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The SV40 large T antigen and adenovirus E1a oncoproteins interact with distinct isoforms of the transcriptional co-activator, p300

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p300 is a nuclear phosphoprotein likely to be involved in the control of cell growth. Here we show that SV40 large T antigen (Tag) forms a specific complex with p300. In various Tag-expressing cell lines, the affinity of Tag for p300 was restricted to a newly identified unphosphorylated but ubiquitinated form of the protein. Further, Tag did not associate with p300 in an SV40 Tag-producing cell line (REV2) in which the original transformed phenotype (SV52) is reverted. Biochemical studies demonstrate that both the phosphorylation and the ubiquitination profile of p300 are altered in REV2 with respect to the wild-type fully transformed SV52 parental cells, wherein Tag–p300 complexes are readily detected. In contrast to Tag, the adenovirus early expression product E1a interacts with both phosphorylated and unphosphorylated forms of p300. In addition, when REV2 cells were infected with adenovirus, E1a–p300 complexes were detected, suggesting that the p300 expressed in REV2 has lost the affinity for Tag, but not for E1a. We then compared the ability of Tag and E1a to affect the transcription levels of the cAMP-responsive promoter (CRE), which is modulated *in vivo* by p300, in REV2 cells. We found that Tag repressed the CRE promoter in all of the cell lines in which Tag–p300 complexes were detected, but not in REV2 cells. In contrast, E1a efficiently inhibited CRE-directed transcription in this cell line. The data thus indicate that the different specificities exhibited by Tag and E1a towards the various forms of p300 are reflected *in vivo* as a difference in the ability of these viral oncoproteins to modulate the expression of CRE-containing genes.

Keywords: E1a/p300/revertant/Tag/transformation/ubiquitination

Introduction

SV40 large T antigen (Tag) and adenovirus E1a are multifunctional viral oncoproteins implicated in a wide variety of cellular processes including transcriptional activation and repression, blockade of differentiation, stimulation of the cell cycle and malignant transformation (for a review, see Moran, 1993). In recent years, it has become apparent that these activities are the result of complex interactions between viral oncoproteins and various intracellular proteins involved in cell growth control and regulation of transcription. Principal among these targets are the products of several anti-oncogenes, namely p53, pRb and the Rb-related proteins p107 and p130, all of which exhibit properties of negative regulators of cellular proliferation (De Caprio *et al.*, 1988; Levine, 1990; Cobrinik *et al.*, 1992; Dyson and Harlow, 1992).

Another cellular protein of more recent interest is a 300 kDa product, p300, which was identified originally by its ability to associate with E1a (Egan *et al.*, 1988; Whyte *et al.*, 1989; Stein *et al.*, 1990). Recently, a cDNA encoding p300 has been cloned (Eckner *et al.*, 1994). Preliminary studies have suggested that this protein acts as a transcriptional co-activator: indeed, the overexpression of p300 *in vivo* results in transcriptional activation by viral as well as cellular enhancer/promoters (Eckner *et al.*, 1994; Arany *et al.*, 1995; Lundblad *et al.*, 1995). As this effect can be abolished directly and specifically by E1a, p300 function(s) is presumably inhibited upon binding to this viral oncoprotein. The properties of E1a mutants which have lost the ability to interact with p300 also suggest an important role of this latter in cell growth control: E1a-driven cell cycle progression and its transforming potential are both suppressed by deletions of the p300 binding site (see Lillie *et al.*, 1987; Jelsma *et al.*, 1989; Moran, 1993). Thus, p300 may modulate the expression of genes involved in control of the cell cycle and, via its inactivation, E1a, as well as other dominant viral oncoproteins, might alter the growth properties of cells.

Several investigations have shown that deletion of the extreme N-terminal domain of Tag impairs the transforming potential of this protein in selected cell lines, albeit without affecting p53 and pRb binding (Graessmann *et al.*, 1984; Manfredi and Prives, 1990; Marsilio *et al.*, 1991). This suggests that interaction(s) of Tag with cellular target(s) other than p53 and pRb may be necessary to induce malignant transformation. In addition, evidence has been provided that an otherwise transformation-deficient Tag can restore the transforming properties lost by E1a mutants which lack the p300 binding site (Yaciuk and Moran, 1991). As the E1a transforming ability is tightly dependent on its binding to p300, these data indicate that Tag can functionally complement the biological activity(s) derived from E1a–p300 complex formation. Although suggested by this assay, the question of whether Tag and p300 do interact has not been addressed previously. In this study, we show that Tag and p300 are associated *in vivo*, and that this interaction may have a biological relevance in SV40 virus-mediated transformation of cells.

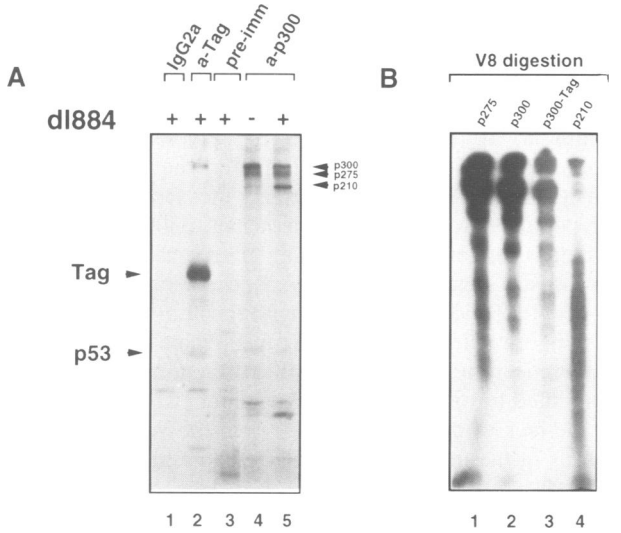

Fig. 1. Complex formation between Tag and p300. (**A**) Immunoprecipitations of ^{35}S-labeled mock-infected (lane 4) or dl884-infected (lanes 1, 2, 3 and 5) CV1 cells. Labeled extracts were immunoprecipitated with the control IgG2a monoclonal antibody (lane 1), the anti-Tag monoclonal antibody pAb419 (lane 2), the rabbit preimmune serum (lane 3) and the anti-p300 polyclonal antiserum (lanes 4 and 5). The relative positions of Tag, p300 and p53 are indicated by arrows. (**B**) dl884-infected CV1 cells were labeled with 5 mCi of [^{35}S]methionine per plate. The p300 included in the anti-p300 immunoprecipitation (lane 2), the 300 kDa protein co-precipitated by the anti-pAb419 (lane 3) and the two protein species indicated as p275 (lane 1) and p210 (lane 4) co-precipitated by the anti-p300 antibody, were visualized by autoradiography, excised from dried gels and subjected to proteolytic digestion with 20 μg of *S.aureus* V8 protease.

Results

SV40 Tag associates with the cellular protein, p300

To determine whether the SV40 Tag forms a complex with p300, anti-Tag and anti-p300 immunoprecipitations were compared. CV1 cells were infected with an SV40 mutant virus (dl884) which does not express the second early product of SV40 virus, small t antigen; the mutant virus was used to exclude any possible effect of small t on Tag–p300 complex formation. Approximately 24 h after the infection, the cells were labeled with [^{35}S]-methionine for 4 h and equal amounts of cell lysate were subjected to immunoprecipitation. The following antibodies were used: pAb419, a monoclonal antibody that recognizes an N-terminal epitope of the SV40 Tag; an anti-p300-specific polyclonal antiserum; and a control, null IgG2a monoclonal antibody. The ability of the antiserum to immunoprecipitate authentic p300 was defined previously by Western analysis and comparative peptide mapping experiments (see Materials and methods). As shown in Figure 1A, lane 2, a 300 kDa protein is co-precipitated specifically with Tag, and it co-migrates with p300 as visualized in the anti-p300 immunoprecipitations (Figure 1A, lanes 4 and 5). In addition, we noted that the anti-p300 immunoprecipitation did not co-precipitate any Tag from the virus-infected cells (Figure 1A, lane 5), even though other p300-associated proteins were detected by the antiserum. To confirm that the 300 kDa polypeptide bound to Tag is indeed p300, partial proteolytic digestion experiments were performed. [^{35}S]Labeled proteins were excised from dried gels and digested with *Staphylococcus aureus* V8 protease. Figure 1B shows that the peptide patterns generated from the 300 kDa species isolated by anti-Tag (lane 3) and anti-p300 (lane 2) immunoprecipitations are virtually indistinguishable. This result demonstrates that SV40 Tag forms a complex with authentic p300. Figure 1A also shows that two polypeptides, named here p275 and p210, are co-precipitated specifically by the anti-p300 antiserum in both mock-infected and virus-infected cells. To test whether p275 and p210 represent protein species related to p300, again the V8 digestion procedure was employed. The pattern obtained with p275 (Figure 1B, lane 1) is similar, but not identical, to that exhibited by p300. Larger deviations are observed when comparing the digestion pattern of p210 with that of either p300 or p275 (compare lane 4 with lanes 1 and 2). This suggests that p210 represents a protein species cross-reacting with the anti-p300 antibody by virtue of its sequence similarity with p300 or, alternatively, a stable product of degradation of a slower-migrating polypeptide, namely p300 or p275. The data thus demonstrate that various p300-related proteins are detected by the anti-p300 antibody, but only p300 is found in association with Tag.

The Tag-bound p300 is un- or underphosphorylated

p300 was described originally as a nuclear phosphoprotein (Yaciuk *et al.*, 1991). It is well known that phosphorylation–dephosphorylation events critically affect the function of many regulatory proteins, such as tumor suppressor gene products, cyclins and transcription factors, by modulating their ability to interact with cellular as well as viral products (for a review, see Meek and Street, 1992). Therefore, we investigated whether the Tag-bound p300 is phosphorylated. dl884-infected or mock-infected CV1 cells were labeled for 4 h with [^{32}P]orthophosphate, and immunoprecipitated with the anti-Tag- and anti-p300-specific antibodies. Surprisingly, the anti-Tag antibody did not appear to co-precipitate any phosphorylated p300 (Figure 2A, lane 1), although Tag itself and other Tag-associated proteins, such as p53, were detectable as phosphorylated species. When parallel unlabeled samples were visualized by Coomassie staining, however, a substantial fraction of p300 was found in a complex with Tag (Figure 2B, lane 1). Meanwhile, phosphorylated p300 was detected in Tag-containing cell extracts, as revealed by the anti-p300 immunoprecipitation (Figure 2A, lane 3). By comparison of the Coomassie staining and the ^{32}P-labeling of anti-p300 immunoprecipitations, it is also evident that neither the total amount (Figure 2B, lanes 3 and 4) nor the overall phosphorylation status of p300 (Figure 2A, lanes 2 and 3) is significantly altered by dl884 infection. Therefore, the lack of association between Tag and phosphorylated p300 is not due to loss of the latter fraction. However, again the anti-p300 antiserum did not co-precipitate Tag from cell extracts of virus-infected cells, as noted above (Figures 1A, lane 5; 2A, lane 3 and 2B, lane 3). This property of the anti-p300 polyclonal antiserum implies that the anti-p300 immunoprecipitation only detects the form(s) of the protein which does not interact with Tag. Thus, our data suggest the existence of at least two populations of p300: one hypophosphorylated form which co-immunoprecipitates with Tag, designated here as p300^{Tag}, and another form, highly phosphorylated

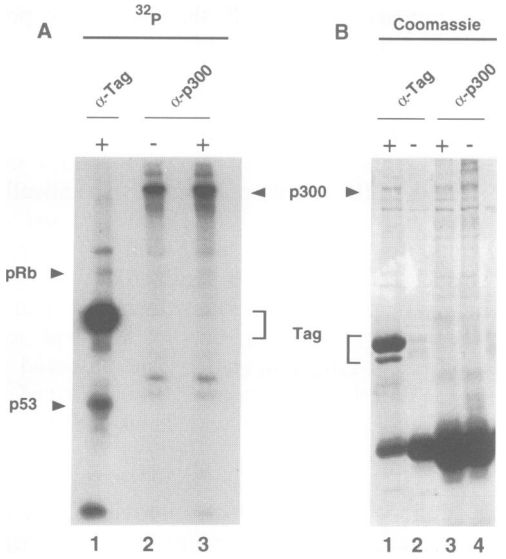

Fig. 2. ^{32}P-Metabolic labeling of anti-Tag and anti-p300 immunoprecipitations. (**A**) CV1 cells were mock infected or infected with dl884, labeled with 3 mCi of [^{32}P]orthophosphate per plate and subjected to the immunoprecipitation procedure as described in Materials and methods. Lane 1 includes the anti-Tag-specific immunoprecipitation; lanes 2 and 3 the immunoprecipitations with the anti-p300 antibody from mock-infected and dl884-infected cells, respectively. In (**B**) the products of anti-Tag- (lanes 1 and 2) and anti-p300- (lanes 3 and 4) specific immunoprecipitations from mock-infected (lanes 2 and 4) or dl884-infected CV1 cells (lanes 1 and 3) were visualized by Coomassie staining. The position of p300 is indicated by the arrow.

(p300^{pho}), which does not interact with Tag but is recognized by the anti-p300 antiserum.

It has been shown that p300 exhibits a high degree of homology to the CREB-binding protein, CBP (Arany *et al.*, 1995). To investigate the differences between the various species of p300 in further detail, the products of anti-Tag and anti-p300 immunoprecipitations were probed with the anti-p300 antiserum or with an anti-CBP-specific antibody in Western blot experiments. As revealed by the comparison of data shown in Figure 3A and B, in the anti-p300 immunoprecipitation, the anti-p300 polyclonal antiserum visualized two closely migrating polypeptides (indicated as *a* and *b* by the arrows in Figure 3A, lane 2), the slower migrating of which (band *a*) also reacted with the anti-CBP antibody (Figure 3B, lane 2). In contrast, neither the anti-p300 nor the anti-CBP antibody detected the 300 kDa species associated with Tag (lanes 3 of Figure 3A and B). In addition, as noted above, the anti-p300 polyclonal antiserum did not co-precipitate Tag in the virus-infected cells (see Figure 1A, lane 5). Since we established, by employing the V8 digestion procedure, that the 300 kDa species included in the anti-Tag and anti-p300 immunoprecipitation are similar, if not identical, there are two possible explanations for these results: (i) critical epitopes of p300 become inaccessible to the anti-p300 antibody following Tag–p300 complex formation; or (ii) the anti-p300 antiserum may recognize predominantly phosphorylated forms of p300. We favor this latter hypothesis given the inability of the antibody to recognize p300^{Tag} in a Western blot. In an attempt to address this question, the following experiment was performed. The membrane shown in Figure 3A was stripped off and incubated with calf intestinal alkaline phosphatase (CIAP) for ~18 h at 37°C, as described in the legend to Figure 3. Following dephosphorylation, the membrane was re-probed with the anti-p300 antibody in a Western blot. The result (shown in Figure 3C, lane 2), demonstrates that the dephosphorylation procedure impaired the ability of the anti-p300 antiserum to recognize the faster migrating polypeptide (band *b*) included in the anti-p300 immunoprecipitation. In contrast, the slower migrating species (band *a*) was still detected after dephosphorylation. Since this latter polypeptide also reacted with the anti-CBP antibody (see Figure 3B, lane 2), this may indicate that the anti-p300 antiserum recognizes CBP and predominantly phosphorylated forms of p300 and this might also be the reason for the lack of detection of p300^{Tag} in the Western blot as well as in the immunoprecipitation experiments.

p300 is modified covalently by ubiquitination

In the past several years it has become apparent that the activity of various proteins involved in control of cell growth, such as transcription factors, cyclins and anti-oncogene products, is modulated by the ubiquitin pathway (for a review, see Varshavsky, 1992). It was shown previously that the *in vitro* translation of a cDNA encoding p300 generates a protein whose molecular weight is lower than 300 kDa (Eckner *et al.*, 1994). This observation raised the possibility that p300 is post-translationally modified *in vivo*. However, as p300^{Tag} and p300^{pho} co-migrate in SDS gels, our data suggest that phosphorylation is unlikely to affect the electrophoretic mobility of this protein. These observations prompted us to investigate whether p300 could be modified covalently by the addition of ubiquitin, a 8.5 kDa protein (Chau *et al.*, 1989). The products of anti-Tag and anti-p300 immunoprecipitations were run on 6.5% SDS gels, transferred to PVDF membranes, and then probed with an ubiquitin-specific antibody in a Western blot (Figure 3D, upper panel). The anti-ubiquitin antibody visualized two polypeptides in both the anti-Tag (lane 3) and anti-p300 immunoprecipitations (lane 2), both of which had the size of 300 kDa as compared with the relative position of the molecular weight standards. These results demonstrate that at least a fraction of p300 expressed in CV1 cells is modified covalently by ubiquitination. In addition, these findings imply that the anti-Tag immunoprecipitation includes a ubiquitinated form of p300.

Adenovirus E1a prevents Tag–p300 complex formation

The adenovirus E1a protein and SV40 Tag interact with a number of common cellular targets (for a review, see Moran, 1993). Although the association of E1a with p300 has been described already, the existence of a population of p300 which is both hypophosphorylated and ubiquitinated has not been reported previously. We next investigated whether E1a interacts with this newly identified form of p300. First, adenovirus serotype 2-infected HeLa cells were metabolically labeled with [^{35}S]methionine (Figure 4A) or [^{32}P]orthophosphate (Figure 4B) and immunoprecipitated with the anti-E1a-specific monoclonal antibody (M73). This showed that, unlike Tag, E1a co-precipitates phosphorylated forms of p300, as described elsewhere (Yaciuk *et al.*, 1991). Next, in a parallel

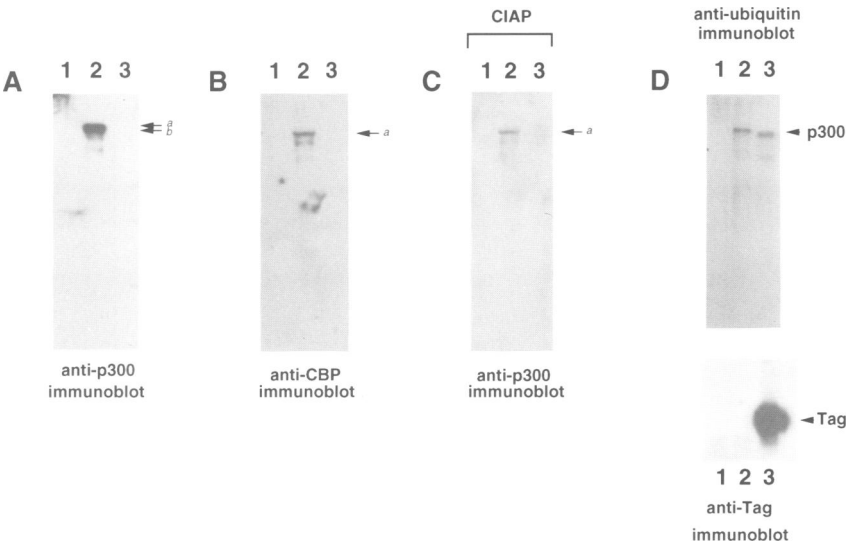

Fig. 3. Anti-p300, anti-CBP and anti-ubiquitin immunoblots of anti-Tag and anti-p300 immunoprecipitations. dl884-infected CV1 cells were lysed and immunoprecipitated in parallel with the control IgG2a monoclonal antibody (lanes 1), the anti-p300 antibody (lanes 2) or the pAb419 antiserum (lanes 3). Immunoprecipitations from ~10^8 CV1 cells were loaded in each lane. Following immunoprecipitations and SDS–PAGE, the gel was transferred on PVDF membrane as described in Materials and methods and probed with the anti-p300 polyclonal antibody in a Western blot (**A**). (**B**) The products of anti-p300 (lane 2) and anti-Tag (lane 3) immunoprecipitations were subjected to Western blot with the anti-CBP specific antibody (UBI). The arrows indicate the positions of the two closely migrating polypeptides (bands *a* and *b*) having the size of 300 kDa. (**C**) The membrane shown in (A) was stripped off, reprobed with [^{125}I]protein A to ensure that the anti-p300 antibody was washed out and subjected to dephosphorylation with CIAP. The membrane was incubated at 37°C for ~18 h, with ~10 µg of phosphatase in dephosphorylation buffer (25 mM Tris–HCl, pH 9; 1 mM $MgCl_2$; 1 mM $ZnCl_2$; 1 mM PMSF). Following the incubation, the anti-p300 antibody was added to the membrane in blocking solution (see Materials and methods) and left overnight at room temperature. Immunocomplexes were revealed with [^{125}I]protein A. (**D**) The products of anti-p300 (lane 2) or anti-Tag (lane 3) immunoprecipitations were loaded on a 6.5% SDS gel which was run for ~24 h at 50 V. After blotting, the membrane was denatured in 6 M guanidine–HCl as described in Materials and methods, washed extensively, and then subjected to the Western blot procedure with the anti-ubiquitin antibody. Immunocomplexes were detected with [^{125}I]protein A. We found that the conditions of this assay, in particular the time of electrophoresis and the denaturing procedure, are critical to obtain reproducible results. In a parallel experiment, the products of anti-p300 and anti-Tag immunoprecipitations (lanes 2 and 3, respectively) and cell extracts immunoprecipitated with the control IgG2a antibody (lane 1) were probed with the anti-Tag antibody in a Western blot.

experiment, unlabeled adenovirus-infected HeLa cells were subjected to M73-specific immunoprecipitation and probed with the anti-p300 and anti-ubiquitin antibodies in a Western blot (Figure 4C and D, respectively). This analysis revealed that the E1a-bound p300 ($p300^{E1a}$) reacted with the anti-ubiquitin antibody (Figure 4D) and with the anti-p300 antibody (Figure 4C), thus indicating that at least a fraction of $p300^{E1a}$ is ubiquitinated. Moreover, since only phosphorylated p300 is co-precipitated overtly by E1a, we could not conclude, by using this approach, that all of $p300^{E1a}$ is phosphorylated. To explore further the individual specificities of Tag and E1a towards p300, we tested whether E1a could compete with Tag, which only co-precipitates un- or underphosphorylated p300, for binding to this protein. The plasmids encoding Tag (CMVTag) and E1a (CMV13SE1a) were transfected, alone or in combination, in CV1 cells. Twenty-four hours after transfection, these cell cultures were labeled with [^{35}S]methionine, followed by immunoprecipitation with the pAb419 and M73 antibodies. Results are shown in Figure 5A. In cells containing only SV40 Tag (lane 1), p300 is found in association with Tag, as previously shown. In contrast, when E1a was co-expressed with Tag, p300 was detected in the anti-E1a (lane 4), but not in the anti-Tag (lane 2) immunoprecipitation. In addition, E1a co-precipitated equal amounts of p300 either in the presence or absence of Tag (compare lanes 3 and 4 of Figure 5A). Similarly, as shown in Figure 5B, the expression of Tag

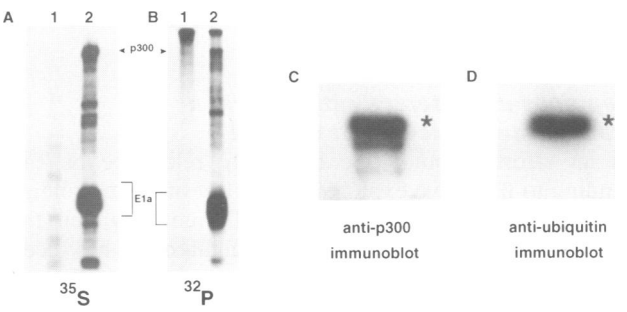

Fig. 4. Detection of the p300 species associated with E1a. HeLa cells were infected with adenovirus type 2, and labeled with 3 mCi/plate of [^{35}S]methionine (**A**) or 3 mCi/plate of [^{32}P]orthophosphate (**B**). Cell lysates from three confluent dishes of HeLa cells were immunoprecipitated, in parallel, with the anti-E1a monoclonal antibody M73 (lanes 2) or with the IgG2a isotype-matched monoclonal antibody (lane 1 of A and B), as a negative control. In (**C**) and (**D**), unlabeled lysates from adenovirus-infected HeLa cells were immunoprecipitated with the M73 antibody and subjected to Western blot with the anti-p300 and the anti-ubiquitin antibodies, respectively. The membrane was first probed with the anti-p300 antibody and autoradiographed. After an overnight exposure, the membrane was stripped off and subjected to Western blot with the anti-ubiquitin antibody as described in Materials and methods. The exposure time of the anti-ubiquitin Western blot was ~16 h. In other experiments (not shown), the anti-p300 and anti-ubiquitin immunoblots were performed independently on equal amounts of cell extracts and similar results were obtained. The asterisks indicate the position of p300.

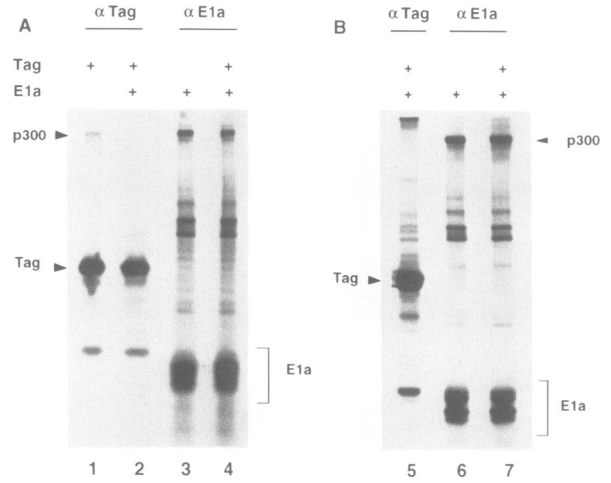

Fig. 5. Co-expression of Tag and E1a in CV1 and 293 cells. (**A**) CV1 cells were transfected with the plasmid encoding Tag (CMVTag) (lane 1) or with the E1a-expressing vector (CMV13S-E1a) alone (lane 3), or in combination (lanes 2 and 4). Twenty-four hours after transfection, the cells were labeled with 1 mCi of [^{35}S]methionine, lysed and equal amounts of cell extracts were immunoprecipitated with the pAb419- (lanes 1 and 2) or the M73- (lanes 3 and 4) specific antibodies. (**B**) The CMVTag plasmid was transfected in 293 cells (lanes 5 and 7). Twenty-four to 36 h after transfection, cells were labeled with [^{35}S]methionine and immunoprecipitated with the pAb419 (lane 5) or the M73 (lane 7) antibodies. Lane 6 contains the anti-M73 immunoprecipitation from non-transfected 293 cells, showing that the amount of p300 which is found in a complex with E1a is unmodified either in the presence or absence of Tag.

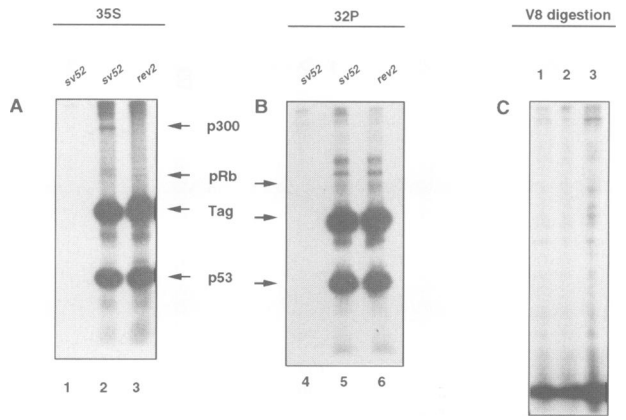

Fig. 6. Comparison of anti-Tag immunoprecipitations in SV52 and REV2. Cultured dishes of SV52 (lanes 1, 2, 4 and 5) and REV2 (lanes 3 and 6) were metabolically labeled with [^{35}S]methionine (**A**, lanes 1–3) or [^{32}P]orthophosphate (**B**, lanes 4–6), and immunoprecipitated with the anti-Tag antibody (lanes 2, 3, 5 and 6) or with the isotype-matched IgG2 monoclonal antibody (lanes 1 and 4). (**C**) Demonstration that Tag associates with pRb in SV52 and REV2 cells. ^{35}S-Labeled pRb species isolated from anti-Tag immunoprecipitations derived from SV52 (lane 1) and REV2 (lane 2) cells, together with the pRb product derived from the anti-pRb- (lane 3) specific immunoprecipitation, were subjected to partial proteolytic digestion with V8 protease.

in 293 cells, which constitutively produce adenovirus early expression products, again resulted in loss of Tag–p300, but not E1a–p300 complex formation (compare lane 5 with lane 7 of Figure 5B). The simplest explanation for these data is that E1a interacts with p300 with higher affinity than Tag and that it binds both hypophosphorylated and phosphorylated forms of p300.

Tag does not interact with p300 in a transformation-revertant cell line

In an effort to study the biological relevance of Tag binding to p300, we explored the ability of these proteins to interact in a Tag-expressing cell line in which the transformed phenotype of the parental cells has been reverted (REV2). The revertant cells were isolated from SV40-transformed rat embryo fibroblasts (SV52) as previously described (Bauer *et al.*, 1987). DNA sequencing experiments revealed that the REV2 cells contain wild-type SV40 DNA that, when injected in the grand-parental cells (REF52), still has the capability to induce transformation (Bauer *et al.*, 1987). This observation indicated that the loss of Tag transforming properties is sustained by a cellular, and not viral, alteration (Deppert *et al.*, 1991). Such cells thus comprise a suitable system to explore the cellular pathway(s) implicated in the maintenance of the transformed phenotype. Therefore, we investigated whether the properties of these cell lines may include differences in Tag–p300 interaction. REV2 and SV52 cells were labeled either with [^{35}S]methionine or [^{32}P]orthophosphate for 3 h and cell lysates were subjected to immunoprecipitation with the anti-Tag antibody, pAb419. Representative results from this set of experiments are shown in Figure 6. As expected, in the fully transformed cells, Tag was found in association with p300 (see lane 2 of Figure 6A), indicating that this interaction is conserved among different cell types and during the transformation process. When the cells were labeled with [^{32}P]orthophosphate, phosphorylated p300 was undetectable in the anti-Tag immununoprecipitation (Figure 6B, lane 5). Again, the affinity of Tag for p300 is restricted to the un- or underphosphorylated forms of the protein in rat embryo fibroblasts as well as in CV1 cells. However, p300 was not detected by the anti-Tag-specific antibody in the cells exhibiting the revertant phenotype (Figure 6A, lane 3). Significantly, only the interaction of Tag with p300 is lost in REV2, while p53 and other Tag-associated proteins are still co-precipitated by the pAb419 antiserum (compare lanes 2 and 3 and lanes 5 and 6 of Figure 6A and B). As shown in Figure 6C, partial proteolysis of pRb species isolated by anti-Tag immunoprecipitations from SV52 (lane 1) and REV2 (lane 2) cells generated digestion patterns identical to that obtained by digestion of pRb from the anti-pRb immunoprecipitation (lane 3). This result demonstrates that Tag associates with pRb in both SV52 and REV2 cells. To rule out the possibility that absence of Tag–p300 association in REV2 is due to defective p300 synthesis or to accelerated turnover, two sets of experiments were performed. ^{35}S-Labeling of anti-p300 immunoprecipitations and Western blot experiments on total cell extracts (upper and lower panels, respectively, Figure 7A) showed that the expression levels of p300 in REV2 and SV52 are similar; pulse–chase experiments (Figure 7B) indicated that the half-life of p300 in REV2 is largely comparable with that exhibited in SV52 cells.

Analysis of post-translational modifications of p300 in REV2 and SV52

The finding that Tag stably associates only with un- or underphosphorylated but ubiquitinated p300 suggests that

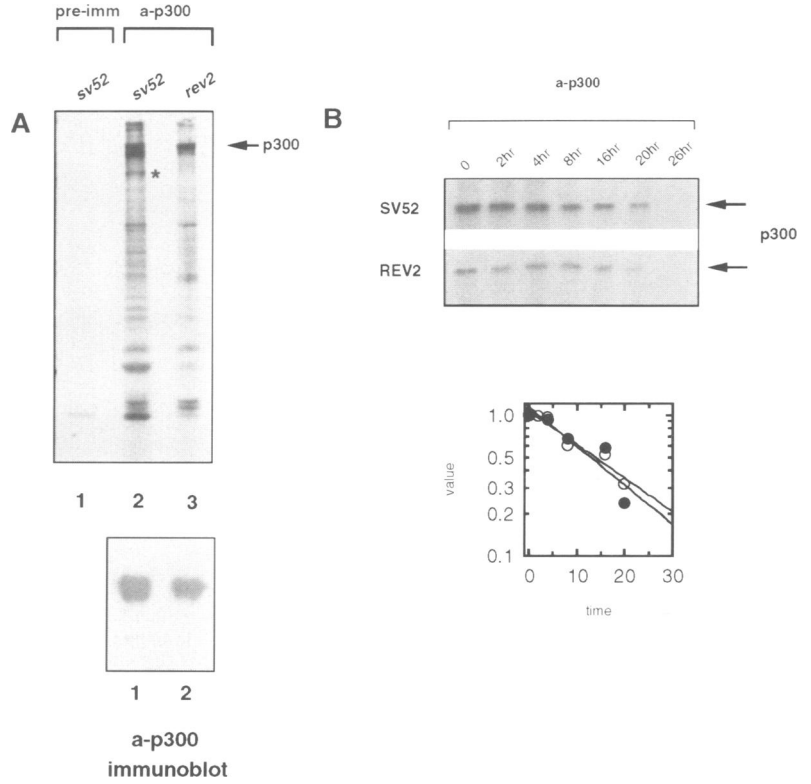

Fig. 7. Analysis of the expression levels and half-life of p300 in SV52 and REV2 cells. (**A**) Upper panel: ^{35}S-labeled SV52 and REV2 cells (lanes 2 and 3, respectively) were immunoprecipitated with the anti-p300 antiserum and subjected to SDS–PAGE. Lane 1 contains the control immunoprecipitation with the pre-immune serum on cell extracts derived from SV52 cells. The position of p300 is indicated by the arrow. The asterisk indicates the position of a protein which is detected in the anti-p300 immunoprecipitation from SV52 but not REV2 cells. Preliminary V8 digestion experiments indicate that this protein corresponds to the p300-related protein described above (see Figure 1B), p210. Lower panel: total cell extracts of SV52 (lane 1) and REV2 (lane 2) were run on a 6.5% SDS gel, transferred to PVDF membranes, and subjected to the Western blot procedure with the anti-p300 antibody. (**B**) Pulse–chase of ^{35}S-labeled p300. Cells were grown at 70% confluency, harvested in methionine-free media for ~2 h and then pulsed in 300 μCi of [^{35}S]methionine for 1 h. Following this incubation, the cells were washed twice with media containing an excess of methionine, and chased for the indicated times. Cell extracts were prepared as described in Materials and methods, and immunoprecipitated with the anti-p300-specific antibody. One dish (100 mm) of SV52 and REV2 was used for each time point. Due to the flat morphology, REV2 cells reached confluency at a lower cell number compared with SV52 and, therefore, total protein content in the cell lysates was lower for REV2 cells than for SV52. The position of p300 is indicated by the arrows. The diagram visualizes the results. The half-life of p300 in both SV52 (open circles) and REV2 (closed circles) is ~14 h. Results are representative of three experiments. For quantitation, the autoradiograms were scanned with a CID3710D monochrome video camera (Cidtec), and analyzed with the NIH Image program, version 154β.

post-translational modifications of this protein affect Tag–p300 complex formation. Thus, the phosphorylation and ubiquitination status of p300 in the revertant and fully transformed cells were compared. ^{32}P-Labeled p300 obtained from the anti-p300 immunoprecipitations performed on SV52 and REV2 cells was excised from the gel shown in Figure 8A, and subjected to partial proteolytic digestion with V8 protease. As can be seen (Figure 8B), the group of peptides designated as (1) is more phosphorylated in REV2 than in SV52 (lanes 2 and 1 respectively). In contrast, the peptides of group (3) appear hyperphosphorylated in SV52 and hypophosphorylated in REV2. Longer exposures of the gels confirmed this observation. Figure 8C shows that the peptide patterns generated by proteolytic digestion of parallel ^{35}S-labeled samples are virtually indistinguishable in the two cell lines, indicating that the differences in phosphorylation noted above are not due to an artifact of the digestion procedure.

To compare the ubiquitination pattern of p300 in REV2 and SV52 cells, the following approach was employed. Unlabeled anti-p300 immunoprecipitates were run on SDS gels together with ^{32}P-containing samples; autoradiography of phosphorylated p300 served as a guide to excise non-radioactive bands which were then subjected to partial proteolytic digestion with V8 protease. Following electrophoresis, these gels were transferred to PVDF membranes and probed with the anti-ubiquitin antiserum in a Western blot. Immunocomplexes were detected with [^{125}I]protein A. The results shown in Figure 8D demonstrate that, in REV2, the anti-ubiquitin antibody visualizes a group of peptides (indicated as 4) which are not detected in SV52. Significantly, all of the peptides visualized by the anti-ubiquitin antibody also reacted with the anti-p300 antiserum (not shown). Together, these results demonstrate that the revertant cells produce a form of p300 whose phosphorylation and ubiquitination status is modified with regard to the wild-type fully transformed cells.

The ability of E1a to prevent Tag–p300 association and the consequent deduction that it interacts with both phosphorylated and un- or underphosphorylated p300, might indicate that post-translational modifications of this protein are not critical for E1a–p300 complex formation. To test further that this could be the case, we next investigated whether E1a is capable of binding to this protein in both REV2 and SV52 which, as we showed

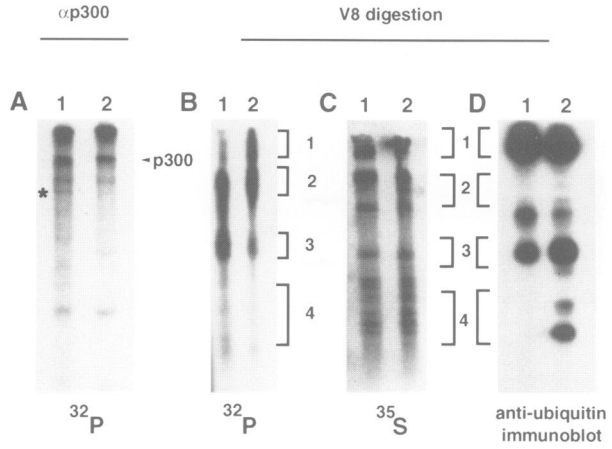

Fig. 8. Analysis of p300 phosphorylation and ubiquitination. (**A**) and (**B**) ^{32}P-Labeled SV52 and REV2 cells (lanes 1 and 2 of each panel, respectively) were immunoprecipitated with the anti-p300 antiserum and run on a 6.5% SDS gel. After autoradiography (A), p300 was localized on the dried gel, excised and subjected to proteolytic digestion with *S.aureus* V8 protease (B), as previously described. The asterisk refers to the same protein as described in the legend to Figure 7A. (**C**) Parallel ^{35}S -labeled samples. Boxes and numbers indicate the groups of peptides of interest. (**D**) Unlabeled anti-p300 immunoprecipitations from ~10^8 SV52 (lane 1) and REV2 (lane 2) cells (10 dishes at 80% confluency) were run on a 6.5% SDS gel together with ^{32}P-containing samples. Autoradiography of phosphorylated p300 served as a guide to excise the unlabeled bands which were subjected to partial digestion with V8 protease. After electrophoresis, the gel was transferred on PVDF membranes, and probed with the anti-ubiquitin antibody in a Western blot as described in Materials and methods.

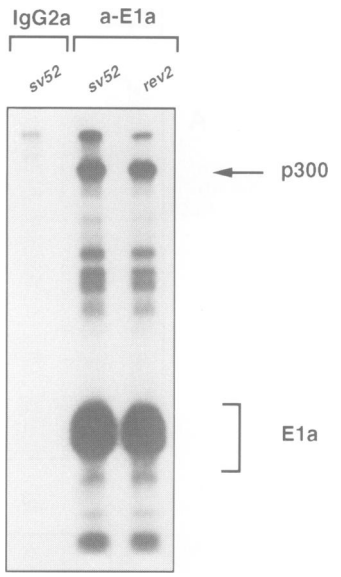

Fig. 9. Demonstration that E1a interacts with p300 in both SV52 and REV2 cells. Anti-E1a immunoprecipitation in SV52 (lane 2) and REV2 cells (lane 3). The cells were labeled with [^{32}P]orthophosphate, lysed and immunoprecipitated with the M73 antibody (lanes 2 and 3). Lane 1 contains a cell extract of SV52 cells immunoprecipitated with the IgG2a monoclonal antibody, as a negative control. The positions of p300 and E1a are indicated.

above, produce different isomeric forms of p300. The cells were infected with adenovirus serotype 2 and, 24 h later, labeled with [^{32}P]orthophosphate followed by immunoprecipitation with the M73 antibody. As shown in Figure 9, E1a was found in association with p300 in both adenovirus-infected REV2 and SV52 cells. This result supports the idea that E1a is less discriminating than Tag towards the different post-translationally modified forms of p300.

Tag represses CRE-directed transcription in SV52 but not in REV2 cells

Recently, it has been shown that p300 acts as a transcriptional co-activator, capable of stimulating transcription from cAMP-responsive (CRE) promoters (Arany *et al.*, 1995; Lundblad *et al.*, 1995). The transcriptional regulation of the CRE by p300 is thought to be dependent on its ability to bind phosphorylated CREB, and it requires the activation of the protein kinase A (PKA) pathway which, by increasing the phosphorylation of critical residues on the CREB activation domain, favors CREB–p300 interaction (Arias *et al.*, 1994). Compelling evidence has been provided that strongly supports this model. Further, recent investigations have now shown that the transcriptional activity of p300 is inhibited directly and specifically by E1a, thus supporting the idea that p300 function is somehow inactivated by the interaction with the viral oncoprotein (Eckner *et al.*, 1994; Arany *et al.*, 1995; Lundblad *et al.*, 1995). To examine the effect of Tag on a transcriptional event linked *in vivo* to p300 activity, we used a transient transfection assay that evaluates the transcriptional activation of a CAT reporter gene under the control of the CRE promoter (CREtkCAT) (Arias *et al.*, 1994). CV1 cells were transfected with CREtkCAT together with a Tag-expressing vector in which the expression of Tag is driven by the cytomegalovirus (CMV) promoter (CMVTag), or with the backbone plasmid (CMVO). Stimulation with forskolin, a strong activator of PKA (Seamon *et al.*, 1981; Tratner *et al.*, 1992), was used to drive maximal CRE-directed transcription. Representative results of this set of experiments are shown in Figure 10A. The basal CAT activity in CV1 cells varied depending on the confluence of the cells and the concentration of the serum. However, when Tag was co-transfected together with the reporter gene, a strong reduction of the CAT levels was observed (compare lanes 1 and 2 of Figure 10A). A strong increase of CAT expression from the CRE was induced following treatment of the cells with forskolin in the absence of Tag (lane 3, Figure 10A), as shown elsewhere (Arany *et al.*, 1995; Lundblad *et al.*, 1995). In contrast, treatment with forskolin failed to stimulate CRE transcription in Tag-expressing cells (compare lanes 3 and 4). These data thus demonstrate that Tag represses CRE-directed transcription.

As will be shown in more detail elsewhere, the transcriptional modulation of the CRE, as well as of AP1-regulated promoters by Tag, is specific in that it requires amino-terminal sequences of the protein specifically involved in the interaction with p300 (M.L.Avantaggiati, manuscript in preparation). Therefore, we next tested whether lack of Tag–p300 association in REV2 versus SV52 cells may reflect a functional change in the ability of Tag to modulate CRE-directed transcription in these cell lines. Transient transfection assays were thus performed in SV52 and REV2 cells. The results are shown in Figure 10B. First, it should be noted that the basal levels of transcription from the CRE are significantly lower in SV52 (black bar

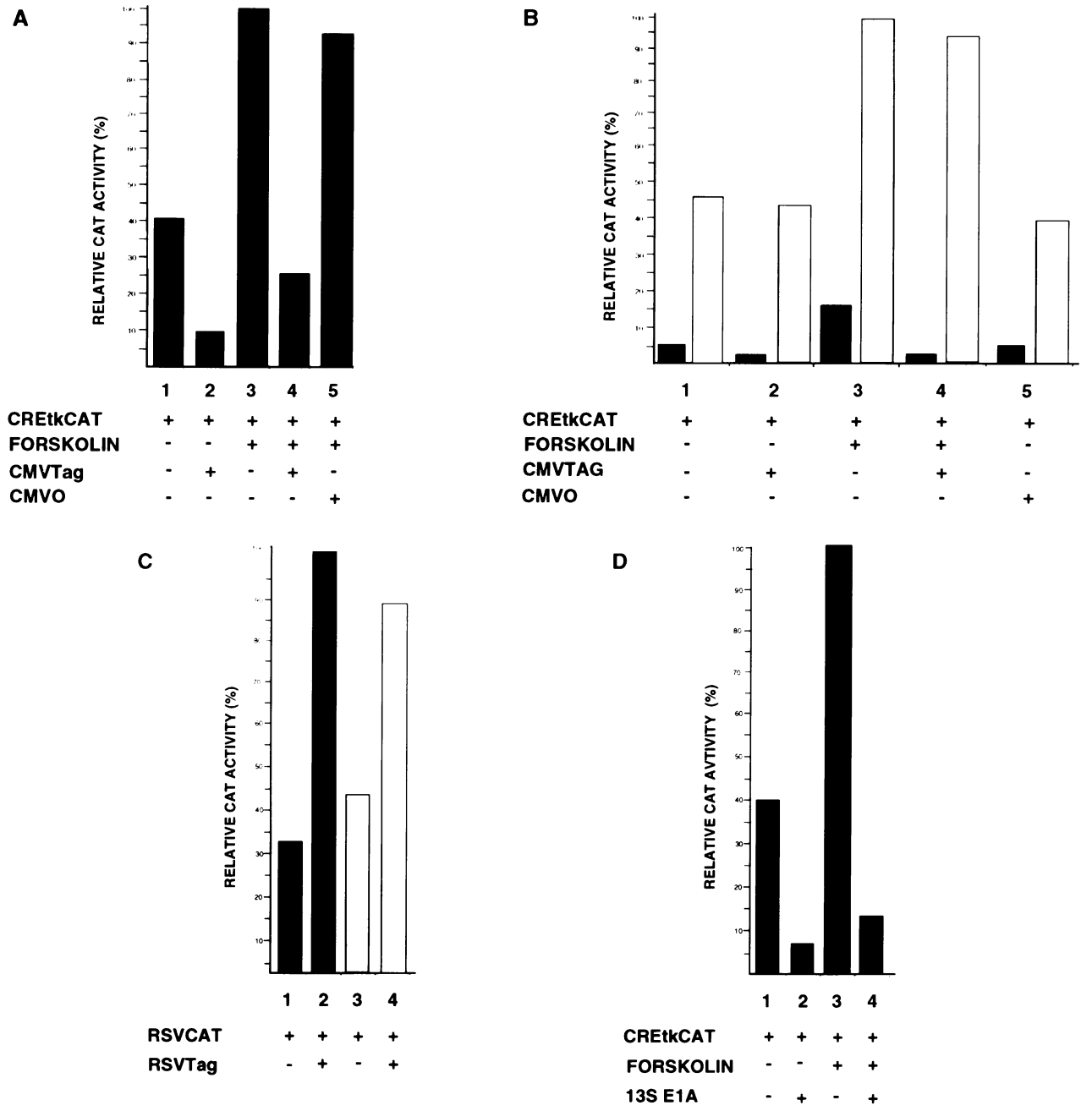

Fig. 10. Tag represses CRE-directed transcription. (**A**) CV1 cells were transfected with lipofectamine (Promega) by using 1 μg of the reporter plasmid CREtkCAT (lanes 1–5), containing the CRE, together with 2 μg of the CMVTag-expressing vector (lanes 2 and 4) or with the backbone plasmid CMVO (lane 5). Where necessary, the pUC19 plasmid was added to bring the total levels of the transfected DNA to equal amounts for each dish. After 8–12 h, the medium was removed and replaced with fresh 10% DMEM. Following an additional incubation of 12 h, the cells were stimulated with 50 μM forskolin (lanes 3, 4 and 5) or similar amounts of dimethylsulfoxide were added to the dishes. CAT levels were determined 2 h later. Protein concentration was equalized by the Bradford method. Typically, 50 μg of total cell extracts were used for the reaction. The levels of acetylation were determined by cutting out the appropriate portions of the thin-layer chromatography plate and determining the radioactivity in a liquid scintillation counter. The higher value obtained with forskolin treatment of the cells (lane 3) was considered as 100. Data shown are representative of four experiments. (**B**) SV52 (black bars) and REV2 (white bars) cells were plated at 5×10^5 cells/dish. Cells were transfected with 0.5 μg of the reporter vector (lanes 1–4), with 1 μg of the CMVTag-expressing vector (lanes 2 and 4), or with the backbone plasmid CMVO (lane 5); similar concentrations of the pUC19 plasmid were added to the transfection reactions to equalize total DNA content (lanes 1 and 3). The transfection procedure was as described above, with the exception that incubation with lipofectamine was carried out for 4 h. At this time, fresh medium was added to the plates and cells were incubated for an additional 12 h. After this time, cells were stimulated with 50 μM forskolin (lanes 3 and 4), and CAT levels were usually determined 2 h later. (**C**) Co-transfection experiments were performed on SV52 (black bars, lanes 1 and 2) or REV2 (white bars, lanes 3 and 4) cells by using as a reporter the RSVCAT plasmid. 0.5 μg of the reporter (lanes 1–4) were transfected together with 1 μg of the CMVTag plasmid (lanes 2 and 4) or 1 μg of the pUC19 plasmid. (**D**) REV2 cells were plated at 60% confluence and transfected with the CREtkCAT vector as described above (lanes 1–4) or with the plasmid encoding 13SE1a (pCMV13SE1a) (lanes 2 and 4). Following 4 h of incubation with the lipofectamine and 12 h in 10% DMEM, cells were stimulated with forskolin. CAT assays were carried out as described previously.

of Figure 10B, lane 1) than in REV2 (white bar, lane 1). Second, and most importantly, treatment of the cells with forskolin did not increase significantly the transcription levels of the CREtkCAT in SV52 (black bar, lane 3), while efficient activation was obtained with identical concentrations of forskolin in REV2 cells (white bar, lane 3). These results indicate that the transcriptional stimulation of the CRE by agonists of the PKA pathway is impaired in SV52 versus REV2 cells, and are consistent with Tag repressing the CRE promoter in the former but not in the latter. Next, to study the effect of overexpression of Tag on the CRE-directed transcription, the plasmid encoding the viral oncoprotein (CMVTag) was co-transfected in REV2 cells together with the reporter plasmid. As shown in Figure 10B, exogenous Tag did not inhibit significantly the levels of CAT transcription in untreated and forskolin-treated REV2 cells (white bars, lanes 2 and 4, respectively). Therefore, Tag loses the ability to repress the CRE promoter in REV2 cells. Transfection of Tag in SV52 cells in the absence or presence of forskolin stimulation (black bars, lanes 2 and 4) did not modify substantially the basal levels of transcription of the CRE promoter that, as noted before, is repressed constitutively in this cell line. To test whether this effect is specific, and is not due to an alteration of the general transcriptional properties of the viral oncoprotein in this cell line, the transactivation potential of Tag was explored by using the Rous sarcoma virus (RSV) promoter, which is positively modulated by Tag (Alwine, 1985). As shown in Figure 10C, the basal transcriptional levels of the RSVCAT reporter gene were similar in SV52 and REV2 cells (black and white bars of lanes 1 and 3, respectively), and in both cases overexpression of Tag stimulated RSV-dependent transcription severalfold (lanes 2 and 4, respectively).

It has been shown recently that E1a's ability to repress the CRE promoter is linked tightly to its binding with p300. Since, unlike Tag, E1a interacts with p300 in REV2 cells, we then wondered whether the different profile of interaction of these viral oncoproteins with p300 in this cell line would also be reflected as a difference in their ability to modulate CRE-directed transcription. To address this question, the E1a-expressing vector, CMV13S-E1a, was transfected in REV2 cells together with the reporter plasmid CREtkCAT. Results are shown in Figure 10D. As expected, repression of the CRE promoter was driven by E1a in both untreated (compare lanes 1 and 2) and forskolin-treated REV2 cells (compare lanes 3 and 4). In contrast, an E1a deletion mutant incapable of binding p300 (Y.Nakatani, unpublished observations), failed to repress the CRE promoter in REV2 cells (not shown). Taken together, the data presented above indicate that the different specificities exhibited by Tag and E1a towards the various isoforms of p300 are reflected, *in vivo*, as a difference in the ability of these viral oncoproteins to repress the CRE promoter.

Discussion

In this study we have demonstrated that Tag forms a specific complex with p300. In addition, we have shown that changes in both the phosphorylation and ubiquitination profile of p300 correlated with loss of Tag–p300 complex formation in a transformation-defective, Tag-expressing cell line (REV2).

SV40 Tag interacts with a hypophosphorylated but ubiquitinated form of p300

Our data demonstrate that p300 is co-precipitated specifically by Tag in various cell lines, including cells permissive for viral replication and cells in which the stable expression of the viral oncoprotein results in transformation. In all of the cell lines examined, Tag exclusively co-precipitated a discrete hypophosphorylated fraction of p300. The co-existence of hypo- and hyperphosphorylated p300 in these cell extracts (Figures 2 and 8), and the reproducible lack of association of Tag with the latter, indicates that Tag may be discriminatory towards the variously post-translationally modified forms of p300, and that phosphorylation might prevent Tag–p300 association. Alternatively, the interaction of Tag with phosphorylated p300 might occur, with dephosphorylation induced by SV40 as a pre-requisite for stable association.

The idea that phosphorylation of p300 inhibits Tag binding is also suggested by data obtained on the revertant cells (REV2). We demonstrated that Tag–p300 complexes are selectively lost in this cell line (Figure 6), and that the phosphorylation state of p300 is modified in REV2 with respect to the wild-type fully transformed cells (SV52) (Figure 8), wherein these complexes are detected. Lack of Tag–p300 association is unlikely to be caused by changes in the biochemical properties of the viral protein, as suggested by the fact that the binding pattern of Tag with other targets, including p53 and pRb (Figure 6), is fully conserved. Therefore, a possible explanation for these findings is that the revertant cells have selected a population of p300 whose phosphorylation state renders it incapable of interacting with Tag. This alteration does not affect, however, the ability of p300 to bind E1a, since E1a–p300 complexes were detected in REV2 cells infected with adenovirus (Figure 9). That E1a is less discriminative than Tag towards p300 can also be deduced from several results presented in this study. Co-expression of the viral oncoproteins in two different cell lines correlated with loss of Tag–p300, but not E1a–p300 association. In addition, E1a was capable of binding p300 in both REV2 and SV52 which, as we showed, respectively produce two different forms of p300. These observations suggest that the affinity of E1a for p300 is higher than that exhibited by Tag and that post-translational modifications of this protein may not be critical for E1a–p300 complex formation. We still do not know, however, whether Tag directly interacts with p300, whereas it has been shown that E1a does so (Eckner *et al.*, 1994; Lundblad *et al.*, 1995). Therefore, the molecular basis for the more discriminatory behavior of Tag may also lie in its requirement for a third component.

We also demonstrated that a significant fraction of p300 is post-translationally modified by ubiquitination. Ubiquitination is a covalent modification occurring when one ubiquitin molecule (8.5 kDa) is linked to a target protein, usually by the formation of an isopeptide bond through the C-terminal glycine residue of ubiquitin and the ε-amino group of a lysine residue in the acceptor protein. Ubiquitin modification of certain cellular proteins plays an important role in a variety of biological processes,

the best characterized of which is proteasome-mediated degradation (for a review, see Varshavsky, 1992). The viral oncoprotein E6 of papilloma virus is known to inactivate the anti-oncogenes p53 and pRb, by targeting them for ubiquitin-mediated degradation (Scheffner *et al.*, 1990, 1992). The finding reported here, that p300 is ubiquitinated, as well as the observation that both Tag and E1a interact with this form of the protein, raises the question of whether a similar mechanism may operate in the case of p300. It is also intriguing that multiubiquitinated forms of p300, which are not detected in SV52 cells, do appear in the cells exhibiting the revertant phenotype. It is known that multiubiquitination is a pre-requisite for proteosome-mediated degradation. Multiubiquitinated proteins can be targeted for degradation directly, or in *trans*, through protein–protein interactions (Chau *et al.*, 1989; Gregori *et al.*, 1990). If this latter mechanism acts in the case of p300, it could be lost in REV2 cells, since the ability of this protein to interact with Tag and, probably with other cellular targets, is altered in these cells. In an attempt to address this question, the half-life of p300 in SV52 and REV2 cells was compared. No difference was revealed in the two cell lines (Figure 7B). Similarly, the turnover of the protein appeared unmodified in uninfected versus SV40-infected CV1 cells (not shown). Thus, these results suggest that Tag does not induce degradation of p300. However, we have shown that the anti-p300 antibody reacts only weakly with the unphosphorylated fraction of p300, $p300^{Tag}$. Therefore, Tag-induced modifications of p300 might not be readily detectable with currently available antibodies.

The conclusions that can be drawn from these data, thus, are: (i) that p300 can be post-translationally modified by ubiquitination and phosphorylation; (ii) that phosphorylation appears to be a strong determinant for Tag–p300 interaction either directly or indirectly (through the recruitment of a third component); and (iii) that phosphorylation and ubiquitination events on p300 molecules are both modulated within the cells, as indicated by the different profile of the protein in REV2 and SV52 cells, which suggests the existence of multiple populations of p300.

Possible role of Tag–p300 complexes in SV40-mediated transformation

The transforming properties of DNA tumor viruses are thought to function through interactions with cellular regulatory proteins. An important observation reported here is that the loss of Tag's ability to bind p300 correlates with the reversion of the transformed phenotype in REV2 rat embryo fibroblasts. These data suggest that Tag–p300 complex formation may be an important step in the maintainance of the transformed phenotype by SV40 virus, at least in the cell type that we studied. Significantly, it was shown previously that adenovirus can re-transform REV2 cells (Bauer *et al.*, 1987) and, as we have shown here, E1a interacts with p300 in this cell line. The ability of E1a to re-establish the transformed phenotype could be considered independent, in this situation, of its binding to pRb, as this protein is complexed to Tag in REV2 cells (Figure 6). These observations strengthen the possibility that re-transformation of REV2 cells by E1a may, rather, occur through its binding to p300. If this is true, the RG2 mutant adenovirus, encoding E1a proteins incapable of binding p300, should display a reduced ability to transform REV2 cells compared with the wild-type virus. Preliminary experiments suggest that this may be the case.

A general notion is that several proteins, directly or indirectly involved in cell growth control, become inactive in their regulatory function(s) upon binding to a dominant viral oncogene (for a review, see Weinberg, 1990). By analogy with this model, one or more of the growth regulatory activities of p300 might be altered following its interaction with Tag or with E1a. This interpretation would be consistent with the different phenotype of SV52 and REV2 cells, with the lack of Tag–p300 complexes in REV2 cells, and with the re-transformation of this cell line by adenovirus. However, a question left open by these findings is what might be the significance of a fraction of p300 which does not interact with Tag, even in situations in which the association of the viral protein with unphosphorylated p300 does occur. In all of the cell lines examined, the existence of highly phosphorylated, non-Tag-bound, p300 was indeed revealed by the anti-p300 immunoprecipitation. If we assume that only binding to p300 by Tag is required for its inactivation, then the Tag non-bound p300 should still be capable of some anti-oncogenic function, thereby inhibiting transformation. The data might thus suggest that hyperphosphorylated p300 represents a constitutively inactive form of the protein, but this would contrast with the reversion of the transformed phenotype in REV2 cells, which presumably only express phosphorylated p300. Therefore, an alternative explanation for our results is that the Tag–p300 complex in SV52 cells acts as a dominant positive regulator of cell growth while, in the absence of Tag binding, as in REV2, p300 suppresses cell proliferation.

The cDNA encoding p300 was cloned recently, and the protein was shown to exhibit properties of a transcriptional adaptor, capable of increasing transcription from several regulatory elements, including the CRE. This effect of p300 was inhibited directly and specifically by E1a, through its binding with p300. Previous results obtained in our laboratory indicated that Tag's ability to modulate transcription from the AP1 consensus elements (TRE), and from the CRE promoters, requires amino-terminal sequences of the viral protein involved in its binding with p300 (M.L.Avantaggiati, manuscript in preparation). We have shown here (Figure 10A) that Tag represses the CRE promoter in both untreated and forskolin-treated CV1 cells, indicating that the transcriptional activity of p300 might be inhibited by the viral oncoprotein or, alternatively, that the Tag–p300 complex acts as a repressor of CRE transcription. Thus, we next wondered whether lack of Tag association with p300 in REV2 cells might be reflected in a functional change in the ability of Tag to modulate CRE-directed transcription in this cell line. The data show that the basal levels of transcription from the CREtkCAT reporter vector are significantly lower in SV52 than in REV2 cells. Furthermore, treatment with forskolin did not increase the transcription levels of the CREtkCAT in SV52, while efficient activation was obtained in REV2 cells. These results thus demonstrate that transcriptional stimulation of the CRE promoter by an agonist of the PKA pathway is impaired in the cells exhibiting the transformed phenotype versus the revertant cells. This is

consistent with Tag repressing the CRE promoter in the former but not in the latter. In agreement with this interpretation, overexpression of Tag did not inhibit the CRE transcription in REV2 cells, while efficient repression by Tag was observed in all of the cell lines wherein Tag–p300 complexes were detected (Figure 10, and data not shown). We also demonstrated that E1a repressed CRE-directed transcription in REV2 cells. Given that E1a's ability to inhibit the CRE promoter is so tightly linked to its ability to interact with p300, the new information emerging from these findings is that, in contrast to Tag, this effect of E1a is independent of the changes in the phosphorylation profile of p300, as exhibited in REV2 cells. Therefore, if, as suggested elsewhere, the E1a-induced repression of the CRE promoter reflects an inhibition of p300 function, it appears that both unphosphorylated and phosphorylated p300 are 'active' with regard to the transcriptional regulation of the CRE. Alternatively, the E1a-mediated repression of the CRE promoter in REV2 cells may be achieved through the interaction of this viral protein with a functional homolog of p300, i.e. CBP. As the binding regions of E1a for these two proteins do overlap, we could not rule out this possibility. In addition, our attempts to compare the effects of exogenous CBP and p300 in SV52 and REV2 cells on CRE-directed transcription yielded inconclusive results, as the effects of the endogenous proteins are probably difficult to overcome. Therefore, both these interpretetations are left open by our results.

While this manuscript was under review, it was reported that changes in the phosphorylation status of p300 correlate with terminal differentiation of embryonal carcinoma (EC) F9 cells (Kitabayashi *et al.*, 1995). The authors speculated that differently phosphorylated forms of p300 might reflect a dual role of this protein in positive and negative regulation of transcription, with unphosphosphorylated p300 being correlated with a repressive activity of the protein towards c-*jun*-regulated promoters in undifferentiated cells. We have now shown that changes in the phosphorylation and ubiquitination profile of p300 correlate with the reversion of the transformed phenotype in REV2 cells. Our data strongly imply that transformation by SV40 virus may require the formation of Tag–p300 complexes which act as repressors of transcription of critical genes. This effect is achieved by Tag targeting unphosphorylated forms of p300. Furthermore, the discriminatory behavior exhibited by phosphorylated and unphosphorylated p300 in REV2 and SV52 cells, respectively, versus its viral targets, Tag and E1a, could reflect a differential recruitment of p300 by its intracellular partners as well. It will be of interest to investigate how changes in the phosphorylation pattern of p300 affect the activity of this protein, and whether they can be modulated in response to cellular stimuli in various signal transduction pathways.

Materials and methods

Cells and viral stocks

Monolayers of African green monkey kidney cells, CV1, HeLa, 293, SV52 and REV2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Virus stocks were harvested from lysates of cells infected with a low multiplicity of virus and titers were determined on CV1 cells. To prepare serum-free virus stock, cells were infected with 0.1 p.f.u. per cell in serum-free medium. When cytopathic effects were apparent by extensive vacuolization, cells and culture supernatants were collected together and subjected to four cycles of freezing and thawing in ethanol/dry ice.

Plasmids and transfections

Transfections of CV1 and 293 cells were performed by using lipofectamine (GIBCO BRL). Typically, 60% confluent cell cultures were pre-incubated in DMEM containing 2% fetal bovine serum (FBS) 12–20 h before the transfection procedure, which was carried out according to the manufacturer's instructions. Unless otherwise indicated, the cells were usually incubated with lipofectamine overnight, washed extensively with phosphate-buffered saline (PBS), followed by a further incubation for ~12–18 h in DMEM supplemented with 10% FBS. The CREtkCAT was a kind gift from M.Montminy (Arias *et al.*, 1994); the CMVTag was generated by Vasily Ogryzko and it has been described elsewhere (Ogryzko *et al.*, 1994). The pCMV13SE1a-expressing vector was provided by J.Nevins (Nevins, 1981).

Purification of p300

The E1a-associated p300 was purified from 125 plates of 293 cells by co-immunoprecipitation with E1a. Cells were grown to confluence in 10% DMEM, lysed in 1 ml of lysis buffer per plate, and immunoprecipitated with the anti-E1a-specific antibody, M73, covalently coupled to protein A (Oncogene Science) for 1 h at 4°C. Proteins were eluted by heating the samples at 100°C for 10 min in SDS sample buffer (50 mM Tris–HCl at pH 6.8, 1% SDS, 10 mM β-mercaptoethanol, 0.0025% bromophenol blue). The volume of the eluate was then reduced 10-fold by rotary evaporation. Additional β-mercaptoethanol was added to a final concentration of 100 mM, and samples were loaded on a 4–12% SDS gradient minigel (Novex). After electrophoresis, proteins were stained for 30 s with Coomassie Brilliant Blue. The band corresponding to p300 was excised from the gel and electroeluted in 1% SDS, 120 mM Tris–HCl, pH 7.9. The ability of this antibody to recognize authentic p300 was evaluated by comparison with V8 protease digestion of the p300 species immunoprecipitated by either E1a in 293 cells or by the anti-p300 antiserum produced in the laboratory of Elisabeth Moran (Yaciuk *et al.*, 1991).

Infection, metabolic labeling and immunoprecipitation

Confluent cultures of CV1 or HeLa cells were infected with the SV40 small t deletion mutant virus (dl884) and adenovirus type 2, respectively, at a final concentration of 25 p.f.u./cell. Similar concentrations of adenovirus were used to infect REV2 cells. At different times post-infection (12–36 h), the medium was removed and cells were pre-incubated with either Pi-free or methionine-free DMEM for 30–90 min. For detection of Tag–p300 complexes, ~10^8 cells were used for each immunoprecipitation reaction. This usually corresponds to six dishes of 150 mm diameter. Cells were labeled with 3 mCi/ml of [^{32}P]orthophosphate (Amersham) for 4–6 h, or 0.3–3 mCi of [^{35}S]methionine (ICN) for 3 h, in P_i- or methionine-free DMEM respectively, supplemented with 5% dialyzed FCS. After labeling, cells were washed extensively with ice-cold PBS and lysed in 5 ml/10^8 cells of lysis buffer (20 mM $NaPO_4$ pH 7.8; 250 mM NaCl; 0.1% NP-40; 5 mM EDTA; 1 mM dithiothreitol), supplemented with freshly prepared protease and phosphatase inhibitors [50 μM calpain inhibitor (Sigma), 5 mM ethylmethylmaleimide (Sigma), 50 μM hemin (Sigma), 10 mM sodium fluoride, 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and leupeptin, aprotinin and pepstatin at 10 μg/ml each]. The pH of the lysis buffer was re-adjusted to 7.8 after the addition of the protease inhibitors. After 30 min of incubation on ice, cell extracts were clarified by centrifugation at 12 000 r.p.m., and the supernatant was collected in a fresh tube. The pellets were re-incubated with 2 volumes of lysis buffer supplemented with 0.01% SDS, final concentration, vigorously vortexed and incubated for an additional 10 min. At this concentration, SDS is a weak inhibitor of isopeptidases. Extracts were then clarified by centrifugation at 12 000 r.p.m.; the supernatants derived from these two extractions were pre-cleared twice with an excess of protein A, and then subjected to immunoprecipitation with the specific antibodies. After 1 h of incubation at 4°C, protein A immobilized on Trysacril (Pierce) was added to the immunocomplexes, followed by an additional incubation of ~2 h. Immunocomplexes were washed 3–6 times in lysis buffer, eluted in 2× SDS sample buffer, and loaded on 6.5% SDS gels, which typically were run overnight.

Antibodies

Polyclonal antisera to p300 were obtained by immunizing two rabbits with microgram quantities of gel-purified p300. The maximal titer of the antibody was reached ~1 month after the second booster. The anti-Tag antigen antibody is an IgG2a mouse monoclonal (Oncogene Science, SV40 Tag Ab1). The protein A-conjugated M73 antibody, used for purification of p300, was provided by Oncogene Science. The monoclonal M73 antibody was a kind gift from Antonio Giordano. The control, isotype-matched IgG2a monoclonal antibody, was supplied by Pharmigen. The anti-ubiquitin polyclonal antiserum was provided by Sigma. The anti-CBP monoclonal antiserum was provided by UBI.

Western blot analysis of proteins

Cell extracts of 10^8 CV1 or HeLa cells were prepared as described above, immunoprecipitated with specific antibodies, then resolved on 6.5% SDS–PAGE. The immunoprecipitations processed for Western blots with the anti-ubiquitin antibody were usually run for 24 h at 50 V. Gels were transferred to PVDF membranes (Millipore, Bedford, MA) at 0.5 A for 4.30 h at 4°C. After blotting, membranes were first incubated in 1× Tris-buffered saline (TBS; 20 mM Tris, pH 7.5; 0.5M NaCl; 5% bovine serum albumin) for 6 h, and then overnight with the anti-p300 antiserum diluted 1:1000 in 0.5× TBS. Immunocomplexes were detected with [^{125}I]protein A (Amersham). The Western blot with the anti-ubiquitin antibody was carried out as follows: after electroblotting, the membranes were pre-incubated with denaturing buffer (6 M guanidine-HCl, 20 mM Tris–HCl, pH 7.5; 5 mM β-mercaptoethanol; 1 mM PMSF) for 30–60 min at 4°C and washed extensively in PBS. The anti-ubiquitin antibody (Sigma) was dissolved in 10 ml of PBS and diluted to 1/20 (v/v) in blocking solution. When the same membrane was probed sequentially with two different antibodies, the membranes were stripped according to the manufacturer's instructions, re-probed with [^{125}I]protein A, and autoradiographed to verify that the first antibody was washed out.

V8 protease digestion

Cells were labeled with 0.5–1 mCi of [^{35}S]methionine, immunoprecipitated as described above and subjected to SDS–PAGE. After autoradiography, the bands of interest were excised from the dried gels and subjected to partial proteolytic digestion with V8 protease. Digestion was carried out as described elsewhere (Cleveland *et al.*, 1977) at the concentrations indicated in the figure legends.

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Note added in proof

The interaction between SV40 Tag and p300 described here has also been observed by R.Eckner *et al.*, submitted.