The histone acetyltransferases CBP/p300 are degraded in NIH 3T3 cells by activation of Ras signalling pathway

Sara SÁNCHEZ-MOLINA*, José Luis OLIVA†, Susana GARCÍA-VARGAS†, Ester VALLS*, José M. ROJAS† and Marian A. MARTÍNEZ-BALBÁS*[1]

*Instituto de Biología Molecular de Barcelona, CID, Consejo Superior de Investigaciones Científicas (CSIC), Parc Cientific de Barcelona (PCB), Josep Samitier 1–5, 08028 Barcelona, Spain, and †Unidad de Biología Celular, Centro Nacional de Microbiología, Instituto de Salud Carlos III, 28220 Majadahonda, Madrid, Spain

The CBP [CREB (cAMP-response-element-binding protein)-binding protein]/p300 acetyltransferases function as transcriptional co-activators and play critical roles in cell differentiation and proliferation. Accumulating evidence shows that alterations of the CBP/p300 protein levels are linked to human tumours. In the present study, we show that the levels of the CBP/p300 co-activators are decreased dramatically by continuous PDGF (platelet-derived growth factor) and Ras signalling pathway activation in NIH 3T3 fibroblasts. This effect occurs by reducing the expression levels of the CBP/p300 genes. In addition, CBP and p300 are degraded by the 26 S proteasome pathway leading to an overall decrease in the levels of the CBP/p300 proteins. Furthermore, we provide evidence that Mdm2 (murine double minute 2), in the presence of active H-Ras or N-Ras, induces CBP/p300 degradation in NIH 3T3 cells. These findings support a novel mechanism for modulating other signalling transduction pathways that require these common co-activators.

Key words: acetylation, cAMP-response-element-binding-protein-binding protein/p300 (CBP/p300), histone acetyltransferase activity (HAT activity), murine double minute 2 (Mdm2), NIH 3T3 cell, Ras pathway.

INTRODUCTION

For many years, acetylation of the histone N-terminal tails has been associated with transcriptional activation [1]. Histone acetylation is performed by HATs (histone acetyltransferases), whereas the reverse deacetylation process is conducted by HDACs (histone deacetylases) [2]. Different HAT families have been characterized among CBP [CREB (cAMP-response-element-binding protein)-binding protein) and p300, the GNAT (GCN5-related N-acetyltransferase) superfamily, the MYST (named after MOZ, Ybf2/Sas3, Sas2 and Tip60) family, nuclear receptor co-activators, TAF_{II} (TATA-box-binding-protein-associated factor) 250 and the TFIIIC (transcription factor IIIC) family [2]. The global co-activators CBP and p300 not only exhibit histone acetyltransferase activity [3,4], but also acetylate non-histone proteins, such as transcription factors [5–10], thereby highlighting the potential importance of HAT activity in CBP/p300 function. CBP/p300 also acts as a transcriptional co-activator [11,12], by binding numerous transcription factors. Viral oncoproteins, such as SV40 (simian virus 40) T antigen or E1A, also specifically target these proteins [13]. As transcriptional co-activators, CBP/p300 are involved in multiple signal-dependent transcriptional events. Many signals from the environment are thought to induce gene transcription by activating intracellular biochemical pathways [Ras, BMP (bone morphogenetic protein), TGF-β (transforming growth factor β), etc.], which control the ability of transcription factors to recruit CBP/p300 to specific promoters. CBP and p300 have been postulated to integrate the different signals of gene expression by serving as essential co-factors of STAT1 (signal transducer and activator of transcription 1), Smad, AP-1 (activator protein 1) and c-Myc transcription factors. In this way, they modulate gene-specific responses to simultaneous activation of two or more signalling transduction pathways, driven by competing transcription factors based on limiting amounts of CBP/p300 [14]. Given that many transcriptional activators, which respond to different cellular pathways, can recruit CBP/p300 to a promoter, control of either CBP/p300 protein levels (by regulating CBP/p300 mRNA levels or protein degradation) and/or CBP/p300 activity may be of general importance for signal-regulated transcription.

Ras proteins operate as molecular switches in signal transduction cascades controlling cell proliferation, differentiation, or apoptosis, and, like all G-proteins, are controlled by a regulated GTP/GDP cycle [15]. The mammalian genome contains three *ras* genes that encode highly related proteins of 21 kDa termed H-Ras, N-Ras and K-Ras with its two variants, K-Ras4A and K-Ras4B, generated from two alternative fourth exons. Each of the three human *ras* proto-oncogenes can give rise to a transforming oncogene via single base mutations. Mutations at codons 12, 13 or 61 significantly downgrade the GTPase ability of the resulting mutant Ras proteins, which are thus rendered constitutively active and able to transform mammalian cells. Once activated, Ras proteins interact with effector proteins, mainly Raf, PI3K (phosphoinositide 3-kinase) and Ral-GDS (guanine nucleotide dissociation stimulator) [16], resulting in the activation of downstream signalling pathways, such as the Raf/MEK [MAPK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase) kinase]/ERK, PI3K/Akt or the Ral-GDS/Ral A

Abbreviations used: ALLN, *N*-acetyl-L-leucyl-L-leucylnorleucinal; CREB, cAMP-response-element-binding protein; CBP, CREB-binding protein; CS, calf serum; DAPI, 4′,6-diamidino-2-phenylindole; ERK, extracellular-signal-regulated kinase; GDS, guanine nucleotide dissociation stimulator; GST, glutathione S-transferase; HA, haemagglutinin; HAT, histone acetyltransferase; HDAC, histone deacetylase; MAPK, mitogen-activated protein kinase; Mdm2, murine double minute 2; MEK, MAPK/ERK kinase; P/CAF, p300/CBP-associated factor; PDGF, platelet-derived growth factor; PI3K, phosphoinositide 3-kinase; RA, retinoic acid; Ral-BD, Ral-binding domain; RTS, Rubinstein–Taybi syndrome; siRNA, small interfering RNA; TAF_{II}, TATA-box-binding-protein-associated factor; TGase, transglutaminase; TK, thymidine kinase.

[1] To whom correspondence should be addressed (email mmbbmc@ibmb.csic.es).

pathway [17,18] leading ultimately to the transcriptional activation of genes [15]. The high degree of sequence homology, coupled with the highly similar capability of mutated forms of H-Ras, the two K-Ras isoforms (4A and 4B) and N-Ras to cause transformation of NIH 3T3 fibroblasts and other cell types, supports the idea that all Ras proteins have the same role *in vivo*. However, growing evidence suggests the possibility of non-redundant roles for the three Ras homologues [15,18–20]. The embryonic lethality seen in the *Kras*, but not *Hras* or *Nras*, knockout mice also lends credence to this idea [21].

Furthermore, CBP/p300 genes are altered in various human tumours [22,23], which is consistent with studies on $CBP^{+/-}$ mice, suggesting that CBP works as a tumour suppressor in the haematopoietic system [24]. Moreover, human patients with RTS (Rubinstein–Taybi syndrome), which results from CBP heterozygosity, also experience an increased incidence of malignancy [25,26]. The profound effects of CBP/p300 suppression, both in cell culture and in intact animals [24], allow us to predict that pathways leading to decreased CBP/p300 levels might sensitize cells to stimuli promoting apoptosis, cell proliferation or differentiation.

We undertook the present study to explore the relation of CBP and p300 proteins with continuous mitogenic stimuli and the putative signalling mechanisms controlling these HAT proteins, including the Ras pathway.

MATERIALS AND METHODS

Cell lines, transfections and antibodies

NIH 3T3 fibroblasts, CV1 and CV1COS cell lines were maintained in DMEM (Dulbecco's modified Eagle's medium) (Invitrogen) supplemented with 10 % CS (calf serum) and 10 % foetal bovine serum respectively (Invitrogen). Stable transfections were carried out using the calcium phosphate precipitation technique [20,27]. Transfected cell lines were also selected in medium supplemented, as appropriate, with 750 μg/ml Geneticin (Invitrogen). Transient transfections were performed with the Lipofectamine™ reagent (Invitrogen). Transfected cells were washed after 14 h and harvested 36 h after transfection. Finally, they were washed once in PBS. Proteasome inhibition was achieved by treatment with MG132 or ALLN (*N*-acetyl-L-leucyl-L-leucylnorleucinal) (Sigma). PDGF (platelet-derived growth factor) and the monoclonal antibodies anti-tubulin and anti-FLAG were purchased from Chemicon and Sigma–Aldrich respectively. Monoclonal antibodies against phospho-MAPK (p-ERK1/p-ERK2) and phospho-Akt proteins were purchased from New England Biolabs. Rabbit polyclonal antiserum against MAPK (ERK1/ERK2), anti-CBP (A-22, raised against a peptide not conserved on p300; mapping at the N-terminus of human CBP; does not cross-react with p300), anti-p300 (C-20, raised against C-terminus of human p300; does not cross-react with CBP), anti-Mdm2 (murine double minute 2), anti-polyubiquitin and anti-Akt were obtained from Santa Cruz Biotechnology; anti-Ral monoclonal antibody was purchased from Transduction Laboratories, and anti-HA (haemagglutinin) and anti-AU5 monoclonal antibodies were from the Berkeley Antibody Company.

DNA constructs

The plasmids pCEFL-KZ-AU5, pCEFL-KZ-AU5-H-Ras V12, pCEFL-KZ-AU5-K-Ras V12 and pCEFL-KZ-AU5-N-Ras V12, were described previously [20,27]. pCDNA3-HA-CBP has been described elsewhere [4,9]. pCDNA3-GAGA and pCDNA3-Flag-p300 were gifts from Dr J. Bernués (Instituto de Biología Molecular de Barcelona, CSIC) and Dr S. Pons (Instituto de Investigaciones Biomedicas de Barcelona, Barcelona, Spain) respectively. pCDNA3-CBP (ΔMdm2) (1098–2441) was a gift from Dr L. Vandel (Centre de Biologie du Developpement, UMR5547 du CNRS/Universite Paul Sabatier, Toulouse, France). pXJ-Mdm2 (expressing mouse Mdm2) and pXJ Mdm2 C462A were gifts from Dr B. Wasylyk (CNRS/INSERM/ULP, Illkirch, France) and Dr D. Trouche (Laboratoire de Biologie Moléculaire Eucaryote, UMR 5099 CNRS, IFR 109, Toulouse, France) [28]. pCMV-collagenase-luciferase and pCDNA3-HA-ubiquitin were gifts from Dr C. Caelles [Instituto de Recerca Biomédica (IRB), Barcelona, Spain] and Dr T. Thonson (Instituto de Biología Molecular de Barcelona, Barcelona, Spain) respectively.

Cell extract preparation

Total cell extracts were prepared in IPH buffer (50 mM Tris/HCl, pH 8, 150 mM NaCl, 5 mM EDTA and 0.5 % Nonidet P40) [4] by keeping the cells on ice for 20 min, followed by centrifugation at 8000 ***g*** for 10 min at 4 °C. All buffers contained protease inhibitors (Boehringer). Analysis of steady-state levels for CBP and p300 were conducted as follows: for endogenous CBP/p300, NIH 3T3 fibroblasts were lysed directly by boiling in Laemmli sample buffer or extracted using IPH buffer as indicated above; for transfected CBP/p300, cells were lysed in 100 μl of luciferase lysis buffer (Promega). Luciferase activity was measured according to the manufacturer's instructions. A volume of 50 μl of 4× Laemmli sample buffer was then added to the cells to prepare the total cell lysates. After boiling and centrifugation at 8000 ***g*** for 5 min, a defined amount of lysate (according to the luciferase activity) was collected and then adjusted to 25 μl with 1× Laemmli sample buffer, before being subjected to immunoblot analysis.

Cell proliferation assays

The measurement of doubling time and saturation density was carried out by a modification of a method described previously [11]. NIH 3T3 transfectants were seeded at a density of 4×10^4 cells/60-mm-diameter plate in 10 % CS-containing DMEM. Cells were counted 24 h later; this time point was considered day 0. The remaining cultures were incubated either in low- (0.5 %) or high- (10 %) serum-containing DMEM and replenished every 2 days. Cells were trypsinized and counted using a haemocytometer every 2 days. Some of the cells grown under high-serum conditions were allowed to reach confluence and were counted 8 days later.

Bacterial expression of fusion proteins

The plasmid pGEX-Ral-BD containing the Ral A-binding domain fused to GST (glutathione S-transferase) was kindly provided by Professor J. L. Bos (Department of Physiological Chemistry, University Medical Center Utrecht, Utrecht, The Netherlands). All GST-fusion proteins were purified [from *Escherichia coli* BL21 (DE3) harbouring these plasmids] following a method described previously [20].

Pull-down assays

Transfected cells were lysed in cold lysis buffer containing 25 mM Hepes, pH 7.5, 1 % Nonidet P40, 0.25 % (w/v) sodium deoxycholate, 10 % (v/v) glycerol, 1 mM EDTA, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM Na_3VO_4, 25 mM NaF, 1 mM PMSF and 10 μg/ml each of leupeptin, aprotinin, pepstatin A and trypsin inhibitor. Nucleus-free supernatants were incubated with GST–Ral-BD (Ral-binding domain) on glutathione–Sepharose beads and analysed as described previously [20].

HAT assays

In vitro acetylation assays were performed as described previously [29].

Immunoprecipitation and immunoblot analyses

Immunoprecipitation and immunoblot analyses were performed as described previously [20]. Quantifications from immunoblots were performed using Gene Tools from the Syngene program.

Reverse transcription–quantitative real-time-PCR

Total cellular RNA extracts were obtained using Ultraspec RNA Isolation system (Biotecx); subsequently, total RNA was reverse-transcribed to cDNA with the Omniscript reverse transcription kit (Qiagen). Differences in the RNA content from the four cell lines were determined by real-time PCR using the ABI 7700 sequence detection system and SYBR Green master mix protocol (Applied Biosystems). PCRs were carried out in triplicate using fixed amounts of template DNA from each cell line at 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. A standard curve was made for each amplicon by plotting the number of cycles at which the fluorescence crossed the threshold (crossing values) against increasing amounts of DNA template. All PCR products were approx. 50 bp long to increase the efficiency of real-time reactions. All experimental values were normalized to those obtained for tubulin.

Immunocytochemistry

Cells on coverslips were fixed in 4 % (w/v) paraformaldehyde in PBS for 30 min at room temperature (25 °C) and permeabilized with methanol for 10 min. After blocking with 3 % (w/v) BSA in PBS and 0.1 % (v/v) Tween 20 for 1 h at room temperature, coverslips were incubated with a 1:200 dilution of anti-HA antibody in PBS and 3 % (w/v) BSA for 2h, followed by incubation for 1 h with Cy3-conjugated goat anti-(mouse IgG), used at 1:250 dilution in PBS and 3 % (w/v) BSA. After every incubation with the antibody, coverslips were washed thoroughly with PBS and 0.05 % (v/v) Tween 20 four times for 10 min each at room temperature. The nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole).

Reporter gene analysis

NIH 3T3 cells and Ras V12-overexpressing cell lines were transfected with 2 μg of pCMV-collagenase-luciferase and 1 μg of TK (thymidine kinase)–*Renilla* luciferase in the presence or absence of pCDNA3-HA-CBP or pCDNA3-Flag-p300. Whole-cell extracts were used in the luciferase assays.

RNA interference

Target sequences for siRNAs (small interfering RNAs) for Mdm2 were as follows: sense sequences 5′-GGUUAUAUGACGAGAAGCAtt-3′, 5′-GGAGCACAGGAAAAUAUAUtt-3′, 5′-GGACUUUGGAAGUGUAUGUtt-3′, Mdm2 and non-specific control were synthesized by Ambion. The siRNAs were transfected twice using Jet-Pei™ (Polyplus-Transfection) at 24 h intervals.

RESULTS

CBP/p300 HAT activity and protein levels specifically decrease in NIH 3T3 fibroblasts overexpressing hyperactive Ras

We analysed the CBP and p300 proteins levels in NIH 3T3 fibroblasts at different times upon PDGF receptor stimulation, by immunoblot analysis using antibodies that specifically recognize either CBP or p300. In agreement with previous studies [30,31], this mitogenic stimulus induced ERK and Akt activation (Figure 1A). However, we also detected a significant decrease (60–80 %) of CBP and p300 levels (Figure 1B). Since ERK and Akt are activated by signalling pathways downstream of Ras, we analysed CBP/p300 co-activators following Ras activation. To this end, we used as a model NIH 3T3 cell lines stably transfected with H-Ras, N-Ras or K-Ras4B mutated at position 12, Ras V12 (which causes Ras proteins to become constitutively active) [20]. These transfectant cell lines displayed transforming activity (Figure 2) characterized by morphological alterations, disruption of actin stress fibres with high numbers of membrane ruffles and lamellipodia [20], cell growth alterations with significantly shorter doubling times and higher saturation densities than NIH 3T3 cells transfected only with the vector (control cells) (Figure 2A), and activation of the different Ras-dependent pathways (Figure 2B). We found a sharp decrease of CBP and p300 protein levels after H-Ras V12 and N-Ras V12 overexpression and a slight decrease after K-Ras4B V12 overexpression (Figure 3A, left-hand panel).

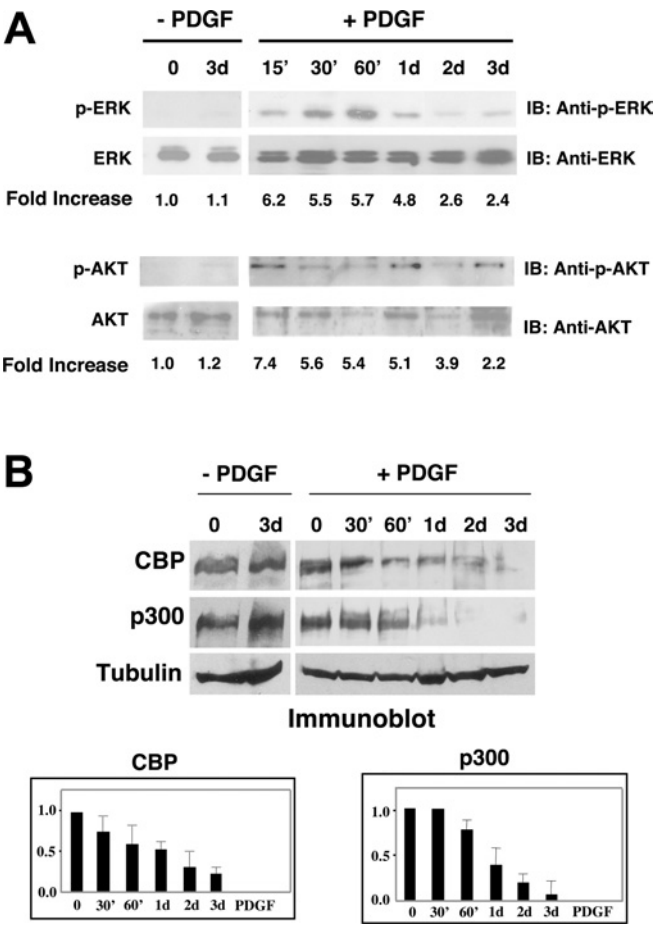

Figure 1 PDGF stimulation decreases CBP/p300 levels in NIH 3T3 cells

NIH 3T3 fibroblasts were stimulated with PDGF (1 mg/ml) for 0, 15 (15′), 30 (30′), 60 (60′) min, 1 day (1d), 2 days (2d) or 3 days (3d). Total cell extracts cells were prepared, the total protein amount was adjusted and analysed as (**A**) and (**B**). (**A**) The ERK (p42 and p44 proteins) and Akt phosphorylation levels were determined using specific anti-phospho- and full antibodies. The fold increase values of p-ERK and p-Akt are the means for three separate assays (in each case with a S.D. lower than 15 % of the average). (**B**) The CBP and p300 levels were monitored by immunoblot analysis using antibodies against CBP, p300 and tubulin (as an internal control). The diagrams shown relative levels of CBP and p300 after PDGF stimulation of NIH 3T3 cells. Results are means ± S.D. for three independent assays. IB, immunoblot.

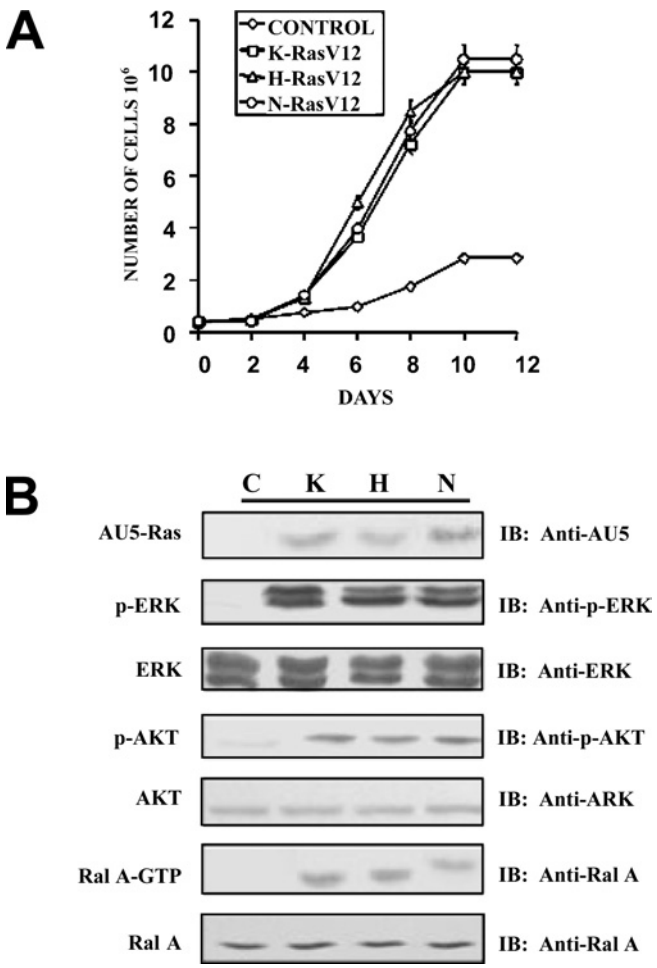

Figure 2 Ras V12-expressing cell lines display transforming activity

(**A**) Cell doubling times of NIH 3T3 clones overexpressing different H-, K- or N-Ras V12 mutants, or transfected only with the vector, were measured as described in the Materials and methods section. Doubling time was determined by counting cells every 2 days. Results are means ± S.D. for three independent experiments where each sample was analysed in triplicate. (**B**) The expression levels of AU5–Ras V12 proteins of the above NIH 3T3 clones were detected by immunoblotting (IB) with monoclonal anti-AU5 (top panel); cell extracts from NIH 3T3 fibroblasts transfected with the vector pCEFL-KZ-AU5 (control, C) were included as a negative control. All cells were serum-starved for 18 h and then the ERK (p42 and p44 proteins) and Akt phosphorylation levels were determined using specific anti-phospho- and full antibodies. Ral A-GTP was recovered from cell lysates by binding to immobilized GST–Ral-BD and detected by immunoblotting with the corresponding anti-(Ral A) monoclonal antibody. The expression levels of the endogenous Ral A protein were detected by immunoblotting of the cell extracts with the corresponding anti-(Ral A) monoclonal antibody. Results are from a representative of four separate experiments.

The CBP and p300 proteins were not absent from the H- and N-Ras V12-transformed cell extracts, since longer exposure allowed the detection of both CBP and p300 (Figure 3A, right-hand panel). In contrast with the observed fall in CBP/p300 levels, TAF_{II} 250, P/CAF (p300/CBP-associated factor) or HDAC1 levels did not change upon Ras V12 (H-, N- or K-Ras4B) overexpression (results not shown). To confirm that the decrease in CBP/p300 levels is not only a consequence of differences in cell proliferation, we analysed the CBP/p300 levels in CV1 and CV1 T-antigen-transformed (CV1COS) cell lines. T antigen induces proliferation mainly by inactivating pRb (retinoblastoma protein); this inactivation results in an activation of E2F-regulated genes that leads to cell proliferation [32]. We find (Figure 3B) that both CV1 T-antigen-transformed and non-transformed cells have similar CBP/p300 levels. This points to Ras pathway activation as being responsible for the observed decrease in CBP/p300 levels. According to these results, CBP and p300 HAT activities also decreased (by 50–80 %) following Ras V12 overexpression (Figure 3C). The activity of CBP/p300 HAT is similar for H-, K- and N-Ras V12; however, the CBP/p300 levels are higher in the case of K-Ras V12, suggesting the existence of an additional negative regulation of CBP/p300 HAT activity by K-Ras V12, as has already been proposed for other oncogenic proteins [33,34] (see also Figure 3D).

To investigate whether Ras V12 expression not only inhibits CBP/p300 HAT activity, but also inhibits CBP/p300-induced transcriptional activity, NIH 3T3 and Ras-overexpressing cell lines were transiently transfected with collagenase–luciferase vector (which is co-activated by CBP/p300), and the transcriptional activity of this promoter was analysed in the presence or absence of overexpressed CBP/p300. Figure 3(D) shows that CBP co-activates the collagenase promoter in NIH 3T3 control cells; however, this effect was very reduced in Ras V12-overexpressing cell lines. To confirm these results, we have analysed the effects of Ras pathway activation on the expression of an endogenous target gene, the TGase (transglutaminase) gene. It has been shown previously that EGF represses the RA (retinoic acid)-mediated induction of TGase gene in NIH 3T3 cells [35]; we decided to assess whether RA is still able to induce TGase gene in a H-Ras V12-overexpressing cell line. Real-time experiments in Figure 3(E) show that, after RA treatment, the TGase RNA levels increase in NIH 3T3 control cells, but not in H-Ras V12-overexpressing cells. Taken together, these results demonstrate a specific loss of CBP and p300 following overexpression of hyperactive Ras on NIH 3T3 cells, resulting in a fall in their transcriptional and HAT activities.

CBP/p300 are degraded through the ubiquitin–proteasome pathway in NIH 3T3 fibroblasts overexpressing Ras V12

We investigated the possible causes of CBP/p300 loss. Real-time reverse transcription–PCR analysis was performed, and the results revealed that CBP and p300 RNA levels were slightly lower in Ras-overexpressing cells than in control cells, mainly in the case of K-Ras V12 (Figure 4A). However, this decrease was not as dramatic as was the CBP and p300 decrease in protein levels, particularly in the case of H-Ras V12 and N-Ras V12, suggesting that the reduction in CBP/p300 protein levels could be due to an additional mechanism(s), most likely proteolytic processing. We therefore tested different protease inhibitors for their ability to reverse CBP and p300 degradation. The proteasome inhibitor ALLN reversed the CBP/p300 decrease observed by H-Ras V12, K-Ras V12 and N-Ras V12 overexpression (Figure 4B). Similar results (not shown) were obtained after treating the cells with MG132, another proteasome inhibitor. These results suggested that CBP/p300 may be degraded by the proteasome pathway in Ras V12-overexpressing NIH 3T3 fibroblasts. To confirm these results, we investigated whether the CBP/p300 half-life was shorter in Ras V12-overexpressing cells than in NIH 3T3 control cells. Unfortunately, we could not consistently detect endogenous CBP/p300 in the absence of proteasome inhibitors (see, e.g., Figure 3A, lanes 2 and 4). As the decrease of CBP/p300 levels was more dramatic for H-Ras V12 and N-Ras V12, we focused on the NIH 3T3 cells overexpressing these proteins to analyse the molecular mechanisms involved in the effects on CBP/p300.

We therefore tested whether CBP/p300 is ubiquitinated in these cells. To this end, NIH 3T3 cells were transfected with HA–ubiquitin and FLAG–p300 expression vectors in the presence or

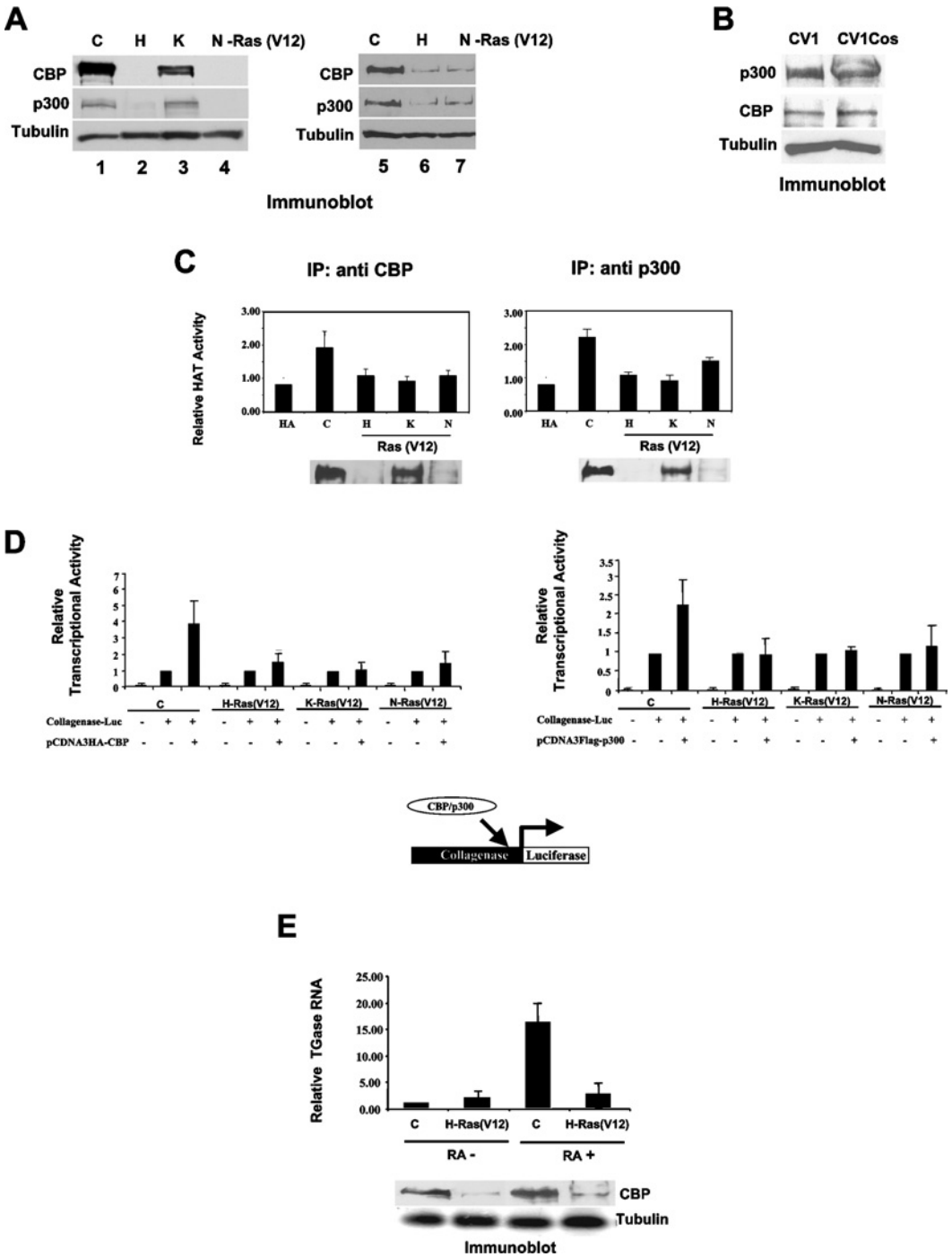

Figure 3 CBP/p300 levels and HAT activity decrease in NIH 3T3 fibroblasts overexpressing hyperactive Ras

(**A**) Total cell extracts from NIH 3T3 cells (control cells, C) and NIH 3T3 H-, K- and N-Ras V12 -overexpressing cells were prepared, the total protein amount was adjusted, and the CBP and p300 levels were monitored by immunoblot analysis using antibodies against CBP, p300 and tubulin (as a loading control). CBP and p300 are not absent from the extract, since longer exposure permits the detection of these proteins. Similar results were obtained when NIH 3T3 cells or NIH 3T3 Ras-overexpressing cells were lysed directly by boiling in Laemmli sample buffer. The results shown are representative of at least six independent experiments. (**B**) Total cell extracts from CV1 cells and CV1COS (T-antigen-expressing cells) were prepared, the total protein amount was adjusted, and the CBP and p300 levels were determined by immunoblotting analysis using antibodies against CBP, p300 and tubulin (as a loading control). (**C**) Total cell extracts from NIH 3T3 cells (control cells, C) and NIH 3T3 cells stably transfected with H-, K- and N-Ras mutated at position 12 (V12) and AU5-tagged were prepared, the total protein amount was adjusted, and CBP and p300 were immunoprecipitated (IP) using antibodies recognizing CBP and p300 proteins and HA protein (as a negative control). The HAT activity associated with the immunoprecipitated proteins was determined in an *in vitro* HAT assay. Results are means ± S.D. for three independent assays. Immunoblots using antibodies against CBP and p300, performed in parallel to the HAT assay, are shown beneath the histogram. (**D**) NIH 3T3 and H-, K- and N-Ras V12-overexpressing cells were transfected with 2 μg of collagenase–luciferase reporter and 2 μg of TK–*Renilla* luciferase reporter, in the presence or absence of pCDNA3-CBP or pCDNA3-p300 expression vectors. Whole-cell extracts were used in the luciferase assays. Results are means ± S.D. for at least eight independent transfections. The activity derived from the collagenase–luciferase reporter was normalized to 1.0 and the other activities are expressed relative to this value. (**E**) NIH 3T3 and H-RasV12-overexpressing cells were treated or not with 5 μM RA for 2 days in the presence of 1 % (v/v) CS. Total cellular RNA was obtained and the TGase RNA content from the two cell lines was determined by real-time PCR using SYBR Green. TGase RNA values were normalized to those obtained for tubulin RNA. Results are means ± S.D. for two independent experiments performed in triplicate. Immunoblots using antibodies against CBP and tubulin (as a control) of NIH 3T3 and H-Ras V12-overexpressing cells treated or not with RA are shown beneath the histogram.

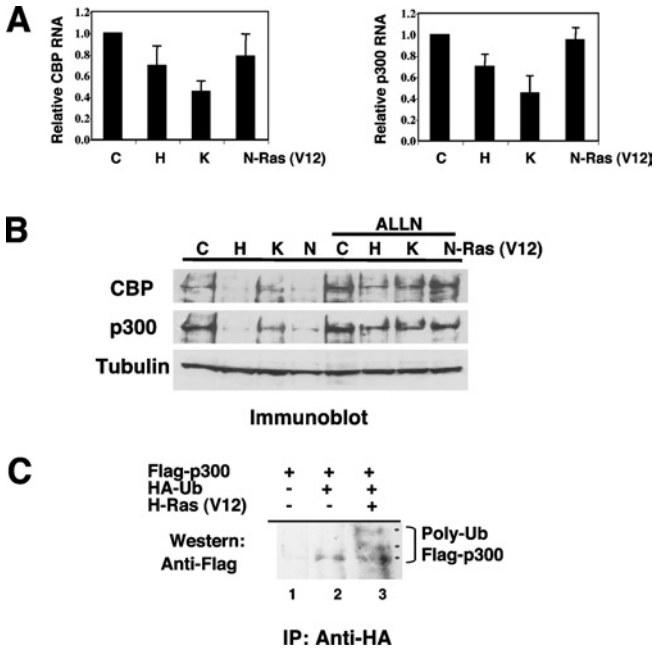

Figure 4 RNA and protein levels of CBP/p300 decrease in NIH 3T3 fibroblasts overexpressing hyperactive Ras

(**A**) Total cellular RNA from NIH 3T3 cells (control cells, C) and NIH 3T3 H-, K- and N-Ras V12-overexpressing cells was obtained and reverse-transcribed to cDNA. Differences in the RNA content from the four cell lines were determined by real-time PCR using SYBR Green. CBP and p300 RNA values were normalized to those obtained for tubulin RNA. Results are means ± S.D. for four independent experiments performed in triplicate. (**B**) Total cell extracts from NIH 3T3 cells and NIH 3T3 H-, K- and N-Ras V12-overexpressing cells treated with ALLN proteasome inhibitor were prepared, and the levels of CBP and p300 were analysed by immunoblot. Total protein amounts were loaded into the gels; subsequently, immunoblotting was conducted using an antibody against tubulin. (**C**) Whole-cell extracts from NIH 3T3 cells transfected with FLAG–p300, HA–ubiquitin and H-Ras V12 expression vectors and treated with MG132 for 12 h were used to immunoprecipitate ubiquitinated proteins, using anti-HA antibody. The immunoprecipitated proteins were analysed by the presence of polyubiquitinated p300 by immunoblotting using an antibody against the FLAG tag.

absence of H-Ras V12. HA–ubiquitinated proteins were immunoprecipitated, and the amount of ubiquitinated FLAG–p300 was determined by immunoblotting using anti-FLAG antibody. The level of p300 polyubiquitination increased, and some new bands appeared following H-Ras V12 overexpression (Figure 4C), suggesting that p300 is degraded in these cells through the ubiquitin–proteasome system.

Mdm2 induces degradation of CBP/p300

Since Mdm2 is responsible for the degradation of several HATs (Tip60, P/CAF) [36,37] and inhibits CBP/p300-mediated p53 acetylation by its interaction with these two proteins [38–40], we hypothesized that Mdm2 could be responsible for CBP/p300 degradation in NIH 3T3 cells overexpressing Ras V12. To test this hypothesis, we transfected NIH 3T3 cells with FLAG-tagged p300 expression plasmid, TK–*Renilla* luciferase reporter and GAGA expression plasmid as an internal control, in either the presence or the absence of Mdm2- and H-Ras V12-expressing vectors. After standardization using luciferase activity for transfection efficiency, we analysed total extracts for the expression levels of p300, Mdm2 and GAGA proteins by immunoblotting with antibodies against FLAG, Mdm2 and GAGA respectively. Co-expression of H-Ras V12 and Mdm2 led to a significant reduction in FLAG–p300 levels (Figure 5A, lane 4), whereas the amount of GAGA remained unaffected. To confirm these results,

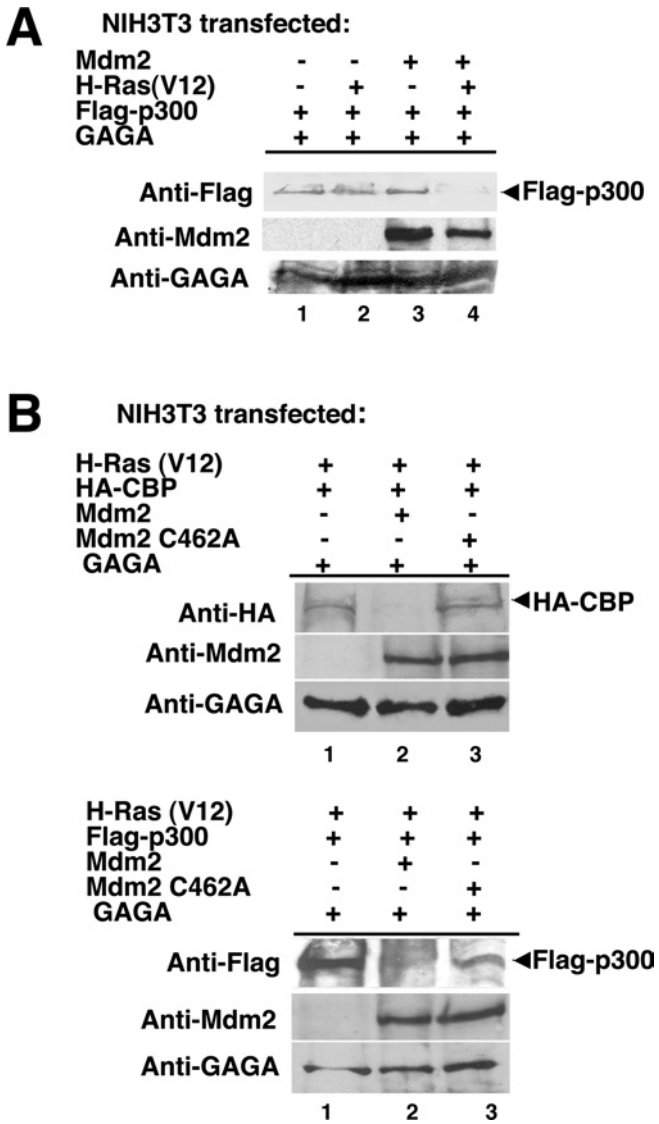

Figure 5 Mdm2 and Ras V12 co-expression decreases CBP/p300 levels in NIH 3T3 cells

(**A**) NIH 3T3 cells were transfected with 100 ng of pCMV luciferase reporter vector, 1 μg of pCDNA3-GAGA, 1 μg of pCDNA3-Flag-p300, in the absence or presence of either 2 μg of pXJ Mdm2 (lanes 3 and 4) or 100 ng of pCEFL-KZ-AU5-H-Ras V12 (lanes 2 and 4). The amount of promoters in the transfection was kept constant by the addition of empty vectors. Luciferase activity was measured 36 h after transfection. Total cell extracts were prepared and analysed by immunoblot using specific antibodies directed against FLAG (to detect exogenous FLAG–p300), Mdm2 and GAGA. (**B**) NIH 3T3 cells were transfected with 100 ng of pCMV-luciferase reporter vector, 1 μg of pCDNA3-GAGA, 1 μg of pCDNA3-HA-CBP or pCDNA3-Flag-p300 and 100 ng of pCEFL-KZ-AU5-H-Ras V12, in the absence (lane 1) or presence of either 2 μg of pXJ Mdm2 (lane 2) or 2 μg of pXJ Mdm2 C462A (lane 3). The amount of promoters in the transfection was kept constant by the addition of empty vectors. Luciferase activity was measured 36 h after transfection. Total cell extracts were prepared and analysed by immunoblotting using specific antibodies directed against HA or FLAG (to detect exogenous CBP or p300 respectively), GAGA and Mdm2.

we analysed the CBP and p300 degradation induced by an Mdm2 mutated (C462A) in the RING-finger domain without any ubiquitin ligase activity. The results showed that wild-type Mdm2 induced a sharp decrease of CBP and p300 levels, whereas in the case of C462A mutant Mdm2, the effects were significantly reduced (Figure 5B, compare lanes 2 and 3). Similar results were obtained for N-Ras V12 (not shown).

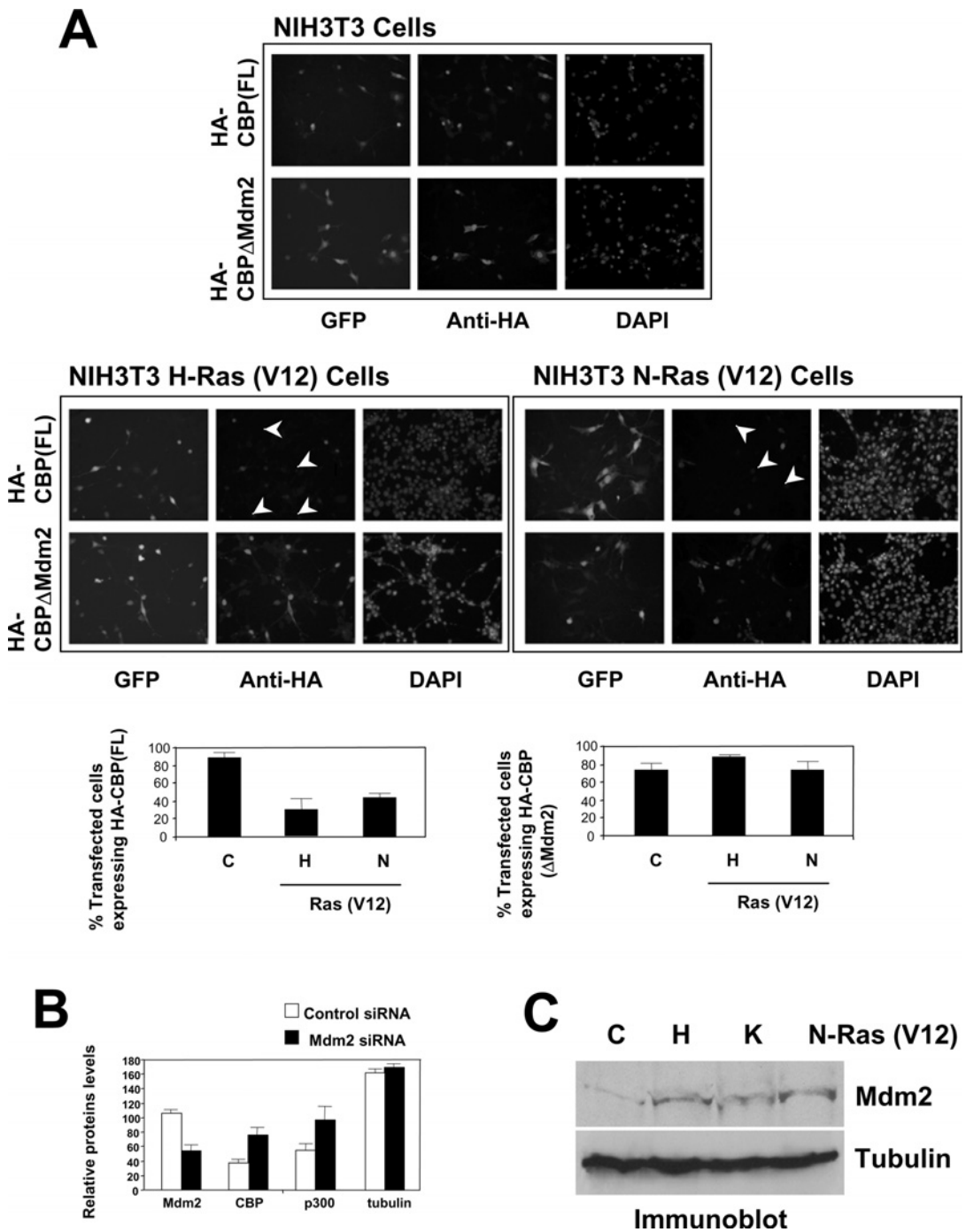

Figure 6 Mdm2 decreases CBP/p300 in NIH 3T3 fibroblasts overexpressing hyperactive Ras

(**A**) NIH 3T3 cells, NIH 3T3 H-Ras V12 and NIH 3T3 N-Ras V12 cells lines were transfected with GFP (green fluorescent protein) and either pCDNA3 HA-CBP (FL) or pCDNA3 HA-CBP (ΔMdm2). At 36 h after transfection, cells were fixed and HA–CBP was visualized by indirect immunofluorescence using the anti-HA antibody. Transfected cells were detected by the presence of green fluorescence from GFP. The presence of nuclei was followed by DAPI staining. White arrowheads indicate transfected cells with undetectable levels of HA–CBP. The percentage of transfected cells expressing HA–CBP (either FL or ΔMdm2) in NIH 3T3 (control cells, C) or H-Ras V12 and N-Ras V12 cells is shown in the histograms. Results were obtained after counting at least 200 transfected cells from three independent experiments. (**B**) NIH 3T3 H-Ras V12 cells were transfected with 6 μg of control siRNA duplexes or siRNA duplexes specific for Mdm2 protein. Total cell extracts were prepared 80 h after transfection, and the levels of Mdm2 and CBP/p300 were analysed by immunoblotting. The diagrams show the relative protein levels obtained from two independent experiments. (**C**) Total cells extract from NIH 3T3 (control cells, C) or H-, K- or N-Ras V12 overexpressing cells were analysed by immunoblotting for the presence of Mdm2 using a specific antibody directed against Mdm2. This same blot was also probed with anti-tubulin antibody as a loading control.

CBP and Mdm2 interact through the N-terminal domain of CBP [38]. To investigate whether CBP degradation requires the CBP–Mdm2 interaction domain, we analysed the HA–CBP full-length (FL) and HA–CBP deletion mutant (ΔMdm2, which lacks the Mdm2-interacting region) protein levels in NIH 3T3 controls cells as well as in H-Ras V12- or N-Ras V12-overexpressing cells. Our results demonstrate that both proteins CBP (FL) and CBP (ΔMdm2) were easily revealed by indirect immunostaining with anti-HA antibody in NIH 3T3 control cells (Figure 6A). However, only the CBP (ΔMdm2) mutant was clearly detected in

all transfected Ras V12-overexpressing cells, whereas CBP (FL) was only visible in a small number of these cells (35–43 % of transfected cells; Figure 6A).

To confirm these results, we knocked down Mdm2 expression in NIH 3T3 H-Ras V12 cells using siRNAs. These cells were transfected with siRNA pool control, or with siRNA pool covering different portions of the mouse Mdm2 coding sequence. Endogenous Mdm2 was decreased by 40–60 % using this siRNA pool (Figure 6B). Under these conditions, the endogenous levels of tubulin were not affected. However, decreased Mdm2 expression correlated with an increase of the CBP and p300 protein levels (Figure 6B), suggesting that in NIH 3T3 H-Ras V12 cell lines Mdm2 contributes to degrade CBP and p300. Since our results indicated that Mdm2 may be involved in CBP/p300 loss in Ras V12-transformed fibroblasts, we tried to assess whether these cell lines exhibited increased levels of Mdm2 protein relative to NIH 3T3 control cells. Consistent with the above proposed role of Mdm2 in CBP/p300 degradation, we found that Mdm2 levels increased in NIH 3T3 fibroblasts overexpressing also Ras V12 (Figure 6C), as shown previously by others [15].

DISCUSSION

The findings of the present study show that CBP/p300 is regulated by proteasome-mediated proteolysis in Ras V12-overexpressing NIH 3T3 cells. We found that Mdm2 induces CBP/p300 degradation in the presence of Ras active proteins. These data also show that CBP/p300 themselves are direct targets for MAPK signalling pathway, as has been suggested previously by others [13,41–43].

The observation that Ras pathway activation and CBP/p300 degradation are associated might appear paradoxical. On the one hand, many genes involved in cellular proliferation require CBP/p300 as a co-activators. In fact several reports support this idea showing CBP/p300 activation via MAPK cascade [41–45]. On the other hand, many studies have shown that CBP/p300 family members possess tumour-suppressor-like function. Mice heterozygous for CBP have a greater rate of malignancies than wild-type mice [24]; human patients with RTS, due to CBP heterozygosity, also show an increased rate of malignancies [46]. Recently, it has been shown that suppression of the activated Ras phenotype occurs by two point mutations in the *Caenorhabditis elegans* p300/CBP homologue, cbp-1. One of these mutations results in an increase of HAT cbp-1 activity, suggesting a role for cbp-1 as a negative regulator of Ras signalling [47]. In the same line of evidence, Poizat et al. [48] have shown that activation of the p38 MAPK pathway by doxorubicin and degradation of p300 are coupled. The paradox might be explained by the different effects of Ras activation, depending on the cellular environment: Ras activation leads to senescence induction; when other genetic alterations occur, oncogenic Ras may cause transformation. It is possible that oncogenic Ras may induce senescence only in some cell types or together with other signals. In fact, Deng et al. [49] have shown that p300/CBP play a critical role in the Ras-triggered senescence, and inactivation of these co-activators in conjunction with other genetic alterations can contribute to Ras-induced transformation and tumorigenesis. Then, CBP/p300 play a critical role limiting the oncogenic potential of Ras.

We have detected that continuous mitogenic stimulation induces loss of CBP and p300 in NIH 3T3 fibroblasts, and these effects can be reproduced by overexpression of Ras V12. The loss of CBP and p300 proteins is probably the main cause of the CBP/p300 HAT activity decrease observed after H-Ras V12 and N-Ras V12 overexpression. However, in the case of K-Ras4B V12, an additional negative regulation of CBP/p300 HAT activity probably occurred, as has been described in other oncoproteins [33,34]. Interestingly, our data suggest that the levels of CBP/p300 proteins were different according to the type of Ras V12-overexpressing cells analysed. Therefore, during H- and N-Ras (V12) overexpression, a sharp reduction of CBP/p300 protein levels was observed, particularly in the latter. Furthermore, in the case of K-Ras V12, a clear effect on CBP/p300 RNA levels was observed (currently under study in our laboratory) (Figure 4A), whereas for H and N-Ras (V12) overexpression, the main contribution to the decrease of CBP/p300 levels was protein degradation. Although there is a high degree of sequence homology between Ras proteins, accumulating evidence supports the idea of different roles for the three Ras homologues [15,18], as was suggested by the embryonic lethality observed in the *Kras*, but not in the *Hras* or *Nras*, knockout mice [21]. Moreover, N-Ras and H-Ras proteins can be reversibly palmitoylated at one or two cysteine residues respectively, whereas K-Ras possesses a polybasic domain close to the C-terminal end, variations which may contribute to differences in trafficking [50], membrane association and effector pathway engagement between the three Ras homologues [51]. Indeed, it has been recently demonstrated that the activation of H-Ras and N-Ras proteins (but not K-Ras) in the Golgi apparatus and in the endoplasmic reticulum [52,53] depends upon Ras-GRP (guanine nucleotide-releasing protein) and Ras-GRF (guanine nucleotide-releasing factor) respectively [54,55]. The functional specificity in signalling by the three homologues is also apparent in the differences in the relative ability of H-Ras compared with K-Ras to activate the Raf and PI3K effector pathways [56]. Indeed, it has been suggested that K-Ras activates Rac more efficiently than H-Ras [57], or that H-Ras and K-Ras induce higher activation of NF-κB (nuclear factor κB) than N-Ras [58]. As these results support the idea that the Ras proteins (H-, K- and N-Ras) have distinct behaviours *in vivo*, the different effects on CBP/p300 levels could be another more phenotypic manifestation of this dissimilar role, as occurs with the differing presence of oncogenic *ras* in human tumours [15].

CBP/p300 are components of the transcription machinery and integrate multiple signals to regulate gene expression in response to different signals. CBP/p300 are able to mediate the expression of genes implicated in many responses, such as proliferation, cell-cycle arrest, differentiation, transformation, depending on the extra- and intra-cellular signals. Regulation of CBP/p300 might be then critical to mediate specific transcriptional responses. Since there are a large number of signal transduction pathways requiring the activity of CBP/p300 co-activators, the availability of these proteins is a critical step in many biological processes. In fact, a direct antagonism between promoters that require CBP/p300 as co-activators has been described previously [14,59]. In particular, it is known that agonists of the Ras pathway promote the interaction of CBP with the MAPK p90 ribosomal S6 kinase, which thereby inhibits the activation of CREB-responsive elements by cAMP [60]. Therefore the alterations in CBP/p300 levels that we have described here following Ras overexpression in NIH 3T3 cells may interfere with other signal transduction pathways through competition for limiting concentration of CBP/p300, thus altering their specificity for selected promoters.

In accordance with the present results, it would be very interesting to determine whether responses to other signalling pathways are affected in Ras V12-overexpressing NIH 3T3 cells in a CBP/p300-dependent manner, and the putative relationship between loss of CBP/p300 protein levels and the transforming effects mediated by hyperactive Ras. Moreover, it would also be helpful to examine whether CBP/p300 are similarly targeted by

the proteasome degradation pathway in human tumours induced by Ras oncogenes.

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