Concomitant Increase of Histone Acetyltransferase Activity and Degradation of p300 during Retinoic Acid-induced Differentiation of F9 Cells*

Franck Brouillard‡ and Chantal E. Cremisi§
From the Unité Propre de Recherche 9079, CNRS, Ligue Nationale Contre le Cancer, Institut Andre Lwoff, 7 rue Guy Moquet, 94800 Villejuif, France

Received for publication, July 3, 2003, and in revised form, July 24, 2003 Published, JBC Papers in Press, July 29, 2003, DOI 10.1074/jbc.M307123200

The p300 and closely related CBP histone acetyltransferases (HAT) function as global transcriptional co-activators that play roles in many cell differentiation and signal transduction pathways. Despite their similarities, p300 and CBP have distinct functions during retinoic acid-induced differentiation of mouse F9 embryonal carcinoma cells. F9 cells constitute a well established model system for investigating the first steps of early development and retinoic acid signaling ex vivo. p300, but not CBP, was shown to be essential for F9 differentiation. In this study we have investigated the regulation of p300 during F9 differentiation. We report a dramatic decrease of p300, but not CBP protein levels, after 48 h of retinoic acid treatment. p300 is degraded via the ubiquitin-proteasome pathway. Although the large majority of p300 is degraded, its global HAT activity stays constant during F9 differentiation, which means that its specific HAT activity increases considerably. p300 is strongly phosphorylated in both undifferentiated and differentiated F9 cells; its HAT activity, however, is independent of phosphorylation before differentiation and becomes dependent on phosphorylation during differentiation. Furthermore, we show that protein kinase A affects p300 HAT activity both in vivo and in vitro as well as p300 phosphorylation in differentiated cells. Thus, we show that p300 is differentially phosphorylated in undifferentiated versus differentiated cells and that the changes in phosphorylation affect its HAT activity. Moreover, our study suggests an explanation for the functional switch of p300-mediated repression versus activation during F9 differentiation.

Mouse embryonal carcinoma (EC) cell lines, derived from the stem cells of teratocarcinomas, provide an attractive ex vivo model system for studying the regulation of gene expression during mouse early embryogenesis (1). EC cells have many features in common with the cells of preimplantation embryos; for instance, they are undifferentiated and pluripotent. One of these cell lines, F9, can be induced to differentiate into primitive endoderm-like cells when grown as a monolayer in the presence of retinoic acid (RA) (2).

p300 and CBP belong to a family of transcriptional co-activators that participate in many physiological processes, including embryonic development, proliferation, differentiation, and apoptosis (3). p300/CBP interact with multiple signal-dependent transcription factors, suggesting that these co-activators function as signal integrators by coordinating complex signal transduction events at the transcriptional level (4, 5). p300/CBP have also been proposed to mediate transcription induction via intrinsic and associated histone-acetyltransferase activities, which may facilitate binding of nuclear factors to their target sites by destabilizing promoter-bound nucleosomes (6, 7). The HAT activity of p300/CBP is able to modify not only histones but also sequence-specific transcription factors (3). A close correlation between HAT activity and activation of transcription has been observed in the analysis of wild type or mutated CBP/CBP domains fused to the Gal4 DNA binding domain (8). The activation of transcription in response to the addition of thyroid hormone to chromatin-bound thyroid receptor also requires p300 HAT activity (9). Similarly, HAT of p300 was shown to make an important contribution to glucocorticoid receptor transcription (10). Additionally, p300/CBP may also mediate target gene activation through association with active polymerase II complexes, thereby bridging upstream transcription factors with the general transcription machinery (11).

However, although p300/CBP are structurally and functionally related transcriptional co-activator proteins, they present several functional differences that have important consequences. For example, distinct roles for p300 and CBP were found during RA-induced F9 differentiation in a study showing that p300, but not CBP, is absolutely required for RA-induced differentiation of F9 cells (12). Moreover, a report investigating in detail the HAT domain of p300 and CBP demonstrates that they are not equivalent; several identical mutations introduced in p300 and CBP differentially impair their respective HAT activity (13). Confirming these data, several other recent studies have highlighted functional differences between CBP and p300. An interesting study found that p300 plays critical roles in growth suppression and transcriptional regulation in epithelial cells and that p300 and CBP perform nonoverlapping functions in these cells (14). Distinct roles for CBP and p300 have been found in hematopoietic stem cell self-renewal (15) and in adipocyte differentiation (16). In this study we investigated the regulation of p300 expression and of its HAT activity during RA-induced F9 differentiation keeping in mind that the differentiation of EC cells mimics the early steps of development.

We show here that the p300 protein levels decrease dramatically during RA-induced F9 differentiation and that p300 pro-
tein is degraded via the ubiquitin-proteasome pathway. Most importantly, our results also show that the specific activity of p300 HAT is markedly up-regulated during differentiation and that the PKA pathway is involved in this increase. We, therefore, propose a model that could explain the switch of p300 function from repressor to activator during F9 differentiation by the differential effects of PKA activation and the concomitant degradation of p300.

EXPERIMENTAL PROCEDURES

Cell Culture—Mouse F9 EC cells were grown in high glucose Dulbecco's modified Eagle's medium (Invitrogen) containing 12.5% fetal calf serum supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin in 0.1% gelatin-coated dishes. Primary endoderm-like differentiation was induced by adding 0.2 μM all-trans-RA (Sigma) to the medium. Typical morphological signs of differentiation were clearly seen by light microscopy. For metabolic labeling experiments, cells were incubated for 2 or 4 h with 50 μCi/ml Tran^35S-label (ICN) in methionine and cysteine-free high glucose Dulbecco's modified Eagle's medium or with 300 μCi/ml [3H]orthophosphate (ICN) in phosphorus-free high glucose Dulbecco's modified Eagle's medium.

Immunoprecipitation—Cells were washed and harvested in cold phosphate-buffered saline solution (PBS) and then lysed for 10 min at 4 °C in lysis buffer containing 50 mM Tris, pH 7.5, 5 mM EDTA, 0.1% Nonidet P-40 (Calbio CA-630 from Sigma), and 250 mM NaCl supplemented with 30 mM sodium pyrophosphate, 10 mM sodium fluoride, 0.1 mM sodium orthovanadate, and protease inhibitor mixture (Molecular Diagnostic). The lysates were subjected to three freeze-thaw cycles and 4 °C in lysis buffer containing 50 mM Tris, pH 7.5, 5 mM EDTA, 0.1% Nonidet P-40, and 1% sodium deoxycholate.

Transient Transfection—F9 EC cells, plated on 6-cm Petri dishes, were transfected with 4.5 μg of pUbi-HA, 8 μl of Plus reagent, and 12 μl of LipofectAMINE reagent according to the manufacturer's instructions (Invitrogen). After 5 h in the presence of the DNA-liposome complexes, cells were not with RA and cultured for 20 h, at which time MeSO or MG-115 (50 μM) were added, and culture was continued for an additional 12 h. MG-115 was preferred to MG-132 because it is more specific and less toxic for F9 cells.

A Phosphatase Treatment—p300 was immunoprecipitated from 1000 μg of whole cell lysate as described above. After incubation with the RW128 antibody, p300 immune complexes were divided in two equal portions: each of them was incubated with the same volume of protein A/G beads, washed 4 times with complete lysis buffer, once with lysis buffer without phosphatase inhibitors, and finally, once with the λ phosphatase reaction buffer 1× (50 mM Tris-Cl, pH 7.5, 0.1 mM Na3EDTA, 5 mM diithiothreitol, 0.01% Brij35). One of the 2 aliquots of p300 immune complexes was incubated for 30 min at 30 °C in presence of 2 μl of a phosphatase (Bioblot) in a 50-μl final volume of 1× reaction buffer. The other aliquot was incubated in 50 μl of reaction buffer containing 2 μl of the enzyme dilution buffer (100 mM NaCl, 50 mM Tris-Cl, pH 7.5, 0.1 mM MnCl2, 0.1 mM EDTA, 2 mM diithiothreitol, 50% glycerol). At the end of the incubation period, immunocomplexes were washed 3 times with 1 ml of lysis buffer containing phosphatase inhibitors to neutralize and eliminate the λ phosphatase.

In Vitro Phosphorylation—For in vitro phosphorylation, immunoprecipitated p300 proteins were washed as previously described in immunoprecipitation section and once more in PKA kinase buffer (20 mM Tris-Cl, pH 7.6, 10 mM magnesium acetate) and resuspended in 50 μl of a solution supplemented with 1 mM diithiothreitol, 20 μM ATP, and 10 μCi of γ[32P]ATP (ICN, 4,000 Ci/mmol). After the addition of 10 units of purified PKA catalytic subunit (Sigma), reactions were allowed to proceed for 30 min at 30 °C, then stopped by extensive washing with cold lysis buffer. Control assays were performed in the presence of 10 mM PKI (Sigma), a specific inhibitor of PKA.

HAT Assay—HAT assays were performed as previously described (22). Briefly, a synthetic peptide (Chiron), corresponding to the N-terminal amino acid sequence of histone H4 (SIGRGGKGGKLGGK-GAKHRKVKLR) coupled through a SGS linker sequence to a biotin moiety, was used as a substrate. After immunoprecipitation, p300 immune complexes were washed once with HAT buffer (50 mM Tris pH 7.6, 1 mM EDTA, 10 mM sodium butyrate, 20 mM NaF, 0.1 mM Na3VO4, and protease inhibitors) and then mixed with the H4 histiylated peptide (30 μM final concentration) in 30 μl of HAT buffer containing 100 nCi of [3H]acetyl-CoA (2.3 GBq/mmol, ICN). Samples were incubated at 30 °C for 45–60 min with frequent agitation, and 500 μl of HAT buffer were added. After centrifugation for 1 min at 8000 ×g, pellets corresponding to the immunoprecipitates were saved, diluted in 1× lysis buffer and stored at −20 °C for subsequent Western blot analysis. Supernatants were incubated with 10 μl of prewashed streptavidin-agarose beads (Sigma) for 30 min at 4 °C on a rotating wheel. After washing with radioimmune precipitation assay buffer, bound radioactivity was counted using a liquid scintillation counter (LKB).

Western Blotting—Proteins resolved on SDS, 5% polyacrylamide gels were transferred on polyvinylidene difluoride filters (Bio-Rad) by electroblotting. Proteins resolved on Tris acetate gels (NuPage, Invitrogen) were transferred to polyvinylidene difluoride following the manufacturer's instructions.

Filters were blocked in 5% lowfat dried milk and dissolved in PBS containing 0.1% Tween 20 (PBS-T) overnight at 4 °C. After washing in PBS-T, the filters were incubated for 1 h at room temperature or overnight at 4 °C with either anti-CBP (Santa Cruz Biotechnologies), anti-p300 (NM-11 from Pharmingen or N-15 from Santa Cruz Biotechnologies), anti-EndoA (Troma-1, a monoclonal antibody, a gift of R. Kemler), or anti-tubulin antibodies (monoclonal antibody from Sigma) as required.

After further washing in PBS-T, the filters were incubated with horseradish peroxidase-conjugated antibodies against rabbit IgG or mouse IgG (sigma), diluted 1/2000 and 1/3000, respectively, for 1 h at room temperature, and then washed extensively in PBS-T. The immune complexes were visualized with the ECL detection system (PerkinElmer Life Sciences) or by autoradiography.

RESULTS

p300 Protein Levels Decrease during RA-induced Differentiation of F9 Cells—F9 cells were cultured for 24 h before the addition of RA and then cultured for an additional 24, 48, 72, or 96 h as indicated, after which p300 protein levels were analyzed by Western blotting of equal amounts of whole cell extracts with a p300-specific polyclonal antibody, N15. p300 was found to be decreased by −50% at 48 h (Fig. 1A, compare p300 lanes 1 and 2) and by about 80% at 96 h (Fig. 1A, compare p300 lanes 1 and 5), whereas CBP levels were stable during the differentiation process. The increase of differentiation marker such as EndoA (EndoA, Fig. 1A) and the appearance of the characteristic flat triangular morphology (2) (data not shown) attested that the F9 cells were well differentiated. To confirm these results immunoprecipitations were performed with the specific p300 polyclonal antibody RW128 (17) and revealed with the monoclonal antibody NM11 (Fig. 1B). Together these results show that p300 protein levels strongly and selectively decrease during F9 differentiation.

Degradation of p300 Protein by the Ubiquitin-Proteasome Pathway—To investigate the basis for the reduction of p300 protein levels during F9 differentiation, we first evaluated the rate of p300 synthesis in both untreated and RA-treated F9 cells by comparing the amount of [35S]labeled methionine and cysteine incorporation detected in p300 immunoprecipitates after a 4-h pulse. The results shown in Fig. 2A reveal that the amount of newly synthesized p300 found in both undifferentiated and differentiated cells is similar, indicating that the rate of p300 protein synthesis is not responsible for the decrease of p300 protein levels observed during differentiation. Similar results were obtained after a 2-h pulse (data not shown).

We then asked whether a post-translational event involving protein degradation occurs during differentiation. The protea-
some pathway was shown to be involved in the degradation of many regulatory proteins including tumor suppressors and transcription factors in particular during F9 differentiation (18–21). We, therefore, tested whether this was also the case for p300. F9 cells were induced to differentiate by RA treatment for 92 h. Half of the cultures were treated with the potent proteasome inhibitor MG-115 during the last 12 h before p300 immunoprecipitation (Fig. 2B). Once again, RA-induced differentiation resulted in a dramatic decrease of p300 levels (Fig. 2B, compare lanes 1 and 2). Treatment with MG-115 clearly inhibited this decrease of p300 levels (Fig. 2B, compare lanes 2 and 3). These data indicate that the 26 S proteasome plays a critical role in p300 degradation during F9 differentiation.

The formation of ubiquitin-protein conjugates by covalent addition of multiple ubiquitin molecules is requisite for efficient recognition and degradation of the target protein by the 26 S proteasome. To determine whether p300 is a substrate for ubiquitination during F9 differentiation, F9 cells were first transfected with a plasmid expressing HA-tagged ubiquitin multimers, pSG5-HA-Ub, and then treated by RA for 48 h, with MG-115 present for the last 12 h. HA-tagged ubiquitin serves as an efficient substrate for ubiquitin-conjugating enzymes in cells, thereby facilitating detection of ubiquitin-protein conjugates by covalent addition of multiple ubiquitin molecules.

**Fig. 1. Decrease of p300 protein levels during RA-induced differentiation of F9 cells.** F9 cells were cultured 24 h, and differentiation was induced by the addition of RA (0.2 μM final concentration). A, Western blotting analysis. 40 μg of whole cell extract prepared from F9 cells at 24, 48, 72, and 96 h after RA addition were separated on a 4–12% Tris acetate gel. Blotting was performed with anti-CBP (A22 polyclonal Santa Cruz), anti-p300 (N15 polyclonal, Santa Cruz), anti-tubulin (DM1A), and anti-Endo A antibodies. B, immunoprecipitation analysis. 500 μg of whole cell extract were immunoprecipitated with 0.7 μg of polyclonal antibody RW128 in lysis buffer. After washing, immunoprecipitates were resolved on a 5% SDS, polyacrylamide gel and immunoblotted with the monoclonal antibody NM11 (Pharmingen). The curve represents a densitometric analysis of the results. The p300 bands in B were analyzed with the NIH image program version 1.63. Data points are the average of four independent experiments.

**Fig. 2. Degradation of p300 protein by the ubiquitin-proteasome pathway during F9 differentiation.** A, [35S]methionine and [35S]cysteine incorporation in p300 immunoprecipitates (IP). After 24 h in culture medium, F9 cells were cultured with RA for 24, 48, and 72 h and pulse-labeled for 4 h with Tran35S-label (ICN) before lysis. p300 was immunoprecipitated using RW128 antibody, and proteins were resolved on a 5% SDS-PAGE gel before autoradiography. B, effect of the proteasome inhibitor MG-115 on p300 protein levels. F9 differentiation was induced by a 78-h RA exposure, and cells were treated with Me2SO (DMSO) or MG-115 for 16 h before being lysed. p300 was immunoprecipitated using RW128 antibody, and proteins were resolved on a 5% SDS-PAGE gel before autoradiography. B, effect of the proteasome inhibitor MG-115 on p300 protein levels. F9 differentiation was induced by a 78-h RA exposure, and cells were treated with Me2SO (DMSO) or MG-115 for 16 h before being lysed. p300 was immunoprecipitated using RW128 antibody, and proteins were resolved on a 5% SDS-PAGE gel before autoradiography. B, effect of the proteasome inhibitor MG-115 on p300 protein levels. F9 differentiation was induced by a 78-h RA exposure, and cells were treated with Me2SO (DMSO) or MG-115 for 16 h before being lysed. p300 was immunoprecipitated using RW128 antibody, and proteins were resolved on a 5% SDS-PAGE gel before autoradiography. B, effect of the proteasome inhibitor MG-115 on p300 protein levels. F9 differentiation was induced by a 78-h RA exposure, and cells were treated with Me2SO (DMSO) or MG-115 for 16 h before being lysed. p300 was immunoprecipitated using RW128 antibody, and proteins were resolved on a 5% SDS-PAGE gel before autoradiography.
HAT Activity and Degradation of p300 during F9 Differentiation

gates. p300 was immunoprecipitated with RW128 antibody and sequentially immunoblotted with the 12CA5 anti-HA antibody and the NM11 anti-p300 antibody. When immunoprecipitated p300 was immunoblotted with anti-HA antibodies (Fig. 2C, lanes 1–3), a smear of high molecular products could be detected in cells treated with RA and the MG-115 inhibitor (Fig. 2C, lane 3). This smear, which is usual after such treatment, corresponds to polyubiquitinated forms of p300. Similarly, higher molecular weight forms of p300 are visible on a longer exposure of the Western blot probed with p300 antibody (Fig. 2C, lane 3). Taken together these experiments indicate that p300 is ubiquitinated and degraded by the proteasome pathway during F9 differentiation.

The Specific HAT Activity of p300 Dramatically Increases during F9 Differentiation—As already mentioned, a key property of p300 that can contribute to its co-activator function is its HAT activity (8–10). We, therefore, analyzed p300 HAT activity during F9 differentiation. p300 was immunoprecipitated from equal amounts of whole cell extract from F9 cells cultured without or with RA for 24, 48, 72, and 96 h, and then HAT activity was assayed. We used a quantitative assay (22) in which the transfer of radiolabeled acetyl groups to a synthetic peptide substrate corresponding to the H4 histone N-terminal tail can be directly detected and measured. As a negative control we performed an immunoprecipitation with an irrelevant mouse IgG antibody. In this case, 200–300 cpm were obtained at the end of the assay. As a positive control we used a synthetic peptide corresponding to the HAT domain of CBP. About 10^5 cpm were routinely observed, whereas with p300 immunoprecipitated from F9 cells, 1–2 × 10^4 cpm were measured, indicating that the assay was not performed under saturating conditions. In addition, the amount of p300 immunoprecipitated was systematically controlled after the enzymatic assay by Western blot analysis of the immunoprecipitates. After immunoprecipitation, stringent washing conditions were used to ensure that no other HAT proteins binding to p300, such as PCAF (23) or SRC-1/A/C/IR (24), were co-immunoprecipitated. Interestingly, as shown in Fig. 3A, the results indicate that the levels of p300 HAT activity stay remarkably constant during differentiation despite the fact that p300 protein levels decrease. We, therefore, conclude that the specific HAT activity of p300, defined as the p300 enzymatic activity normalized to the p300 protein content, dramatically increases during F9 differentiation by about 5-fold at 96 h. These results could be interpreted as indicating that in F9 cells there are at least two subpopulations of p300, one having HAT activity and persisting during the differentiation process, whereas the other is devoid of this activity and is degraded. To gain insight into this question, we measured the p300 HAT activity immunoprecipitated from differentiated F9 cells treated with the proteasome inhibitor MG-115. After MG-115 treatment, the amount of p300 protein increases (Fig. 3B, compare second and third lanes); nevertheless, the levels of p300 HAT activity do not change significantly, indicating that the p300 species accumulated in RA-treated F9 cells after MG-115 treatment have no HAT activity. These results could thus, be consistent with the idea that there are two subpopulations of p300 in F9 cells.

p300 HAT Activity Becomes Phosphorylation-dependent during F9 Differentiation—p300 is a phosphoprotein that is actively phosphorylated in both proliferating and quiescent cells (25). We, therefore, tested whether the p300 HAT activity was affected by phosphorylation during the differentiation process. p300 was immunoprecipitated from untreated and RA-treated F9 cells. Aliquots of each immunoprecipitate were either mock- or λ phosphatase-treated, then tested for HAT activity and analyzed by Western blotting. Fig. 4A shows that whatever the origin of p300 from F9 stem cells or differentiated F9 cells, after phosphatase treatment p300 migrates much more rapidly, indicating that p300 was largely phosphorylated in both undifferentiated and differentiated F9 cells. Interestingly, the HAT
activity of p300 is completely independent of the phosphorylation state in F9 stem cells, whereas it becomes progressively dependent on phosphorylation during differentiation (Fig. 4A). At 96 h, one observes a decrease of about 50% of the HAT activity of p300 after phosphorylation treatment. Taken together, these results indicate that although p300 was largely phosphorylated both before and during F9 differentiation, the precise pattern of p300 phosphorylation must change during differentiation, directly increasing its intrinsic HAT activity.

To get more information on the phosphorylation state of p300 during differentiation, we performed metabolic labeling with \(^{32}P\)orthophosphate during the last 3 h of culture in F9 cells incubated with RA for 0, 24, 48, 72, and 96 h. Standardized aliquots containing same amount of protein were immunoprecipitated using an anti-p300 antibody and then analyzed by autoradiography of the gel. We observed that p300 is largely phosphorylated in proliferating F9 cells (Fig. 4B, lane 1) as well as in arrested differentiated cells (Fig. 4B, lanes 4 and 5), in agreement with the previous experiment (Fig. 4A) and a published study (25). The labeling of p300 decreases during differentiation, probably because the amount of p300 proteins also decreases. The bands of labeled p300 (Fig. 4, lanes 1–4) are large and diffuse, suggesting, in agreement with the migration pattern of unlabeled p300 and with previous results (25), that multiple phosphorylated forms of p300 are comigrating. However, it is difficult to distinguish the different phosphorylated species of p300. Taken together these results show that p300 is differentially phosphorylated during F9 differentiation and that its HAT activity becomes dependent upon phosphorylation during this process.

**PKA Pathway Controls p300 HAT Activity during F9 Differentiation**—Because the p300 HAT activity becomes dependent on phosphorylation during differentiation, we investigated the phosphorylation pathway involved in this process. PKA is known to be activated during F9 differentiation (26); we, therefore, tested whether a PKA inhibitor, H-89, could affect the p300 HAT activity. H-89 has been routinely used as a potent, highly selective, and reversible inhibitor of PKA (27, 28). It is an ~1000-fold more efficient inhibitor for PKA than for PKC or other known kinases (27). F9 cells were cultured with or without RA for 48 h, and H-89 (2.5 \(\mu\)M) was added 24 h before the end of the incubation period. The results, presented in Fig. 5A, show that H-89 reduces the HAT activity of p300 purified from RA-treated cells by about 40%. In contrast, H-89 has no effect on the HAT activity of p300 from F9 stem cells. To assess the effect of H-89 on the phosphorylation of p300, we reproduced the experiment and radiolabeled the F9 stem cells with \(^{32}P\)orthophosphate during the last 2 h of H-89 treatment. The results show that the migration of labeled p300 immunoprecipitated from these cells was the same whether or not they were treated by H-89 (Fig. 5B, compare lanes 1 and 2), indicating that in this case p300 was not phosphorylated by PKA. In contrast, the migration of labeled p300 isolated from differentiated F9 cells treated by H-89 is globally slightly faster, and the band much less diffuse than in differentiated control cells not treated by H-89 (Fig. 5B, compare lanes 3 and 4), clearly showing that in this case p300 was underphosphorylated in the presence of the PKA inhibitor. In addition we tested whether PKA can directly phosphorylate p300 and stimulate its HAT activity. We performed an in vitro kinase assay using purified PKA catalytic subunit and p300 immunoprecipitated from F9 differentiated cells and pretreated with \(\lambda\) phosphatase as substrate. After incubation with PKA in the presence of \([\gamma-^{32}P]ATP\), p300 immunoprecipitates were tested for HAT activity, resolved by SDS-PAGE, and transferred onto polyvinylidene difluoride membranes. This procedure allowed us to control for the p300 protein levels in samples by Western blotting and to detect p300 phosphorylation by autoradiography. As shown on Fig. 5C, PKA indeed phosphorylates p300 in vitro (Fig. 5C, lane 2). This phosphorylation is PKA-specific since no phosphorylation was detected when the PKA inhibitor was added to the reaction (Fig. 5C, lane 3). Furthermore, the results of the HAT assays indicated that the HAT activity of p300 was markedly stimulated by PKA in vitro (Fig. 5, compare the values of lanes 2 with lanes 1 and 3). The involvement of other kinases was also tested, i.e., such as PKC, p38, and MEK (mitogen-activated protein kinase/extracellular signal-regulated kinase kinase), by using the inhibitors bisindolylmaleimide VI, SB203580, and PD98059, respectively. None of them affect the specific HAT activity of p300 (data not shown). Taken together all these results provide strong evidence that PKA is...
responsible for the direct phosphorylation of p300 and subsequently affects its HAT function.

DISCUSSION

We demonstrate here that the acetyltransferase p300 is strongly and selectively degraded through the ubiquitin-proteasome pathway during RA-induced F9 differentiation. Concomitantly, the HAT-specific enzymatic activity of p300 dramatically increases during differentiation and becomes partially dependent upon phosphorylation, notably by PKA.

The ubiquitin-proteasome pathway is the major system in eukaryotic cells for selective degradation of regulatory proteins and transcription factors (18). During RA-induced F9 differentiation, several proteins are known to be specifically degraded in a RA-dependent manner through this pathway; that is, the retinoid acid receptors $\gamma 2$ and $\alpha 1$ and the retinoid X receptor $\alpha 1$ (19) as well as components of the basal transcription machinery such TATA-binding protein and TATA-associated factors 135 (20). Interestingly, all these proteins are required for normal F9 differentiation. However, what is unique to p300 is the fact that proteolytic degradation correlates with an increase in its specific HAT activity.

Studies of mice deficient in p300/CBP have provided very important and unexpected information (29). Normal mouse development is sensitive to both p300 and $\text{cbp}^-$ mice suffer very early embryonic lethality. Surprisingly, p300$^-$ mice also manifested embryonic lethality despite the presence of normal levels of the highly homologous protein CBP. Importantly, signaling by RA is also compromised in cells lacking p300. In line with this observation, it has also been shown that p300 is absolutely required for RA-induced F9 differentiation (12). All these results highlight the specific role of p300 during early development and RA-induced events. They are, therefore, consistent with the evidence presented here for the selective degradation of p300, but not of CBP, during RA-induced F9 differentiation, with a concomitant increase in the specific activity of p300 HAT.

One of the conclusions from the analysis of p300$^-$ mice was that p300 might be a cellular protein endowed with both growth-suppressing and growth-stimulating activities (29). F9 cells offer an experimental system where this dual function is present. Two published studies report that in F9 stem cells p300 acts as a repressor (30, 31); this function might be related to the very high proliferative activity of these cells. In contrast, in arrested differentiated F9 cells, p300 acts as a co-activator (30, 31) for the expression of specific differentiation genes, and this role is thought to be related to its growth suppressive function.

The first study (30) demonstrated that p300 is involved in the repression of $c$-jun in F9 stem cells and in its transcriptional induction during differentiation. The authors suggested that the phosphorylation of p300 induced by RA or by E1A would determine which of the two functions it will perform. In the mean of four independent experiments; bars indicate S.E. B, p300 phosphorylation after H-89 treatment. F9 cells were cultured as above. 2 h before lysis cells were labeled with $^{32}$P in the continued presence of the drug. p300 was immunoprecipitated and assayed for HAT activity; data are expressed as % of the control (cells without RA). Immunoprecipitates were then analyzed by Western blotting (WB) using NM11 antibody to evaluate the p300 levels. Results are

![Fig. 5. PKA targets p300 phosphorylation and HAT activity during F9 differentiation](image-url)
HAT Activity and Degradation of p300 during F9 Differentiation

39515

this regard it is important to note that E1A expression is known to induce F9 differentiation (32).

Different conclusions were drawn from the other study (31), where a decreased level of p300 was seen in differentiated F9 cells. However, that study did not address the mechanism underlying this down-regulation. The authors concluded that the high levels of p300 seen in F9 stem cells participated in the repression of the SV40 enhancer. It was, therefore, suggested that high levels of p300 protein are important for maintaining the undifferentiated state of F9 cells and that the reduction of endogenous p300 during differentiation by RA or by low level expression of E1A may cause derepression of the cellular genes required for differentiation.

Our results agree, in part, with both of these studies. From our data, we conclude that the selective degradation of p300 and concomitant increase in its HAT-specific activity is a crucial event in F9 differentiation, most likely participating in the activation of p300 and, thereby, contributing to the specific expression of differentiation markers. During F9 differentiation, a selection of some p300 molecules and degradation of the others might occur. In that sense our results tend to support the conclusions of Ota et al. work (31). However, our data clearly show that phosphorylation of p300 by PKA is specifically required for proper activation of its HAT activity (Fig. 5).

Thus we observe, in agreement with Kitabayashi et al. (30), that p300 is differentially phosphorylated in undifferentiated versus differentiated cells. But in contrast to the latter study, using 32P labeling (Fig. 4B) and phosphatase treatment (Fig. 4A) we observe that p300 is already highly phosphorylated in F9 stem cells; this concurs with a previous report showing that p300 is actively phosphorylated in both quiescent and proliferating cells (25). Unlike Kitabayashi (30) we have not observed a clear reduction of electrophoretic mobility of p300 during differentiation either without labeling or with 32P or 35S labeling. This difference cannot be attributed to a low resolution under our gel migration conditions, since we can clearly detect in both undifferentiated and differentiated cells a phosphatase-sensitive form of p300 with decreased electrophoretic mobility (Fig. 4A).

Our results could be explained by the following hypothesis. Induction of the PKA pathway during F9 differentiation leads to phosphorylation of p300 molecules (Fig. 5B), resulting in increased HAT activity (see Fig. 5C) and recruitment to the promoters of specific differentiation genes. These promoter-associated p300 proteins would then be ubiquitinated and degraded by the proteasome pathway as in other experimental systems (33). The situation here could also be analogous to that recently described for Myc, where stimulation of Myc activity and destruction by the proteasome were unexpectedly found to be coupled (34). The activation by PKA would be crucial and could account for the increased specific activity of p300 HAT (Fig. 5C). Treatment with the proteasome inhibitor MG-115 would generate accumulation of ubiquitinated forms of p300, which are no longer enzymatically active. This would explain the decreased specific activity of the enzyme that is observed after MG-115 treatment (Fig. 3B). In undifferentiated F9 cells, p300 would act as a repressor either because it is not phosphorylated by PKA and not active and subsequently not brought to the relevant specific promoter(s) and/or because the large amount of p300 present would compete with limiting amounts of other essential factors. The entire process should be very dynamic, and phosphorylation and degradation are certainly related events, as seen in several other situations (21).

However, our results cannot totally exclude another possibility. In F9 cells, one could have at least two species of p300 proteins; the majority, highly phosphorylated and devoid of HAT activity, which is degraded during differentiation, and a second species, hypophosphorylated and endowed with HAT activity, which is targeted by PKA during differentiation and not degraded. According to this alternative hypothesis, phosphorylation by PKA would directly or indirectly be a signal protecting p300 against degradation by the proteasome-ubiquitin pathway, which is contrary to almost all previous reports. Indeed, phosphorylation is generally required for subsequent degradation by the proteasome pathway (18). In any case, we obviously cannot exclude the intervention of other kinases, although we have reason to believe that PKC, p38, and MEKK1 are not involved (data not shown).

It was reported that proteasome-mediated degradation of p300 can also occur in cardiac cells if the cells are treated by the anticancer agent doxorubicin (35). Sustained sodium butyrate treatment can likewise induce degradation of p300 through the 26S proteasome pathway (10). However, in both of these cases the degradation of p300 correlates with impaired transcriptional activity.

Few studies have investigated the phosphorylation of CBP/p300. An important first study (25) showed that p300 is a nuclear phosphoprotein that undergoes cell cycle phase-specific modification and that p300 is actively phosphorylated in both quiescent and proliferating cells. As discussed above, p300 phosphorylation has previously been studied during F9 differentiation (30); however, most of the reports that followed concerned in vitro phosphorylation of CBP/p300 by different kinases (MEKK1, PKC, Cdc2, Cdk2), used recombinant CBP/p300, and did not differentiate between CBP and p300. Thus, the physiological significance of the phosphorylation has remained unclear, although published work using fibroblast cells (36) showed that CBP phosphorylation and CBP HAT activity are linked and cell cycle-dependent. To our knowledge, our approach is the first to demonstrate a link between phosphorylation of endogenous p300 and its intrinsic HAT activity and, moreover, to demonstrate that this occurs during a physiological process, a differentiation event that could also occur during early development.

Acknowledgments—We are very grateful to L. Pritchard, H. Lehrman, and A. Chauchereau for critical reading of the manuscript. We thank H. Lehrman and V. Ogryzko for helpful discussions and A. Chauchereau for the kind gift of the HA-Ub plasmid.

REFERENCES

1. Martin, G. (1980) Science 209, 768–776
2. Strickland, S., and Mahdavi, V. (1978) Cell 15, 393–403
3. Goodman, R., and Smolik, S. (2000) Genes Dev. 14, 1553–1577
4. Chakravarti, D., LaMorte, V., Nelson, M., Nakajima, T., Schulman, I., Jugunil, H., Montminy, M., and Evans, R. (1996) Nature 383, 99–103
5. Kamei, Y., Xu, L., Henzel, T., Tschirch, J., Kurokawa, R., Glass, B., Lin, S., Heyman, R., Rose, D., Glass, C., and Rosenfeld, M. (1996) Cell 85, 493–511
6. Bannister, A., and Kouzarides, T. (1996) Nature 384, 641–643
7. Ogryzko, V., Schiltz, R., Russanova, V., Howard, B., and Nakatani, Y. (1996) Cell 87, 953–959
8. Martinez-Balbas, M., Bannister, A., Martin, K., Haus-Seuffert, P., Meisterernst, M., and Kouzarides, T. (1998) EMBO J. 17, 2886–2893
9. Li, Q., Inohh, A., Collingwood, T., Unrorn, P., and Wolffe, A. (1999) EMBO J. 18, 5634–5652
10. Li, Q., Su, A., Chen, J., Lefebvre, Y., and Haché, R. (2002) Mol. Endocrinol. 16, 2813–2827
11. Inohh, A., Yang, X., Ogryzko, V., Nakatani, Y., Wolffe, A., and Ge, H. (1997) Curr. Biol. 7, 689–692
12. Kawasaki, H., Eckner, R., Yao, T., Taika, K., Chiu, R., Livingston, D., and Yokoyama, K. (1998) Nature 393, 284–289
13. Bordoli, L., Husser, S., Luthi, U., Netsch, M., Osman, H., and Eckner, R. (2001) Nucleic Acids Res. 29, 4462–4471
14. Suyama, T., Kawabata, M., Ohshima, H., and Ikeda, M. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 14749–14794
15. Takahashi, N., Kawada, T., Yamamoto, T., Goto, T., Taimatsu, A., Aoki, N., Kawasaki, H., Taika, K., Yokoyama, K., Kamei, Y., and Fushiki, T. (2002) J. Biol. Chem. 277, 16921–16927
16. Eckner, R., Ewen, M., Newsome, D., Gerdes, M., DeCaprio, J., Lawrence, J., and Livingston, D. (1994) Genes Dev. 8, 869–884
17. Glickman, M., and Ciechanover, A. (2001) Physiol. Rev. 82, 373–428
19. Kopf, E., Plassat, J., Vivat, V., The, H. d., Chambon, P., and Rochette-Egly, C. (2000) *J. Biol. Chem.* 275, 33280–33288
20. Perletti, L., Kopf, E., Carre, L., and Davidson, I. (2001) *BMC Mol. Biol.* 24
21. Gianni, M., Kopf, E., Baetsen, J., Oulad-Abdelghani, M., Garattini, E., Chambon, P., and Rochette-Egly, C. (2002) *J. Biol. Chem.* 277, 24859–24862
22. Ait-Si-Ali, S., Carlisi, D., Ramirez, S., Upegui-Gonzalez, L., Duquet, A., Robin, P., Rudkin, B., Harel-Bellan, A., and Trouche, D. (1999) *Biochem. Biophys. Res. Commun.* 262, 157–162
23. Yang, X., Ogryzko, V., Nishikawa, J., Howard, B., and Nakatani, Y. (1996) *Nature* 382, 319–324
24. Yao, T., Ku, G., Zhou, N., Scully, R., and Livingston, D. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 10626–10631
25. Piet, A., Gerbaud, P., Anderson, W., and Brion, D. (1985) *Differentiation* 30, 159–164
26. Chijiwa, T., Mishima, A., Hagiwara, M., Sano, M., Hayashi, K., Inoue, T., Naito, K., Toshinka, T., and Hidaka, H. (1990) *J. Biol. Chem.* 265, 5267–5272
27. Muniz, M., Alonso, M., Hidalgo, J., and Velasco, A. (1996) *J. Biol. Chem.* 271, 30935–30941
28. Yao, T., Oh, S., Fuchs, M., Zhou, N., Ch'ng, L., Newsome, D., Bronson, R., Li, E., Livingston, D., and Eckner, R. (1998) *Cell* 93, 361–372
29. Kitabayashi, I., Eckner, R., Arany, Z., Chiu, R., Gachelin, G., Livingston, D., and Yokoyama, K. (1996) *EMBO J.* 15, 3496–34509
30. Ou, M., Eto, K., Ninomiya, Y., and Heda, M. (1998) *Cell Growth Differ.* 9, 989–997
31. Molinari, E., Gilman, M., and Natesan, S. (1999) *EMBO J.* 18, 6439–6447
32. Kim, S., Herbst, A., Tworkowski, K., Salghetti, S., and Tansey, W. (2003) *Mol. Cell* 11, 1177–1188
33. Ait-Si-Ali, S., Ramirez, S., Barre, F., Dkhissi, F., Magnaghi-Jaulin, L., Girault, J., Robin, P., Knibiehler, M., Pritchard, D., Ducommun, B., Trouche, D., and Harel-Bellan, A. (1998) *Nature* 396, 184–186