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p300 Family Members Associate with the Carboxyl Terminus of Simian Virus 40 Large Tumor Antigen

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Several cellular polypeptides critical for growth regulation interact with DNA tumor virus oncoproteins. p400 is a cellular protein which binds to the adenovirus E1A oncoprotein(s). The biological function of p400 is not yet known, but it is structurally and immunologically closely related to p300 and CREB-binding protein, two known E1A-binding transcription adapters. Like p300, p400 is a phosphoprotein that binds to the simian virus 40 large tumor antigen (T). In anti-T coimmunoprecipitation experiments, staggered deletions spanning the amino-terminal 250 amino acids of T did not abrogate T binding to either p400 or p300. A T species composed of residues 251 to 708 bound both p400 and p300, while a T species defective in p53 binding was unable to bind either detectably. Anti-p53 immunoprecipitates prepared from cells containing wild-type T also contained p400 and p300. Hence, both p400 and p300 can bind (directly or indirectly) to a carboxyl-terminal fragment of T which contains its p53 binding domain. Since the p53 binding domain of T contributes to its immortalizing and transforming activities, T-p400 and/or T-p300 interactions may participate in these functions.

The identification of proteins required for the regulation of cell growth has, in part, emerged from the study of DNA tumor viruses, such as the adenoviruses and papovaviruses. Transformation by these agents is mediated by certain viral early proteins, acting individually or in combination. At least part of the mechanism by which viral oncoproteins induce transformation involves their binding to one or more cellular proteins which operate as growth- and/or differentiation-regulating elements (4, 5, 28, 57, 67, 79, 87, 89).

A comprehensive analysis of adenovirus oncoprotein-cellular protein interactions has been conducted (6, 7, 25, 30, 37, 39, 40, 62, 70, 72, 78, 81, 86). Adenovirus E1A and E1B cooperate to transform rodent cells. A nonconserved, amino-terminal segment and two conserved domains of E1A, CR1 and CR2 (CR denotes regions conserved among various adenovirus serotypes), constitute the transforming sequences of this protein. CR1 is composed of residues 40 to 80, and CR2 contains residues 120 to 139. At least two of these units are required for E1A binding to each of the cellular protein targets with which it must interact to deliver a transforming signal.

CR2 contains the motif L-X-C-X-E, which is required for binding to members of the nuclear pocket protein family: pRB, p107, and p130 (reviewed in reference 61). CR2 mutations which impair E1A binding to the pocket proteins can also impair E1A-induced transformation and mitogenesis (25, 39, 41, 55, 71, 72, 78, 80, 81, 92). The notion that pocket proteins contribute to the maintenance of normal cell growth is underscored by the above-noted genetic results and by the knowledge that pRB is a tumor suppressor and an essential component of a cell cycle-regulatory pathway.

CR1 sequences enhance CR2-pocket protein interactions (6, 18, 19, 25, 39, 78, 81). They are also necessary, but not sufficient, for E1A binding to the cellular proteins p300 and CREB-binding protein (CBP) (6, 25, 39, 70, 81). Mutations which compromise E1A binding to p300 or CBP can compromise the ability of E1A to transform cells and to induce quiescent cells to undergo DNA synthesis and replicate (39, 40, 41, 60, 70, 71, 78, 81). p300 is known to be a transcriptional coactivator (2, 21, 51, 54). It likely also promotes differentiation along certain pathways (8, 10, 24, 38, 45, 47, 48, 58, 62, 68).

p400 is another E1A-binding cellular protein (39). The genetics of E1A-p300/p400 binding are in part similar and in part distinct. E1A-p300 binding depends on the integrity of multiple noncontiguous E1A sequence units, i.e., residues 1 to 25 and a segment of CR1 (6, 25, 39, 40, 70, 78, 81). p400 binding requires that E1A residues 1 to 47 remain intact. The deletion of sequences C terminal to this region had little effect on E1A-p400 binding (6, 39). Although p300 and p400 are structurally related proteins (6), and our understanding of p300 function is improving, no biochemical or biological activity has yet been ascribed to p400.

Of the E1A-binding cellular proteins, several bind to other DNA viral oncoproteins. For example, pRB, p107, and p130 bind simian virus 40 (SV40) large tumor antigen (T), human papillomavirus E7, and other polyomavirus T proteins (14, 16, 17, 20, 26, 27, 34, 82). p300 and CBP also bind to SV40 T (3, 22).

p53 can also bind to multiple DNA tumor virus oncoproteins, although it does not bind to E1A. p53 is bound and inactivated by adenovirus E1B (9, 32, 65, 76, 87, 88, 90, 91) and is bound, stabilized, and inactivated by SV40 T (15, 49, 50, 52, 63). It is inactivated by the human papillomavirus E6 protein, which targets p53 for rapid destruction by ubiquitin-mediated proteolysis (66).

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TABLE 1. Origin and characteristics of cell lines used

Cell line	Derivation or cell line origin	Viral oncoproteins expressed (amino acid residues present)	Reference or source
PVU-0	C57BL/6 MEF[a]	wt T (1–708); wt t	12
PVU-1	C57BL/6 MEF	Mutant T (1-105∧Y∧112-708); wt t	12
K1	C57BL/6 MEF	Mutant T (1-708; E107K); wt t	12
B6/*dl*2005	C57BL/6 MEF	wt T (1-708); no t	73
B6/y2xmet 128-70K	C57BL/6 MEF	Mutant T (128-708; K128M); no t	73
B6/S11-S24	C57BL/6 MEF	Mutant T (1-126∧251-708); wt t	73
B6/*dl* 501-550	C57BL/6 MEF	Mutant T (1-500∧RNS∧551-708); wt t	46
B6 S-cl7	C57BL/6 MEF	None	73
Rat-2	Rat-1	None	75
Rat-2 pPVU-0	Rat-2	wt T (1-708); wt t	This study
Rat-2 CAV251-708	Rat-2	Mutant T (251-708); no t	This study
Saos-2	Human osteosarcoma	None	29
U-2 OS	Human osteosarcoma	None	64
FaDu	Human squamous carcinoma	None	ATCC HTB 43
HOS	Human osteosarcoma	None	56
SV80	Human fibroblast	wt T (1-708); wt t	74
293	Human renal cell	wt E1A; wt E1B	31

[a] MEF, mouse embryo fibroblast.

Here, we report that p400 also binds SV40 T and that a common region of T supports binding of both p300 and 400. Surprisingly, this region includes the p53 binding domain and lacks the T/t common region of the protein.

MATERIALS AND METHODS

Cell lines and their maintenance. Most cell lines used in this study have been described previously. Background and descriptive characteristics are provided in Table 1. Saos-2, U-2 OS, and HOS cells were obtained from the American Type Culture Collection. FaDu cells were the kind gift of Erik Flemington. FaDu and all C57BL/6- and Rat-2-derived cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco/BRL) containing 10% qualified fetal bovine serum (FBS; Gibco/BRL). HOS cells were cultured in Eagle's minimal essential medium (Gibco/BRL) containing 10% FBS. All of these lines were incubated at 37°C in a 5% CO_2-containing atmosphere. U-2 OS, Saos-2, 293, and SV80 cells were maintained in DMEM containing 10% Fetal Clone I (HyClone) at 37°C in a 10% CO_2-containing atmosphere. Cultures were passaged at a ratio of 1:10, except for FaDu, Saos-2, and 293 cultures, which were passaged at a 1:5 ratio.

Rat-2 pPVU-0 cells were generated by stable cotransfection of Rat-2 cells with pSV2neo and pPVU-0 (44), an SV40 expression plasmid which encodes SV40 T and t. Similarly, Rat-2 CAV251-708 cells were generated by stable cotransfection of Rat-2 cells with pSV2neo and the previously described plasmid CAV251-708 (11). All transfectants were selected for G418 resistance. The synthesis of appropriately sized T species by the stably transfected cells was monitored by immunoprecipitation and immunoblotting (not shown).

Antibodies. All monoclonal antibodies used in this study were described previously. M73 is a mouse immunoglobulin G2a (IgG2a) antibody which recognizes E1A (36). PAb 419 is a mouse IgG2a antibody which recognizes the amino terminus of SV40 T (35). PAb 423 is a mouse IgG1 antibody which recognizes the carboxyl terminus of T (35). PAb 901, an IgG2a monoclonal antibody specific for the carboxyl terminus of T, was kindly provided by S. S. Tevethia. PAb 421 is a mouse IgG2a antibody which recognizes the carboxyl terminus of both wild-type (wt) and mutant p53 (35). Hybridoma supernatants containing M73, PAb 419, PAb 423, and PAb 421 were the kind gifts of Ed Harlow. Monoclonal antibodies raised against human p300 (RW 102, IgG3; RW 105, IgG1; RW 109, IgG1; RW 128, IgG1; and RW 144, IgG1) were described previously (21). The IgG1 monoclonal antibody AC 238 recognizes the p300-related protein CBP (22). The polyclonal anti-p300 serum, TAP p300L, was raised against E1A-associated p300 as described previously (21). F5D, a mouse IgG1 which recognizes myogenin (83), was the kind gift of Woodring Wright.

Immunoprecipitation. Immunoprecipitates were prepared by using cell cultures that were approximately 90% confluent. In vivo radiolabeling and extract preparation were performed as described previously (53), with several modifications. Media for ^{35}S protein labeling contained 333 μCi of [^{35}S] EXPRE^{35}S^{35}SLABEL (NEN/DuPont) per ml and 5% dialyzed FBS. All monolayers were harvested in 300 μl of EBC (50 mM Tris-HCl [pH 8.0], 120 mM NaCl, 0.5% Nonidet P-40, 4 mM NaF, 2 mM sodium orthovanadate) and rocked at 4°C for 1 h for protein extraction. Protein content of the supernatants was measured by the Bio-Rad Laboratories (Bradford) protein assay with bovine serum albumin as the standard. Fresh protein extracts were used in all immunoprecipitations. The amount of protein used for each experiment is noted in the appropriate figure legends.

Antibody volumes used per 800 μg of cellular protein were 70 μl (M73, PAb 419, PAb 423, and PAb 421) or 100 μl (F5D, RW 102, RW 105, RW 109, RW 128, RW 144, and AC 238) of monoclonal antibody and 2 μl of polyclonal antiserum. Monoclonal antibodies not of the IgG2a subclass were supplemented with 1.5 μl of secondary antibody (affinity-purified rabbit anti-mouse IgG; Cappel). Extract-antibody mixtures were rocked for 2 h prior to the addition of 60 μl of protein A-Sepharose/NETN [NETN is 20 mM Tris-HCl [pH 8.0], 120 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 4 mM NaF, and 2 mM sodium orthovanadate). Proteins were resolved in sodium dodecyl sulfate (SDS)–6% polyacrylamide gels. Prestained protein molecular weight markers (Sigma) were included in each gel. Gels used for fluorography were treated with En^{3}Hance (Amersham).

Immunoprecipitation-reimmunoprecipitation experiments. Increased quantities of protein were used for these experiments, due to the relative inefficiency (approximately 5%) of RW 144 in reprecipitating p400. First-round immunoprecipitations were as described above. Following the fifth wash, pelleted beads were suspended in an equal volume of NETN supplemented with SDS to 2% and dithiothreitol to 10 mM. The suspensions were boiled for 10 min, and NETN was used to dilute the contents of the tubes 10-fold. The Sepharose beads were removed by successive centrifugations, with transfer of the supernatants to fresh tubes after each of two centrifugations. The volumes of monoclonal antibody, secondary antibody, and protein A-Sepharose added for reimmunoprecipitation were twice those indicated above. Binding proceeded overnight at 4°C with rocking. Subsequent washes and gel analyses were as described above.

Immunoblotting. The transfer of gel-resolved proteins to Immobilon polyvinylidene difluoride membranes (Millipore) was performed in transfer buffer (195 mM glycine, 25 mM Tris, 20% methanol, 0.01% SDS) at 4°C and 450-mA constant current for 20 to 24 h. Membrane blocking, washing, and enhanced chemiluminescence (ECL) development were as described previously (46). RW 144 or RW 128 hybridoma supernatant, diluted 1:6 in phosphate-buffered saline (PBS) containing 5% (wt/vol) nonfat dry milk, was incubated with the appropriate membrane at room temperature for 4 h. Control T immunoblots were performed with PAb 901 as previously described (46).

Pulse-chase analysis. Subconfluent cultures of U-2 OS cells were washed twice with PBS and once with methionine-free DMEM. Cells were starved for 1 h at 37°C in methionine-free DMEM containing 5% dialyzed FBS. Starvation medium was replaced with prewarmed, methionine-free DMEM supplemented with 5% dialyzed FBS and 333 μCi of [^{35}S] EXPRE^{35}S^{35}SLABEL (NEN/DuPont) per ml. Cultures were incubated at 37°C for 20 min, washed three times with PBS, and then incubated for the indicated chase time in complete DMEM supplemented with 10% FBS and 3 mg of L-methionine per ml. Cell harvesting, extract preparation, immunoprecipitation, and gel electrophoresis were performed as outlined above. Half-life determination was performed by direct quantification (Betascope 603 blot analyzer; Betagen) of counts per minute present in individual p400 and p300 bands at each chase time point. Values corrected for background counts per minute were used to generate decay curves.

RESULTS

Some monoclonal antibodies generated against p300 also bind to p400. Previous reports suggested that p300 and p400 are structurally related (6), with similar, albeit not identical,

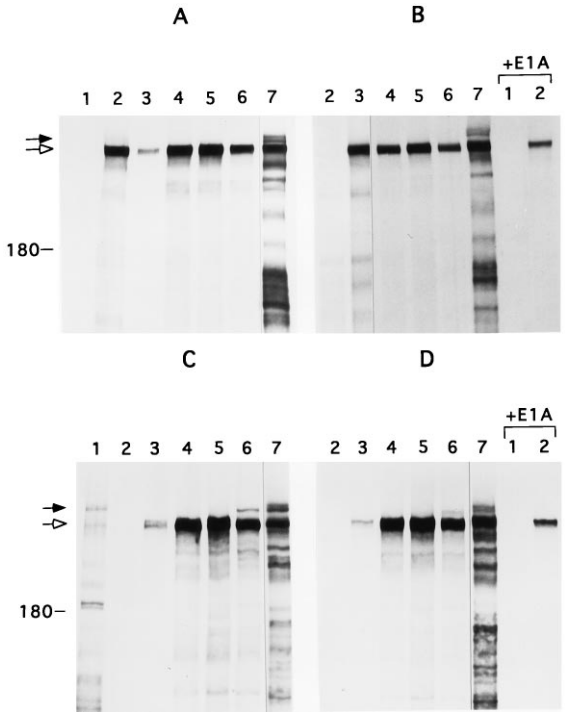

FIG. 1. Immunoprecipitation of p400 by anti-p300 monoclonal antibodies. For each reaction, 500 μg of ^{35}S-labeled cellular protein was analyzed. For lanes marked by brackets, 500 μg of unlabeled 293 cell extract was mixed with the radiolabeled extract to provide E1A in *trans*. This allowed for further confirmation of the identity of immunoprecipitated p300 by virtue of its comigration with E1A-bound p300. Immunoprecipitates were prepared using extracts of human (293 [A] or U-2 OS [B]) or murine B6/*dl*2005 [C] or B6S-cl7 [D]) cells. Antibodies used for immunoprecipitation were PAb 419 (lane 1), M73 (lane 2), TAP p300L (lane 3), RW 102 (lane 4), RW 105 (lane 5), RW 128 (lane 6), and RW 144 (lane 7). The migration positions of p400 and p300 are denoted by the closed and open arrows, respectively. The position of the 180-kDa prestained protein marker is also indicated. Each panel was prepared as a composite of two exposures from the same gel to allow visualization of all relevant protein bands.

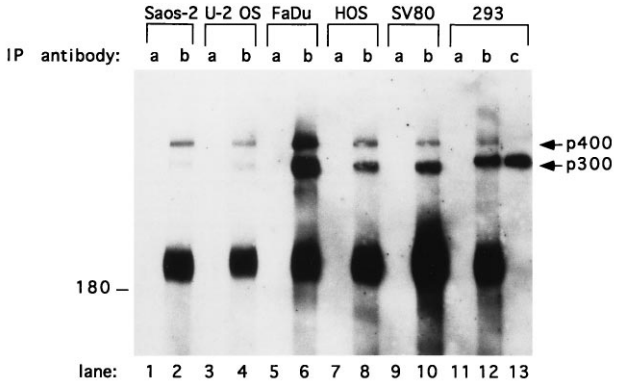

FIG. 2. The anti-p300 monoclonal antibody, RW 144, directly binds p400. The indicated cellular protein extracts were immunoprecipitated (IP) with control monoclonal antibody F5D (lanes a), anti-p300 monoclonal antibody RW 144 (lanes b), or E1A-specific monoclonal antibody M73 (lane c). Immunoprecipitated proteins were detected by RW 144 immunoblotting. The migration positions of p400 and p300 are indicated. The quantities of cellular protein (in micrograms) used for immunoprecipitation were 550 for Saos-2, 600 for U-2 OS, and 800 each for FaDu, HOS, SV80, and 293. Binding reactions for lanes 11 to 13 also received 80 μg of ^{35}S-radiolabeled 293 extract, to allow for comparison of the positions of the immunoblotted and immunoprecipitated p300 and p400. Despite its presence, the radioactivity in the immunoprecipitates electrophoresed through lanes 11 to 13 did not contribute to the immunoblotting signal in these three lanes.

E1A-binding genetics (6, 39). To determine whether the two proteins are also immunologically related, a panel of four monoclonal antibodies generated against human p300 (21) was tested for p400 binding. Two of the monoclonal antibodies, RW 102 and RW 105, immunoprecipitated p300 from human and murine cell extracts without detectably binding p400 (Fig. 1A to D, lanes 4 and 5). A third monoclonal antibody, RW 144 (lanes 7), immunoprecipitated human and murine p300 and p400. It was previously reported that gel-resolved p400 at times migrated as a doublet (6). Both p400 bands were observed in this experiment with RW 144. Antibody RW 128 (lanes 6) recognized human and murine p300, bound the lower band of murine p400, and failed to bind human p400. Interestingly, bands comigrating with p300 and p400 were detected in anti-T immunoprecipitates from the T-producing cell line, B6/*dl*2005 (Fig. 1C, lane 1). This phenomenon is addressed below.

All immunoprecipitates that contained p400 also contained p300. It was possible that p400 was not directly recognized by the antibodies but was coprecipitated through an association with p300. We tested whether RW 144 could bind directly to p400 by Western blot analysis. RW 144 and F5D (antimyogenin) negative control immunoprecipitates were prepared from extracts of various human cell lines and processed for immunoblotting with RW 144. As shown in Fig. 2 (even-numbered lanes), p300 and p400 species from a variety of human cell lines directly bound to RW 144. Therefore, p300 and p400 are immunologically, as well as structurally, related. The identity of the ~180-kDa band in this figure is unknown. Among various possibilities, it might be a p300/CBP/p400 cleavage product.

We recently reported that T directly or indirectly suppresses p300 phosphorylation, whereas adenovirus E1A-expressing 293 cells contain hyperphosphorylated p300 (22). These observations likely explain the differing migration of p300 from various cell lines, which is apparent in Fig. 2. Confirmation of the positions of p300 and p400 was obtained in this and other experiments by including a small amount of radiolabeled cellular protein extract in some immunoprecipitation reactions. This allowed for the detection of radiolabeled p300 and p400 by autoradiography of the relevant immunoblots (data not shown). In all cases, the immunoblotted p300 and p400 proteins colocalized with the immunoprecipitated, radiolabeled p300 and p400 proteins. In repeated experiments, the relative quantities of detectable p300 and p400 varied significantly among the cell lines tested (not shown). Furthermore, although RW 144 bound directly to p400 and p300, it was relatively inefficient in Western blotting experiments (by comparison to RW 128, which efficiently recognized filter-immobilized p300). Because of this technical limitation, RW 144 was not used in further Western blotting analysis.

Pulse-chase analysis was performed to compare the half-lives of p400 and p300. Quantitative analysis indicated that radiolabel incorporation into p300 and p400 peaked at the 15-min chase point. The half-life of p400 in actively growing U-2 OS cells was approximately 1.5 h (Fig. 3). By contrast, the half-life of p300, extrapolated from data in the same experiment, was greater than 6 h. A previous determination of p300 half-life in WI-38 cells was approximately 9 h (85). The significantly different p300 and p400 half-lives imply that the two proteins operate, in part, differently in vivo. On the other hand, both p300 (85) and p400 (data not shown) are phosphoproteins.

p400 binds SV40 T. We and others have shown that p300 and CBP have structural, immunological, and functional similarities, including the ability to bind T (3, 24). Selective inac-

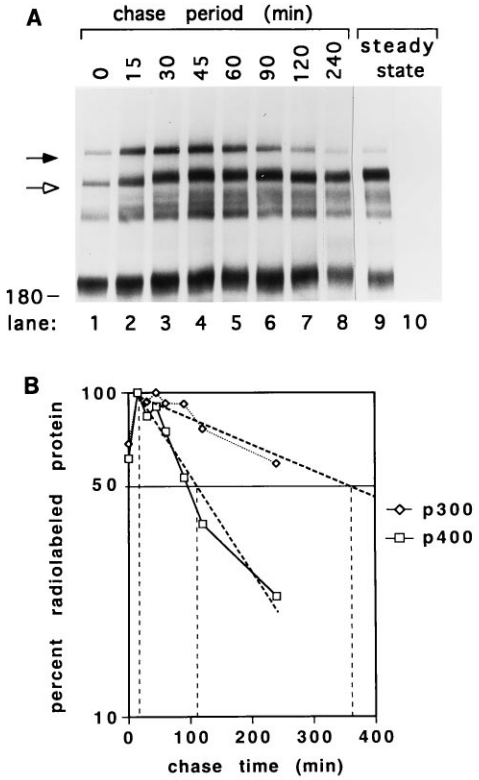

FIG. 3. Comparison of the half-lives of p400 and p300. (A) For pulse-chase analysis, RW 144 immunoprecipitates in lanes 1 to 8 were prepared by using 700 μg of ^{35}S-radiolabeled U-2 OS protein. Immunoprecipitates in lanes 9 (RW 144) and 10 (F5D) were prepared by using 300 μg of protein from cells continuously labeled for 4 h. The figure is a composite, with the exposure time of lanes 9 and 10 four times shorter than that of lanes 1 to 8. p400 and p300 are marked by the closed and open arrows, respectively. The position of the 180-kDa prestained protein marker is also indicated. (B) The half-life determination for p400 and p300 from U-2 OS cells was performed as described in Materials and Methods, using the results shown in panel A.

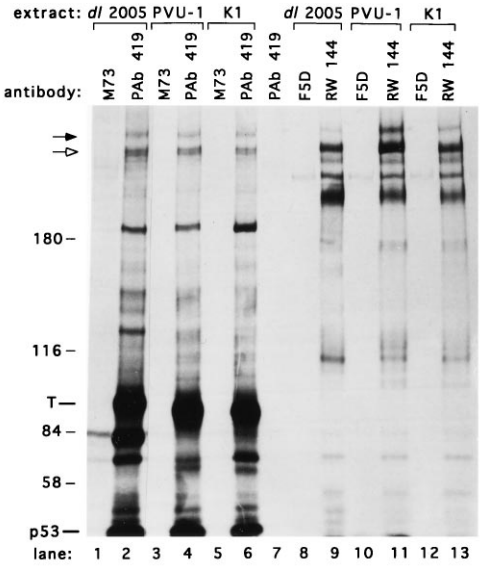

FIG. 4. A functional pRB binding site is dispensable for T binding to p400. The quantities of ^{35}S-labeled cellular protein used per immunoprecipitation were 600 μg (lanes 1 to 7) and 200 μg (lanes 8 to 13). Extracts and antibodies used are indicated. The immunoprecipitate in lane 7 was generated by using B6S-cl7 cell extract, which contains no T. p400 is indicated by the closed arrow, and the CBP/p300 doublet is indicated by the open arrow. The positions of prestained protein markers are indicated at the left, with their apparent masses given in kilodaltons. The positions of T and p53 are also marked. The figure is a composite: lanes 1 and 2 were taken from a longer exposure autoradiogram in order to normalize the T signals in lanes 1 to 6.

tivation of the pRB-binding domain of T failed to abrogate T binding to p300 or CBP, implying that other sequence units contribute to this interaction. Both p300 and CBP bound to T in the absence of small t (3, 22).

Initial observations (Fig. 1C, lane 1) suggested that p400 could also bind to T. To confirm this, and to determine whether a functional pRB binding site was required for T binding to p400, a series of immunoprecipitation assays was performed on cell extracts containing wt and various mutant T species (Fig. 4). As recently shown (22), CBP and p300 are the upper and lower bands, respectively, of a protein doublet which regularly coprecipitates with T. wt T (*dl*2005) coprecipitated p300, CBP, and p400 in the absence of small t (lane 2). These bands were also present in anti-T immunoprecipitates from extracts of cells producing two mutants of T, PVU-1 and K-1 (lanes 4 and 6, respectively). T-coprecipitated p300 and p400 comigrated with RW 144-immunoprecipitated p300 and p400 from the same extracts (compare lanes 2, 4, and 6 with lanes 9, 11, and 13, respectively). None of the bands were immunoprecipitated by the isotype-matched negative control antibodies, M73 and F5D (lanes 1, 3, and 5 and lanes 8, 10, and 12, respectively). Therefore, like p300 and CBP, p400 also associates with T. And since neither PVU-1 nor K1 T contains a functional pRB binding site, this T function is not required for T binding to p300, CBP, or p400.

The carboxyl terminus of T contains sequences that are necessary and sufficient for p400 and p300 binding. A structure-function analysis was undertaken to define the region(s) of T essential for binding to p400 and p300. Structure-function mapping (Fig. 5) was performed by a modification of the immunoprecipitation procedure. Control (F5D) or anti-T (PAb 423) immunoprecipitates were prepared by using radiolabeled protein extracts from murine cell lines stably producing wt T, mutant T, or no T. Immune complexes were dissociated, and the separated proteins were subjected to reimmunoprecipitation using a monoclonal antibody (RW 144) which directly recognizes p300 and p400. Reprecipitated proteins were resolved in SDS-gels and visualized by autoradiography. When a specific positive signal is obtained, this technical approach provides immunological confirmation that T-coprecipitated proteins which comigrate with p300 and p400 are, indeed, p300 and p400.

Both p300 and p400 specifically coprecipitated with T (Fig. 5, lane 2). Neither protein was present in anti-T immunoprecipitates from a spontaneously transformed murine cell line, B6S-cl7, which produces no T (lane 3). Deletion of sequences from the N terminus of T had little effect on its ability to bind endogenous levels of p300 and p400 in vivo: T lacking amino acids 1 to 127 (lane 4) or 127 to 250 (lane 5) coprecipitated both proteins. It can be concluded from these data that residues 1 to 127 and 127 to 250 are individually dispensable for T binding to p300 and p400.

By contrast, deletion of T residues 501 to 550 resulted in the loss of p300 and p400 binding (lane 6). Sequences within this region are therefore important for binding p300 or p400 but may not comprise the binding site per se. In this experiment, control immunoprecipitations showed that the amount of T in the B6/*dl*501-550 immunoprecipitation reaction was approximately 60% of that in B6/*dl*2005 reactions (not shown). In

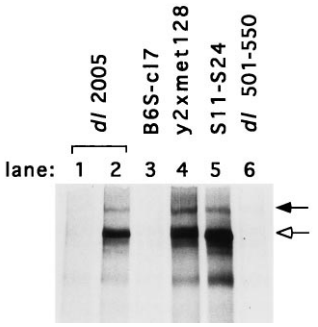

FIG. 5. Genetics of T binding to p400 and p300. Two milligrams of ^{35}S-labeled protein from each of the indicated cell lines was used for immunoprecipitation with control antibody F5D (lane 1) or T-specific antibody PAb 423 (lanes 2 to 6). Immunoprecipitated proteins were dissociated and reimmunoprecipitated with RW 144. p400 and p300 are indicated by the closed and open arrows, respectively.

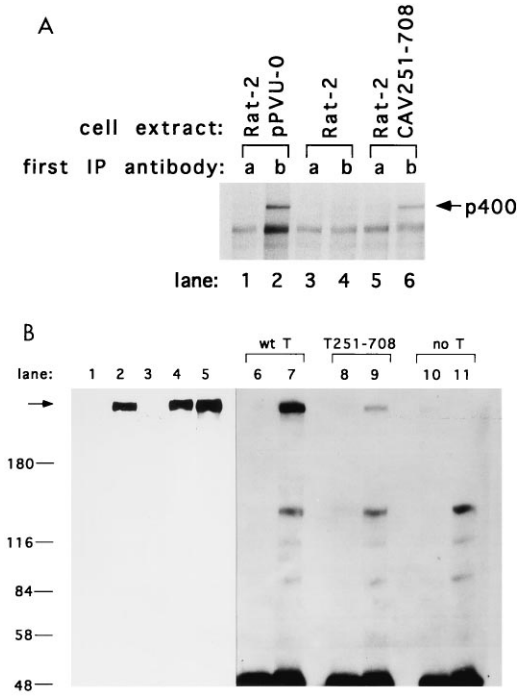

FIG. 6. A carboxyl-terminal fragment of T is sufficient for coprecipitation of p400 and p300. (A) ^{35}S-labeled protein (2.3 mg [lanes 1 to 4] or 2.0 mg [lanes 5 and 6]) from the indicated cell lines was immunoprecipitated (IP) with control antibody F5D (lanes a) or T-specific antibody PAb 423 (lanes b) and reimmunoprecipitated with the p300/p400-specific monoclonal antibody, RW 144. The migration position of p400 is indicated. In this experiment, a background band comigrating with p300 was present in all lanes. (B) Unlabeled cellular extract protein (300 μg [lanes 1 to 5] or 10 mg [lanes 6 to 11]) was immunoprecipitated with various antibodies. Lanes 1 to 5 contain reference control immunoprecipitates for lanes 6 to 11. Lanes 2, 4, and 5 show the positions of RW 144-immunoprecipitated p300 from cells expressing wt T, 251-708 T, and no T, respectively. Lane 1 contains the negative control F5D immunoprecipitate from wt T-expressing cells. Lane 3 contains CBP immunoprecipitated from wt T-expressing cells by the CBP-specific antibody AC 238; it serves here as a specificity control for the anti-p300 immunoblotting antibody. To test for p300 coprecipitation with the various T antigens (lanes 6 to 11), Rat-2 extracts containing the indicated T antigens were immunoprecipitated with either negative control antibody F5D (lanes 6, 8, and 10) or T-specific antibody PAb 423 (lanes 7, 9, and 11). Immunoprecipitated proteins were immunoblotted with the p300-specific antibody, RW 128, and the ECL detection system. The figure was prepared as a composite, in order to compensate for the much stronger p300 ECL signal in control lanes 2, 4, and 5 than in all other lanes. Sizes are indicated in kilodaltons.

repeated experiments, F5D or RW 144 negative control immunoprecipitations for all mutants contained no detectable p300 or p400 (data not shown). Notably, the deletion of T residues 501 to 550 abrogates T binding to p53 (46).

These data suggest that residues 251 to 708 are sufficient for p300 and p400 binding by T. However, it was possible that T contains redundant sites for binding these proteins, with one site mapping within residues 1 to 127 and a second mapping within residues 127 to 250. Mutant T antigens lacking one of these domains would be capable of binding p300 and p400 due to the presence of the second domain. Indeed, redundant binding sites for the core transcription factor, TATA-binding protein have been identified within the N terminus of T (33, 43), and p300 has been reported to bind to TATA-binding protein (1). In this scenario, the effect of the deletion of amino acids 501 to 550 on p300 or p400 binding might be ascribed to gross alterations in T structure, leading to functional inactivation of both amino-terminal binding sites.

To test this possibility, binding of p300 and p400 to a T species composed of residues 251 to 708 (251-708 T) was evaluated. Protein extracts from Rat-2 cells producing high levels of wt T, 251-708 T, or no T were analyzed by either of two approaches: immunoprecipitation-dissociation-reimmunoprecipitation (Fig. 6A) as described above or combined immunoprecipitation-immunoblotting (Fig. 6B). p400 (Fig. 6A, lanes 2 and 6) and p300 (Fig. 6B, lanes 7 and 9) coprecipitated with both wt and 251-708 T. The immunoblotting signal was specific for p300. The RW 128 probe antibody failed to detect CBP (Fig. 6B, lane 3) or p400 (present in lanes 2, 4, and 5). These data also suggest that RW 128 recognizes the lower band of the rodent p400 doublet (Fig. 1C and D) in the native but not denatured state (Fig. 6B). In repeated experiments, RW 128 failed to bind to denatured rodent p400 derived from either rat or murine cell lines (data not shown).

Although residues 251 to 708 are sufficient to bind to p400 and p300, additional T sequences amino terminal to this region may enhance complex formation. This possibility is supported by PAb 901 (anti-T) reprobing of the filter from Fig. 6B (not shown), which demonstrated that the amounts of wt and mutant T in lanes 7 and 9 were approximately equal, whereas the amount of p300 recovered by coimmunoprecipitation with 251-708 T was much less than that recovered with intact T.

Like CBP, p300 and p400 associate with both T and p53. We recently reported that CBP is present in complexes containing T and p53 (22). We wished to determine whether p300 and p400 also complex with p53 in T-expressing cells. Anti-T, anti-p53, and control immunoprecipitates were prepared from [^{35}S]methionine-labeled murine cell extracts containing wild-type T (B6/*dl*2005) or no T (B6S-cl7). As shown in Fig. 7, anti-T (PAb 419 and PAb 423) and anti-p53 (PAb 421) immunoprecipitates from T-producing cells contained p300, CBP, and p400 (lanes 2 to 4). The top band of the p300 doublet is CBP, as determined previously by using a CBP-specific monoclonal antibody (22). Even after prolonged autoradiographic exposure (not shown), none of these proteins appeared as p53-coprecipitating proteins in the absence of T (lane 9). In the SDS–6% polyacrylamide gels used for this analysis, p53 reproducibly migrated faster than the 48-kDa prestained molecular weight marker. Our results demonstrate that p53, p300, CBP, and p400 can complex with T and that T, p300, CBP, and p400 can complex with p53. These results do not indicate whether these multiprotein interactions are binary, ternary, or more complex in nature. Further experimentation is necessary

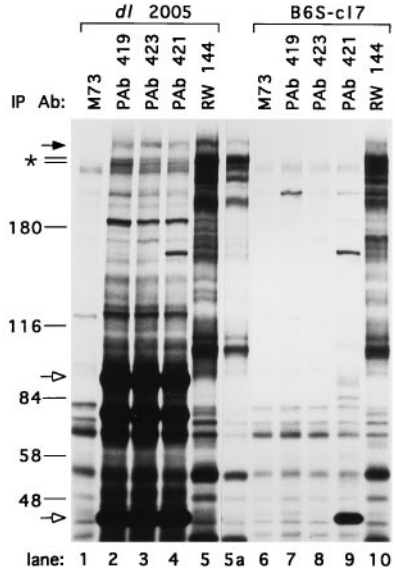

FIG. 7. p400 and p300 coprecipitate with T and with p53. ^{35}S-labeled murine cell extracts containing wt T (*dl*2005) or no T (B6S-cl7) were immunoprecipitated (IP) with antibodies (Ab) M73 (control anti-E1A), PAb 419 (anti-T), PAb 423 (anti-T), PAb 421 (anti-p53), and RW 144 (anti-p300/p400). Protein quantities used were 800 μg (lanes 1 to 4 and 6 to 9) or 250 μg (lanes 5 and 10). All lanes were derived from the same exposure of the gel to film except for lane 5a, which is a shorter exposure of lane 5, provided to allow for the resolution of individual protein bands. p400 is indicated by the closed arrow. CBP and p300 are indicated by the upper and lower dashes, respectively, marked by the asterisk. T and p53 are marked by the upper and lower open arrows, respectively. The bands migrating just beneath the 84-kDa marker, and specifically present in lanes 2 to 4, are apparently proteolytic degradation products of T (69) which react with T-specific antibody, PAb 901 (not shown). The positions of prestained protein markers are indicated, with their apparent masses given in kilodaltons.

to determine whether T and p53 are present together in a single complex with p400 and/or p300.

DISCUSSION

Our results confirm that p400 is a member of the p300/CBP family of transcriptional adapter proteins (2, 6). p300 and p400 are structurally and immunologically related phosphoproteins with similar, but not identical, biochemical characteristics. Both proteins bind to E1A and T, but they differ in half-life and their bindings to E1A are separable genetically.

We have attempted to assign a potential biological function to p400, as well as to p300 and CBP, by identifying the region(s) of T to which they bind. The results of the structure-function analysis reported here were unexpected, given the prior demonstration by Yaciuk et al. (84) that T residues 17 to 27 are critical, in the context of intact T, for modulating a p300-, CBP-, and/or p400-associated function. Although Yaciuk et al. did not conclude that amino acids 17 to 27 contribute to the p300 binding domain of T, other investigators have cited those data as proof that the p300/CBP binding domain of T lies in its amino terminus. Additionally, T antigen contains two independent regions (amino acids 1 to 147 and 251 to 708) with activities that cooperate with an activated *ras* oncogene in transformation assays (11), yet a T antigen lacking amino acids 17 to 27 does not cooperate with *ras* (84). The deletion of amino acids 17 to 27 (*dl*1135) yields a protein which, when synthesized in mammalian cells, is stable but severely compromised for ATPase activity (13), a function which maps to the carboxyl terminus of T (13, 59). Thus, as previously suggested by others (13), it is possible that the *dl*1135 mutation induces structural changes which lead to the loss of multiple T biochemical activities.

In the context of full-length T, residues 17 to 27 clearly modulate a p300-associated function. However, these sequences are dispensable for efficient binding of T to p300, p400, or CBP, since all three proteins coprecipitated with a T species lacking residues 1 to 127 (this report and reference 23). Results presented here demonstrate that a carboxyl-terminal fragment of T is sufficient for p400 and p300 binding. These data do not exclude the possibility of an additional p400/p300 binding site in the amino terminus of T. Indeed, the amount of p400 or p300 coprecipitating with 251-708 T was less than that coprecipitating with an equivalent amount of wt T, suggesting that the T amino terminus enhances p400 or p300 binding. The generation of a p400 cDNA clone will make possible equilibrium binding studies of purified proteins, which should address this question.

Similarly, the data presented here are relevant to results which we recently reported. We observed (22) that another T mutant, T50L7, binds poorly to p300 and CBP. The T50L7 mutation encompasses the pRB binding domain of T and extends beyond it. In the absence of other data, this finding might suggest that the pRB-binding function of T is critical for T binding to p300 family members. However, we have found (reference 22 and this study) that the T mutants K1 and PVU-1, minimally mutated but fully defective for pRB binding, can efficiently coprecipitate radiolabeled p400, p300, and CBP in anti-T immunoprecipitations. Therefore, the pRB-binding function is completely dispensable for T binding to p300 family members. We have shown here that the amino-terminal 127 residues of T, which encompass the T50L7 mutation, can be deleted from T without compromising T-p300 family member interactions in radiolabeling experiments. Therefore, it is likely that the T50L7 mutation, in addition to abrogating T binding to pocket proteins, induces a conformational change in T which reduces its ability to bind to p300 family members.

Interestingly, when anti-CBP immunoprecipitates derived from various T-expressing cells were assayed by immunoblotting for coprecipitated T (22), significantly less pRB-binding-defective T (K1 or PVU-1) coprecipitated with CBP, by comparison with the amount of wt T which coprecipitated with CBP. The differing results from the radiolabeling and immunoblotting experiments may reflect different biochemical aspects of T-p300 family member interactions: the radiolabeling experiments may principally show the binding of newly synthesized proteins, while the immunoblot results may reflect both protein binding and complex stability over time. One interpretation of these results which is consistent with all of the data is that the pocket protein-binding function of T is dispensable for T-p300 family member binding but contributes to the stabilization of the newly formed complexes. This possibility will be tested in future experiments.

Like CBP (22), p300 and p400 both coprecipitated with T and with p53 in T-containing mouse cells. If p300 family member/p53 complexes exist in the absence of T, they were undetectable in the assay that we used (Fig. 7, lane 9), suggesting, at a minimum, that T enhances the formation of p300 family member/p53 complexes. Results of this kind are consistent with a model in which T, p300/CBP/p400, and p53 are all present together in discrete, multiprotein complexes. However, further work is needed to address this possibility directly. Among the questions at hand is whether any p300 family member binds directly to p53, which in turn binds to the known p53 binding region of T.

The genetics of T/p53 complex formation indicate a close link between this process and the ability of T to immortalize

primary mammalian cells (46, 93). T binding to p53 also results in the suppression of the p53 transactivation function (28, 42, 57, 67), a process linked to T transforming activity. What might be the biological significance of complexes containing T, p53, and a p300 family member? If T/p53 complexes serve as a special binding center for other T-associated proteins, p300 family members entering into such complexes might thereby be efficiently sequestered. From what is known of the transformation suppression and differentiation promoting functions of p300 family members (see references in the introduction), their sequestration might be one component of the transforming function of T. However, in addition to sequestering these proteins, the recruitment of p300 family members into complexes containing both T and p53 might lead to new activities for the complex components. Indeed, Wang et al. have already shown that E1A can serve as a binding center for multiple cell proteins (i.e., pRB and p300) in a single complex (77). Although the individual sequestration of p300 and pRB family proteins contributes to certain aspects of E1A-mediated transformation, their data (77) suggest that the induction of cellular proliferation by E1A requires the formation of multimolecular complexes containing E1A, p300, and pRB family members.

It is not yet known whether p53/p300 family member complexes normally exist in cells. If so, it is possible that one or more of the functions of p300 family members is to modulate the function of p53 and/or vice versa. If one speculates that p53/p300 complexes normally exist and T can bind to them, at least one effect of T binding might be to perturb the function of these complexes, which would contain two proteins known to participate in growth regulation and transformation suppression.

Finally, prior reports and results reported here show that T likely binds only a fraction of the total amount of p300 family member proteins present in the extracts analyzed (3, 22) (Fig. 4, 6B, and 7). While there are multiple ways to interpret such a finding, it does lead to the speculation that T-containing complexes have dominant negative functions which interfere with the normal activities of one or more of their cellular components. Future work will undoubtedly provide a test of such an hypothesis.

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