment

offers a potential explanation for the teratogenic properties of the drug. Also, as mentioned above, valproic acid induces differentiation of F9 teratocarcinoma cells in a manner related to its teratogenic properties (Werling et al., 2001). Interestingly, differentiation of these cells is associated with degradation of p300. Unfortunately, this p300 degradation was observed during retinoic acid-induced differentiation of F9 cells. Because valproic acid-induced F9 differentiation was revealed to be different from retinoic acid-induced differentiation, degradation of p300 in the former case remains to be shown. Nevertheless, F9 cell differentiation offers additional support to the important biological relevance of the valproic acid induced degradation of p300 and CBP in early development. Still, even if the degradation of these proteins was revealed to be involved in the embryonic malformations caused by valproic acid, further investigation would be required to elucidate the molecular events taking place downstream of this effect. Indeed, degradation of p300 and CBP would imply altered expression of genes that depend on these coactivators for their transcriptional activation. Hence, the effect of valproic acid, through down-regulation of p300 and CBP, could be to alter the expression of genes involved in cell-cycle control, since malformations are thought to be the result of premature cell differentiation or defective proliferation.

Unfortunately, although they have been shown to be essential, very little is known about the molecular role of p300 and CBP during embryogenesis. Bamforth and colleagues (2001) revealed that lethality and malformations similar to that observed in the p300/CBP null mice were also present in mice embryos null for the CBP/p300-interacting transactivator with ED-rich tail 2 (CITED2). Indeed, defects in neural tube closure and cardiac development were described in Cited2$^{-/-}$ mice. They also demonstrated that

CITED2 was a coactivator for the transcription factor activating protein 2 (TFAP2) while RAR and Pax3 were not, even though all three transcription factors are known to be involved in development of the neural tube (Zhang et al., 1996; reviewed in Juriloff and Harris, 2000). Moreover, this study suggested that transcriptional activation by TFAP2 required p300 or CBP and CITED2 coactivators. Thus, by depleting p300 and CBP protein levels during embryogenesis, valproic acid could possibly affect the expression of genes that require TFAP2 for their activation.

Biological significance of p300 and CBP degradation: tumorigenicity

Valproic acid is a serious candidate for cancer therapy (reviewed in Blaheta et al., 2005). Yet, p300 and CBP are well known for their tumor-suppressor properties. Thus, the mechanisms by which valproic acid produces its anti-cancer effect might be independent of its effect on p300 and CBP. However, in some cases, it seems that degradation of p300 and CBP by valproic acid could be consistent with reduced tumor growth or cancer development. Development of blood vessels is required for tumor growth in order to supply the cells with oxygen and an important factor in this process is the vascular endothelial growth factor (VEGF). Induction of VEGF expression occurs, in part, in hypoxic conditions where the hypoxia-inducible factor-1 (HIF-1) transcription factor is activated and stimulates VEGF production (reviewed in Ferrara, 2004). In a study conducted with the human colon cancer cell line Caco-2, valproic acid was shown to cause a decrease in VEGF production (Zgouras et al., 2004). This decrease was shown to be the result of inhibition of VEGF mRNA production. Interestingly, a study from Arany and colleagues (1996) revealed that coactivation by p300 is required for HIF-1

dependent transcriptional activation. What is more, disruption of the p300/HIF-1 complex by E1A inhibited expression of the VEGF gene. Therefore, inability of HIF-1 to activate the VEGF gene due to degradation of p300 by valproic acid could potentially explain how the drug is able to prevent angiogenesis and thus, limit tumor growth. Another important factor in angiogenesis is the endothelial nitric oxide synthase (eNOS) enzyme (reviewed in Xu et al., 2002 and Lala and Orucevic, 1998). The levels of this enzyme were shown to be decreased upon valproic acid treatment of endothelial cells (Michaelis et al., 2004). This resulted in decreased growth, migration and viability of these endothelial cells, all of which are processes involved in formation of new blood vessels. Consistently, this study also demonstrated *in vivo* inhibition of angiogenesis in both adult and embryonic mice. The molecular basis of this decrease was not identified. Therefore, whether this was due to inhibition of eNOS at the transcriptional, translational or post-translational level remains to be determined. However, in another study conducted with COS-7 and human endothelial cells, p300 was shown to interact with the transcription factor Kruppel-like factor-2 (KLF2) to coactivate the transcription of eNOS (SenBanerjee et al., 2004). Hence, as with VEGF, p300 coactivates the expression of this angiogenic enzyme, and targeting of p300/CBP represents an interesting mean by which valproic acid could inhibit VEGF and eNOS, thus blocking angiogenesis and limiting tumor development.

There is also some evidence that supports a potential role for valproic acid in the treatment of prostate cancer, which is the most frequently occurring type of cancer in males. Indeed, a study by Thelen et al. (2004) revealed that valproic acid caused apoptosis and reduced the levels of the prostate-specific antigen (PSA) in the prostate

cancer cell line LNCaP. Conversely, p300 has been shown to be overexpressed in prostate cancer cells, promoting cell proliferation and cancer progression (Debes et al., 2003). Moreover, p300 was found to be a coactivator involved in the transcription of PSA (Debes et al., 2005). Hence, the relationship between valproic acid and p300 might also be of biological relevance in the context of prostate cancer. Furthermore, in cancers where aberrant acetylation by p300 and CBP is observed, inhibitors, such as valproic acid, of these acetyltransferases could possibly reduce growth and proliferation of transformed cells.

Acetylation of the androgen receptor has been associated with increased growth of prostate cancer cells (reviewed in Fu et al., 2004). Because p300 acetylates this hormone receptor, inhibition of its catalytic activity through degradation by valproic acid might explain the efficacy of this drug in treating this type of cancer. Such is also the case with the estrogen receptor-α (ERα), of which the expression is increased in breast cancer along with expression of some of its coactivators, notably CBP (reviewed in Fu et al., 2004). Another promising anti-cancer agent, curcumin, has been demonstrated to prevent tumor development, reduce growth of tumor cells from a wide range of cancer types and inhibit proliferation of a large variety of cancer cell lines (for review, see Duvoix et al., 2005). Curcumin, also known as diferuloylmethane, is a compound found in the spice turmeric. Interestingly, it has recently been revealed as a potent inhibitor of the p300 acetyltransferase activity (Balasubramanyam et al., 2004). Although strictly correlative at this time, the inhibition of p300 activity, whether through degradation or inhibition of its acetyltransferase domain, might provide a molecular pathway through which agents such as valproic acid or curcumin produce their anti-tumor effect.

Perspectives

Our results have established the molecular basis of the valproic acid-induced degradation of p300 and CBP by presenting evidence of the involvement of the B56γ3 regulatory subunit of PP2A in the ubiquitin-dependent proteasomal degradation of these coactivators. However, many questions remain unanswered and complete understanding of these molecular events will require further dissection of this pathway. Characterization of the exact role of B56γ3 and PP2A on the C-terminal region of p300 and CBP remains unknown. As such, the potential dephosphorylation of specific sites in this region of these proteins will need to be assessed. Ongoing work from our laboratory suggests that ubiquitination does not occur in the C-terminus. This would imply that the lack of the C-terminal region of p300 would therefore not prevent ubiquitination directly, but rather prevent an ubiquitination signaling event occurring in this region. Whatever the role of B56γ3 may be, if and how the events taking place in the C-terminal region of p300 and CBP act as signaling mechanism for their ubiquitination should be examined. Future work will need to focus on elucidating the remaining mysteries of this complex regulatory pathway. Considering the central role of p300 and CBP in critical cellular processes, understanding the upstream regulatory pathways of these transcriptional coactivators will become as important as the accumulating knowledge of their downstream functions.

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