Hepatitis C virus core protein regulates p300/CBP co-activation function.
Possible role in the regulation of NF-AT1 transcriptional activity

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Abstract

Hepatitis C virus (HCV) core is a viral structural protein; it also participates in some cellular processes, including transcriptional regulation. However, the mechanisms of core-mediated transcriptional regulation remain poorly understood. Oncogenic virus proteins often target p300/CBP, a known co-activator of a wide variety of transcription factors, to regulate the expression of cellular and viral genes. Here we demonstrate, for the first time, that HCV core protein interacts with p300/CBP and enhances both its acetyl-transferase and transcriptional activities. In addition, we demonstrate that nuclear core protein activates the NH₂-terminal transcription activation domain (TAD) of NF-AT1 in a p300/CBP-dependent manner. We propose a model in which core protein regulates the co-activation function of p300/CBP and activates NF-AT1, and probably other p300/CBP-regulated transcription factors, by a novel mechanism involving the regulation of the acetylation state of histones and/or components of the transcriptional machinery.

Keywords: Hepatitis C virus; Core protein; p300/CBP; HAT activity; NF-AT; Transcriptional activation domain

Introduction

Hepatitis C virus (HCV) infection is the most frequent cause of chronic liver disease in Western countries and 20–30% of these patients will develop cirrhosis with the risk of hepatocellular carcinoma. The genome of HCV is composed of a positive-stranded RNA of 9.6 kb encoding a polyprotein, of approximately 3010 amino acids, which is processed by viral and cellular proteases to render 10 proteins (Suzuki et al., 1999). HCV core protein spans the first 191 amino acids of the polyprotein (Lai and Ware, 2000). This immature core protein is further processed at its COOH-terminal region to render the mature form of 173 amino acids, which shapes the viral nucleocapsid (Yasui et al., 1998). Analysis of the subcellular localization of core protein has provided interesting but discrepant results. It has been shown that immature (1–191) and mature (1–173) core proteins localize in the cytoplasm, associated with endoplasmic reticulum, mitochondria and/or lipid droplets (Barba et al., 1997; Liu et al., 1997; Marusawa et al., 1999; Okuda et al., 2002). In addition, other authors have also described a nuclear localization of the mature and COOH-terminal truncated forms of core protein (Sabile et al., 1999; Watashi et al., 2001; Yamanaka et al., 2002b; Yasui et al., 1998). There are three nuclear localization signals in the NH₂-terminal region of core protein that could be involved in nuclear translocation (Chang et al., 1994). On
the other hand, the hydrophobic COOH-terminal domain of core appears to be responsible for its cytoplasmic retention (Liu et al., 1997). In some instances, the function of core and its subcellular localization have been associated (Watashi et al., 2001; Yamanaka et al., 2002a).

Besides its structural function, core protein also regulates different cellular processes. In this context, it has been shown that core protein expression activates several signal transduction pathways, leading to activation of transcription factors such as Elk-1, AP1, Sp1, and Egr-1 (Giambartolomei et al., 2001; Kato et al., 2000; Lee et al., 2001; Shrivastava et al., 1998). Other transcription factors, such as p53 and RXR, are activated by core via mechanisms involving protein–protein interactions (Otsuka et al., 2000; Tsutsumi et al., 2002). Modulation of these and other cellular components by core protein alters the gene expression pattern and affects important cellular processes, including apoptosis, cell growth, and transformation (Cho et al., 2001; Hahn et al., 2000; Machida et al., 2001; Moriya et al., 1998; Ray et al., 1998, 2000; Ruggieri et al., 1997; Zhu et al., 1998).

Specific transcription involves the assembly of a multi-protein complex at the gene enhancer/promoter regions, which is composed of specific transcription factors, general transcription machinery and co-activators such as p300/CBP among others. p300/CBP functions as a bridge due to its ability to interact both with transcription factors and the basal transcription machinery (Chan and La-Thangue, 2001; Goodman and Smolik, 2000; Shikama et al., 1997). In addition, p300/CBP has histone acetyltransferase activity, which results in relaxation of chromatin structure to facilitate the transcription process (Chan and La-Thangue, 2001). More recently, it has been described that other proteins involved in transcription may also be targets of this acetyltransferase activity (Barlev et al., 2001; Boyes et al., 1998; Imhof et al., 1997; Lu et al., 2003; Martinez-Balbas et al., 2000). Many regulatory proteins of oncogenic viruses target p300/CBP to control the expression of viral and cellular genes (Goodman and Smolik, 2000); however, this mechanism of transcriptional regulation has not been explored so far for HCV core protein.

Although HCV is mainly hepatotrophic, it has been shown that this virus may also infect cells of the immune system (Lerat et al., 1998; Sung et al., 2003). Thus, it is conceivable that HCV may also induce in these cells the expression of molecules involved in the immune and inflammatory responses. In this regard, it has been shown that core protein activates the IL-2 promoter and nuclear factor of activated T cells (NF-AT)-dependent transcription in T lymphocytes (Bergqvist and Rice, 2001), by a mechanism involving cytosolic calcium mobilization and requiring the C-terminal hydrophobic portion of core (Bergqvist et al., 2003).

NF-AT is a family of transcription factors that includes four related members, NF-AT 1–4. In resting cells, NF-AT proteins are phosphorylated in the NF-AT homology region and retained in the cytoplasm. When cells are activated by calcium mobilization stimuli, the calcium/calcmodulin-dependent phosphatase calcineurine is activated and dephosphorylates NF-AT proteins (Rao et al., 1997). Then, NF-AT proteins translocate to the nucleus where they bind to their specific DNA sequences either alone or cooperatively with transcription factors of the AP-1 family to form composite NF-AT:AP-1 sites (Rao et al., 1997). Once NF-AT proteins have targeted their recognition DNA sequences, they are further functionally regulated by interactions with transcriptional co-activators and by posttranslational modifications (Avots et al., 1999; Garcia-Rodriguez and Rao, 1998, 2000; San-Antonio et al., 2002).

NF-AT1 is the founding member of the NF-AT family and is expressed in several cells of the immune system as well as in other nonimmune tissues (Rao et al., 1997). In addition, NF-AT1 is the only family member that is expressed in resting T lymphocytes (Lyakh et al., 1997). It contains a strong acidic transcription activation domain (TAD) in the first 100 amino acids of the protein, not conserved in other family members (Rao et al., 1997), which is bound and regulated by the co-activator p300/CBP (Garcia-Rodriguez and Rao, 1998).

The aim of this study was to determine whether HCV core was a p300/CBP-regulating protein. Herein, we show that nuclear core protein interacts with p300/CBP, regulates both its transcriptional and histone acetyltransferase activities, and cooperates with calcium-mobilizing stimuli to further induce NF-AT-mediated transcription and the activity of NF-AT1-TAD, apparently through the activation of p300/CBP function.

Results

Direct interaction of core protein with the co-activator p300/CBP

The co-activators p300 and CBP are highly related proteins that present conserved domains and functions, thus hereafter they will be described as p300/CBP, unless otherwise specified. To determine whether core protein interacts with p300/CBP, a GST-based pull-down assay was performed. The fusion protein GST-core 1–191, but not GST, interacted with endogenous p300 from a cellular lysate of HEK293 cells (Fig. 1A). To study the in vivo interaction of these proteins, HEK293 cells were co-transfected with the expression vector pEF-core 1–153, encoding nuclear core protein (data not shown), along with pCI-Flag-p300, encoding Flag-tagged p300, or the control empty vector. Lysates from transfected cells were immunoprecipitated using an anti-p300 mAb and immunoblotted with anti-core or anti-Flag mAbs. The amount of core protein in the precipitates from transfected cells was immunoprecipitated using an anti-p300 mAb and immunoblotted with anti-core or anti-Flag mAbs. The amount of core protein in the precipitates increased dramatically when cells were co-transfected with both expression plasmids (Fig 1B).

To identify the region of p300/CBP involved in the interaction with core protein and to determine whether the
interaction could be direct, a GST-based pull-down assay
was performed, employing different GST-CBP constructs
and in vitro-translated $^{35}$S-core 1–191 protein. GST-fused
sequences 1098–1877, 1098–1620, and 1679–1858 of CBP
interacted with $^{35}$S-core 1–191, whereas no interaction
was observed with the NH$_2$-terminal fragment of CBP spanning
amino acids 1 to 1099 (Fig. 1C). This result suggests that
the core–CBP interaction is direct. However, the possibility
that a protein of reticulocyte lysate, employed for in vitro
translation, is mediating the interaction cannot be excluded.

Sequence alignment of p300 and CBP showed that the
regions of CBP involved in the interaction with core
protein are highly conserved in p300 protein (data not
shown).

Core protein induces the transcriptional and histone
acetyltransferase activities of p300/CBP

The transcriptional activity of the co-activator p300/CBP
may be studied by the Gal4 reporter system using a chimeric

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**Fig. 1.** Direct interaction of core protein with the co-activator p300/CBP. (A) Pull down assay with GST-core 1–191 or GST and a cellular lysate of HEK293
cells. Bound proteins were subjected to Western blot analysis using an anti-p300 mAb. Coomassie brilliant blue staining of 1/10 of the GST proteins used
shows full-length and degradation products of the chimeric protein (bottom). (B) Cellular lysates of HEK293 cells co-transfected with 5 µg of pEF-core 1–153
and 5 µg of pCI-Flag-p300 or a control vector were immunoprecipitated with an anti-p300 mAb. Immunoprecipitates and cellular lysates were analyzed using
anti-core mAb and anti-Flag M2 mAb. (C) Pull-down assay with different GST-CBP constructs and in vitro translated $^{35}$S-labeled core protein 1–191 (top).
One microliter of $^{35}$S-labeled core protein was run as a control (input). Coomassie brilliant blue staining shows 1/10 of the GST proteins used (middle). Schematic representation of the CBP constructs employed and summary of the core-binding activity observed (bottom). p300/CBP domains: RID, nuclear
hormone receptors interaction domain; CH1/2/3, cysteine/histidine rich domains 1/2/3; KIX, kinase inducible domain/CREB-binding domain; Br,
bromodomain; HAT, histone acetyltransferase domain.
Gal4-CBP construct (Chawla et al., 1998). To determine whether the core-p300/CBP interaction affected the transcriptional activity of the co-activator, Jurkat cells were co-transfected with the reporter plasmid pGal4-Luc along with the expression vectors pGal4-CBP and pEF-core 1–153, or the control plasmids. Co-expression of nuclear core protein increased the transcriptional activity of Gal4-CBP, with activations up to fivefold over the values obtained in the absence of the viral protein (Fig. 2A). These results suggest that nuclear core protein interacts with p300/CBP and induces its transcriptional activity.

Given that the region of p300/CBP that interacted with core contained the histone acetyltransferase domain (HAT) (Fig. 1C), the effect of core protein on the HAT activity was analyzed. To this end, HEK293 cells were co-transfected with the expression vectors pCI-Flag-p300 and pEF-core 1–153 or the control empty vector pEF1, and then Flag-p300 was immunoprecipitated with anti-Flag mAb and used for in vitro acetylation assay. As shown in Fig. 2B, the ability of Flag-p300 to acetylate histones was increased when core 1–153 was co-expressed. Similar levels of Flag-p300 were immunoprecipitated both in the presence and absence of core protein (lower panel).

**Nuclear forms of core protein induce NF-AT-dependent transcription**

The ability of core protein to induce the IL-2 promoter in cooperation with different stimuli is abolished by deletion of its COOH-terminal domain (Bergqvist and Rice, 2001), which suggests that core function is dependent of its subcellular localization. To investigate whether this domain of core was required for cooperative activation of NF-AT-dependent transcription, Jurkat cells were co-transfected with pNF-AT-Luc, containing three copies of the composite NF-AT/AP-1 site of the human IL-2 enhancer element, along with expression vectors coding for full-length core (pEF-core 1–191) or COOH-terminal truncated core proteins (pEF-core 1–173 and pEF-core 1–153), which localize predominantly in the cytoplasmic extract and in the nuclear insoluble fraction, respectively (data not shown). The transfected cells were stimulated either with PMA, to activate only the AP-1 element of the composite NF-AT/AP-1 site, or with PMA plus calcium ionophore (hereafter PMA + Io), which provides a full stimulus for this composite site. As expected, PMA + Io induced the reporter plasmid pNF-AT-Luc up to 30-fold, whereas PMA alone was not sufficient to activate this construct (Fig 3A). Only core protein 1–191 cooperated with PMA treatment, with activations of eight- to ninefold. In contrast, both full-length and truncated core proteins (core 1–173 and core 1–153) exerted a cooperative effect with PMA + Io in the activation of the NF-AT enhancer (Fig. 3A). No effect of core protein was observed in the absence of cellular activation (data not shown). In summary, these results indicate that nuclear accumulation of core protein, observed even in core 1–191-transfected cells, synergize with PMA + Io to further induce NF-AT-dependent transcription.

To analyze whether core protein was able to induce the NF-AT1 transactivation domain (TAD) activity once this factor had targeted its recognition sequences, a Gal4-derived reporter system was used. Jurkat cells were co-transfected with the chimeric vectors pGal4-NF-AT1 (1–415), pGal4-NF-AT1 (1–171), or the parental vector pRSV-Gal4-DBD, along with the reporter plasmid pGal4-Luc and either pEF-core 1–191 or the control vector pEF1. Both chimeric proteins Gal4-NF-AT1 (1–415) and (1–171) contained the NH2-terminal TAD of NF-AT1 and activated the reporter plasmid pGal4-Luc (Fig. 3B). As expected, core 1–191 did not stimulate transcription when co-transfected with the control plasmid pRSV-Gal4-DBD (Fig. 3B). In contrast, the transcription of pGal4-Luc was induced by the full-length core protein when co-transfected with both Gal4-
NF-AT1 chimeric proteins, suggesting that the viral protein was able to induce the NF-AT1-TAD activity (Fig. 3B). Interestingly, when the truncated forms of core (1–173 and 1–153) were included in the Gal4 reporter assays, statistically significant higher transactivation abilities of these mutants, compared with core 1–191, were observed (Fig. 3C). In addition, a construct containing core 1–153 fused to an endoplasmic reticulum signal peptide (core 1–153-ER), which showed a partial decrease of nuclear accumulation, displayed a statistically significant parallel reduction of its ability to induce the NF-AT1-TAD (Fig. 3D). To analyze whether the effect of nuclear core protein on NF-AT1 transcription depended on p300/CBP function, the Gal4 reporter assay was performed in the presence of the p300/CBP-inhibiting adenoviral protein E1A. As shown in Fig. 3E, E1A protein inhibited the core-protein-mediated induction of Gal4-NF-AT1 (1–171) transcriptional activity, suggesting that p300/CBP might mediate transactivation function of core.

Core protein interacts indirectly with the NH2-terminal transactivation domain of NF-AT1

To analyze whether core protein was able to interact with the NH2-terminal transactivation domain of NF-AT1 (NF-AT1-TAD), a GST-based pull-down assay was performed. HEK293 cells were transfected with pEF-core 1–191 and cellular lysates were used in pull-down assays with different HA-tagged NF-AT1 fragments fused to GST. As shown in Fig. 4A, core protein interacted with the first 68 amino acids of NF-AT1, which are located within the NH2-terminal TAD of this transcription factor. No interaction was observed when the control proteins GST or GST-HA were employed in the pull-down assay. A similar binding was observed when the pull-down assay was performed with GST-core 1–153 constructs and cellular lysates of HEK293 cells transfected with pEF-HA-NF-AT1 (1–384) (Fig. 4B). To determine whether core-NF-AT1 interaction was direct, a pull-down assay was performed
using in vitro-translated $^{35}$S-labeled core protein 1–191 and GST-NF-AT1 (1–384). As shown in Fig. 4C, in vitro-translated core protein did not bind to NF-AT1 unless the binding was forced in the presence of the thiol-cleavable cross-linker DSP. In agreement with these results, the binding of core to NF-AT1 could not be observed in vivo by co-immunoprecipitation assays (data not shown). Taken as a whole, these results suggested that the induction of the transcriptional activity NF-AT1-TAD by core protein was not mediated by a direct interaction between these two proteins.

**Deacetylase inhibitors induce the NF-AT-dependent transcription and mask nuclear core protein function**

To analyze whether acetylation might regulate NF-AT1-dependent transcription, the activity of Gal4-NF-AT1 (1–171) was assessed in the presence of increasing amounts of the deacetylase inhibitor sodium butyrate. The activity of NF-AT1-TAD increased in a dose-dependent manner by sodium butyrate (Fig. 5A, left panel). Similar activation of NF-AT1-TAD was observed with another deacetylase inhibitor, trichostatin A (Fig. 5A, central panel). In addition, sodium butyrate further induced the expression of the reporter plasmid pNF-AT-Luc over the values obtained with PMA + Io stimulation (Fig. 5A, right panel). These results suggest that NF-AT1-dependent transcription is regulated by acetylation of histones and/or components of the transcriptional machinery.

Interestingly, when the effect of nuclear core 1–153 was analyzed on sodium butyrate-induced Gal4-NF-AT1 (1–171) activity, a gradual mask of core function was observed as the concentration of sodium butyrate increased (Fig. 5A, left panel). It is important to note that the cooperative effect of core and sodium butyrate in the activation of NF-AT1-TAD was synergistic at low concentration of the inhibitor (0.5mM) and tended to be additive as the concentration of sodium butyrate increased. In addition, core 1–153 hardly increased TSA-induced Gal4-NF-AT1 (1–171) activity (Fig. 5A, central panel) or the expression of pNF-AT-Luc induced with PMA + Io and sodium butyrate (Fig. 5A, right panel). To rule out that the apparent loss of core function was due to a reduction of core protein expression or to a change in its subcellular localization, Jurkat cells were transfected with pEF-core 1–153 and treated with sodium butyrate. Similar expression levels and subcellular localization of core 1–153 were observed both with and without sodium butyrate (data not shown). In addition, core–CBP interaction was maintained in the presence of sodium butyrate and only a marginal decrease in core–NF-AT1 interaction was observed, which would not explain the mask of core protein function by deacetylase inhibitors (Fig. 5B). Interestingly, the cooperative activity of PMA and core 1–191 was not blocked by sodium butyrate (Fig. 5C), suggesting that the masking effect of the deacetylases inhibitors was specific for the nuclear function of core. These results, in addition to those
shown in Figs. 2 and 3, suggest that nuclear core protein might activate NF-AT1-dependent transcription by activation of the acetyltransferase activity of p300/CBP, which results in the acetylation of histones and/or unidentified component(s) of the transcriptional complex.

**Discussion**

During the last few years, acetylation has emerged as an important posttranslational modification to regulate the function of proteins involved in transcriptional control, including general and specific transcription factors (Barlev et al., 2001; Boyes et al., 1998; Imhof et al., 1997; Lu et al., 2003; Martinez-Balbas et al., 2000). The co-activator p300/CBP has intrinsic histone acetyltransferase (HAT) activity, which regulates not only the chromatin structure but also the activity of numerous proteins of the transcriptional complex. It has been shown that the HAT activity of p300/CBP may be regulated by different cellular factors and by several viral proteins (Ait-Si-Ali et al., 1998; Deng et al., 2001; Hamamori et al., 1999; Soutoglou et al., 2001; Zhao et al., 2003). Therefore, this co-activator should not be considered as a mere component of the transcriptional complex, whose only regulatory function is to bridge specific transcription factors with the basal transcriptional machinery.

In this study, we demonstrate that HCV core protein interacts both in vivo and in vitro with p300/CBP and induces its transcriptional and HAT activities. Furthermore, we demonstrate that core protein activates the transcriptional activity of the p300/CBP-regulated protein NF-AT1, once this transcription factor has targeted its recognition sequences in the nucleus.
Other viral proteins known to induce NF-AT1 activity through a nuclear localization are Tat of HIV-1 (Macián and Rao, 1999) and HBx of HBV (Carretero et al., 2002). These two viral proteins induce the NF-AT1-TAD activity by a direct protein–protein interaction. In contrast, the interaction of core with NF-AT1 did not appear to be direct. In an attempt to identify the mechanism by which core protein induces the NF-AT1-TAD activity, we employed the adenoviral protein E1A 12S, which inhibits the activity of p300/CBP by interacting with and inhibiting its HAT activity (Goodman and Smolik, 2000; Hamamori et al., 1999) and our results suggested that core protein might enhance NF-AT1 activity by regulating p300/CBP function and the acetylation state of histones and/or components of the transcriptional machinery. First, the HAT activity of p300 is increased in the presence of core protein. And second, there is a gradual mask of nuclear, but not of cytoplasmic, core function in the presence of deacetylases inhibitors. Given that the acetylation state of cellular proteins is the result of counteracting activities achieved by acetyltransferases and deacetylases, an increase in acetylation level may be obtained by stimulating acetyltransferases and deacetylases. Therefore, it can be hypothesized that core protein is not able to further increase the acetylation level induced by deacetylases inhibitors, being the effect of core protein masked by these agents.

In agreement with our results, it has been recently reported that the expression of core protein induces the hyperacetylation of p53 at Lys$^{373}$ and Lys$^{382}$ residues, which are specific targets for p300/CBP HAT activity, leading to enhanced DNA-binding activity of this transcription factor (Kao et al., 2004). Interestingly, the Lys$^{320}$ residue of p53, which is acetylated by PCAF, is not affected by core expression (Kao et al., 2004). Our results provide evidence, for the first time, that both NF-AT-dependent transcription and NF-AT1-TAD activity are regulated by acetylation. It would be worth to analyze whether NF-AT1 and/or other proteins involved in the transcriptional complex built from NF-AT1 could be the target of this posttranslational modification induced by core protein.

Previous studies have shown that core protein synergize strongly with incomplete stimuli, which do not mobilize calcium, to activate IL-2 promoter and NF-AT-dependent transcription in T lymphocytes, suggesting that core protein may compensate the calcium signal (Bergqvist and Rice, 2001). In agreement with this observation, expression of full-length core protein induces an increase in the levels of cytosolic calcium and spontaneous calcium oscillations in T cells (Bergqvist et al., 2003). The effect of core on calcium mobilization appears to be mediated by the insertion of core in the endoplasmic reticulum (ER) membrane, through its COOH-terminal hydrophobic sequence, which causes calcium leakage (Bergqvist et al., 2003). Herein, we show that both full-length and C-terminal truncated versions of core protein, which do not interact with the ER membrane, are able to cooperate with calcium-mobilizing stimuli to further induce NF-AT1-dependent transcription and to increase the activity of NF-AT1-TAD. We propose a novel mechanism for activation of NF-AT1, and probably other transcription factors, by core, involving the interaction of nuclear core with and activation of the co-activator p300/CBP. Our results point to core protein as a new p300/CBP HAT-inducing protein and add new insight to understand the transcriptional regulation by HCV in the infected cells.

Materials and methods

Cell culture and plasmid constructs

The human T lymphoma cell line Jurkat and HEK (human embryonic kidney) 293 cells were grown as previously described (Carretero et al., 2002).

The expression vector pEF1α has been previously described as pcDEF (Zhu et al., 1998). The expression vectors pEF-core 1–191, 1–173, and 1–153 were generated by subcloning, into pEF1α, PCR fragments encoding amino acids 1–191, 1–173, and 1–153 of core protein, respectively, from HCV genotype 1b. The expression vector pEF-core 1–153-ER was obtained by fusing in COOH-terminus of core 1–153 the endoplasmic reticulum signal peptide GWSCIIILFLVATATGAHS from vector pEF/myc/ER (Invitrogen, The Netherlands). The PCR fragment encoding amino acids 1 to 191 of core protein was cloned into pcDNA3.1 vector (Invitrogen), and this vector was employed for in vitro synthesis of core protein.

The plasmids pGST-core 1–191 and 1–153 were constructed by subcloning into pGEX-2T (Amersham Bioscience, UK), fragments coding for core 1–191 or 1–153. The plasmid pCI-Flag-p300, encoding full-length Flag-tagged p300, and the vectors pGST-CBP 1–1099, 1098–1877, 1098–1620, and 1679–1858, coding for the indicated CBP protein amino acid sequences fused to the GST protein, were kindly provided by Dra. A. Aranda (Instituto de Investigaciones Biomédicas, Madrid, Spain). The plasmid pGal4-CBP, coding for a human/mouse CBP chimera fused to the DNA binding domain (DBD) of yeast Gal4 protein, has been previously described (Chawla et al., 1998). The expression vector pE1A, encoding the wild-type E1A 12S adenoviral protein, was kindly provided by Dr. J.C. Lacal (Instituto de Investigaciones Biomédicas). The plasmids pSRVGal4-DBD, pGal4-NF-AT1 1–415, and pGal4-NF-AT1 1–171 have already been described (Luo et al., 1996) and were kindly provided by Dr. A. Rao (Harvard Medical School, Boston, MA). The plasmid pEF-HA-NF-AT1 1–384, encoding a C-terminal deleted HA-tagged murine NF-AT1, has been described elsewhere (Carretero et al., 2002). The plasmids pGST-HA, pGST-HA-NF-AT1 1–68, and pGST-HA-NF-AT1 1–57 have been previously described (Carretero et al., 2002). The construct pGST-HA-NF-AT1 1–384.
was generated by cloning into pGEX-4T-2 (Amersham) a PCR fragment encoding amino acids 1–384 of murine HA-tagged NF-AT1. The reporter construct pNF-AT-Luc contains three tandem copies of a composite NF-AT/AP-1 binding site of the human IL-2 promoter fused to the minimal IL-2 promoter (Durand et al., 1988) and was provided by Dr. G. Crabtree (Stanford University Medical School, Stanford, CA). The reporter plasmid pGal4-Luc, bearing five copies of the Gal4-binding sites upstream of the luciferase gene, was a kind gift from Dr. R. Perona (Instituto de Investigaciones Biomédicas, Madrid, Spain).

Transfection and luciferase assay

Jurkat cells were transfected with the indicated plasmids, employing Lipofectin (Gibco-BRL, Gaithersburg, MD) according to manufacturer’s recommendations. At 24 h after transfection, cells were either left untreated or stimulated with PMA, Iq, sodium butyrate, and/or trichostatin A (TSA) for 12 h. Then, cells were harvested and cellular extracts were assayed for luciferase activity using a Lumat LB9501 luminometer (Berthold, Wildbad, Germany).

Nuclear and cytoplasmic extracts

Jurkat cells were transfected, by electroporation, with 40 μg of expression vector pEF-core 1–191, 1–173, or 1–153 using an electrical pulse of 0.28 kV and 1200 micro-Faradays (μF). At 36 h after transfection, cells were either lysed with buffer Laemmli or separated into cytoplasmic and nuclear soluble fractions, employing the NE-PER Nuclear and Cytoplasmic Extraction Reagents from Pierce (Rockford, IL). After extraction of the nuclear soluble fraction, the remaining insoluble material was resuspended in Laemmli buffer and it is called nuclear-insoluble fraction. Purity of the different fractions was determined by checking in these extracts the presence of the cytoplasmic marker tubulin and of the nuclear marker Sp1, by Western blot assay, employing an anti-tubulin monoclonal antibody from Sigma (St. Louis, MO) and an anti-Sp1 polyclonal antibody from Santa Cruz (Santa Cruz, CA). The presence of core protein in the different proteins was determined by Western blot analysis using an anti-core monoclonal antibody (mAb) from Affinity BioReagents (Goleen, CO).

HEK293 cells were co-transfected with 5 μg of expression plasmid pEF-core 1–153 and 5 μg of expression plasmids pCI-Flag-p300 or the empty vector, using FuGENE 6 transfection reagent (Roche). Cells were lysed in buffer containing 150 mM NaCl, 50 mM Tris–HCl pH 7.4, 0.25% NP-40, 1 mM EGTA, 1 μg/ml aprotinin, and 1 μg/ml leupeptin, and precleared with protein A-Sepharose (Amersham Pharmacia Biotech). Precleared lysates were then immunoprecipitated with anti-p300 mAb from Oncogene. Bound proteins were separated on a SDS-polyacrylamide gel and analyzed by Western blotting using anti-Flag M2 mAb (Sigma) and anti-core mAb.

Histone acetylation assays

HEK293 cells were co-transfected with 5 μg of pCI-Flag-p300 and 5 μg of pEF-core 1–153 or pEF1α, using FuGENE 6 transfection reagent. Cells were lysed and cellular lysates were immunoprecipitated, as described in the previous section, with an anti-Flag M2 mAb. Immunocomplexes were then resuspended in acetylation buffer containing 20 mM HEPES pH 7.4, 1 mM DTT, 10 mM sodium butyrate, 1 μg histones (type IIA from Sigma) and 0.5 μCi [14C]-acetyl coenzyme A (Amersham Pharmacia Biotech) and reactions were performed at 37 °C for 1 h. Then, samples were run in SDS-PAGE. Radioactive signal
was increased by incubating fixed gel with Amplify reagent and visualized by autoradiography.

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References


