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Simian Virus 40 Large T Antigen Alters the Phosphorylation State of the RB-Related Proteins p130 and p107

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p130 and p107 are nuclear phosphoproteins related to the retinoblastoma gene product (pRb). pRb, p107, and p130 each undergo cell cycle-dependent phosphorylation, form complexes with the E2F family of transcription factors, and associate with oncoproteins of DNA tumor viruses, including simian virus 40 (SV40) large T antigen (TAg) and adenovirus E1A protein. The results of recent studies with mouse embryo fibroblasts (MEFs) lacking the retinoblastoma gene (*Rb-1*) have suggested that p130 and p107 may be important targets for SV40 large TAg-mediated transformation (J. B. Christensen and M. J. Imperiale, J. Virol. 65:3945–3948, 1995; J. Zalvide and J. A. DeCaprio, Mol. Cell. Biol. 15:5800–5810, 1995). In this report, we demonstrate that the expression of TAg affects the phosphorylation state of p130 and p107. In cells expressing wild-type TAg, only un(der)phosphorylated p130 and p107 were detected. To determine the domains within TAg that contribute to this effect on the phosphorylation of p130, we performed transient expression assays. While transiently expressed p130 was apparently phosphorylated normally, only un(der)phosphorylated p130 was detected when p130 was coexpressed with TAg. Using this assay, we found that the first 147 amino acids of TAg were sufficient to alter the phosphorylation state of p130. Within this region, the LXCXE domain of TAg, required for binding to the retinoblastoma family of proteins, was necessary but not sufficient to affect p130 phosphorylation. Residues within the first 82 amino acids of TAg were also required. TAg with mutations in the N terminus retained the ability to efficiently associate with p130 but did not affect its phosphorylation state. This demonstrates that the effect of SV40 TAg on p130 is not simply the result of binding and suggests that TAg has a novel effect on p130 and p107 that differs from its effect on pRb.

The retinoblastoma (RB) family of proteins, including pRb, p130, and p107, are targeted by the transforming proteins of certain DNA tumor viruses. The simian virus 40 (SV40) large tumor antigen (TAg), adenovirus E1A, and human papillomavirus E7 oncoproteins all associate with pRb (12, 16, 63). The ability of TAg and E1A to associate with p107 and p130 has also been described previously (15, 19, 28, 65). These oncoproteins have a short region of homology that contains the conserved residues LXCXE (where X is any amino acid). The LXCXE domain is required both for binding to the RB family of proteins and for transformation (8, 17, 45–47, 51, 64).

pRb, p107, and p130 are not only related by sequence but show a number of functional similarities as well. Like pRb, p107 and p130 are capable of inducing growth arrest in certain cell types (11, 70) and can bind to certain members of the E2F family of transcription factors (4, 6, 7, 23, 30, 55, 61). Specific E2F DNA-binding sites are located in the promoters of many genes required for cell cycle progression, including c-*myc* (29), B-Myb (35), dihydrofolate reductase (5, 56), and E2F-1 itself (32, 48). p107 and p130 are also, like pRb, phosphorylated in a cell cycle-dependent manner in G_1 phase (3, 43, 58a). In addition, pRb, p107, and p130 have been shown to associate with cyclins (18, 20, 21, 26, 33, 37, 39, 55) and are likely to be phosphorylated by cyclin-dependent kinases (cdks).

The pRb-, p107-, and p130-E2F complexes can each be disrupted by viral oncoproteins, resulting in the dysregulation of E2F transcriptional activity (1, 2, 49, 62). Since pRb, p107, and p130 all bind to viral oncoproteins via the LXCXE domain, it has been difficult to assess their individual contributions to viral oncoprotein-mediated transformation. It has recently become possible to address this issue because of the availability of cells from RB knockout mice (10, 31, 36). It was demonstrated that TAg with mutations in the LXCXE domain was not capable of transforming RB-deficient mouse embryo fibroblasts (MEFs) (9, 68). This observation suggested that the requirement for an intact LXCXE domain in TAg-mediated transformation may involve more than binding to just pRb. Significantly, wild-type TAg disrupts p130-E2F and p107-E2F DNA-binding complexes (68). These results implicate p107 and p130 as functionally important targets in TAg-mediated transformation.

In this report, we show that TAg alters the phosphorylation states of p130 and p107. Furthermore, we determined that the LXCXE domain and sequences within the amino terminus of TAg are required for this effect.

MATERIALS AND METHODS

Cells. The $RB^{+/+}$ and $RB^{-/-}$ MEFs expressing TAg or the K1 mutant of TAg have been described previously (68). U-2 OS and COS-1 cells were obtained from the American Type Culture Collection. All cell lines were grown in Dulbecco's modified Eagle's medium (Cellgro) supplemented with 10% fetal clone serum (HyClone), 100 U of penicillin per ml, and 100 μg of streptomycin per ml.

Plasmids and transfections. Cells were transfected by the calcium phosphate precipitation method (25). The precipitate was left on the cells 12 h, and the cells were lysed 24 to 48 h after its removal. The plasmid pcDNA1-HA-p130 has been previously described (61). The plasmids pSG5-T, pSG5-K1, pSG5-PVU-1, and pRc/CMV–wild-type E7 have been described previously (68). The TAg mutation ΔH expresses the first 270 amino acids of genomic TAg. pIB147 expresses the first 147 residues of genomic TAg (57). The plasmids EMsb59-64 (residues 59 to 64 of TAg replaced with the sequence NAAIRS) and EMdl65-70 (TAg with residues 65 to 70 deleted) have been described previously (42). The pSG5-EMsb59-64 (cDNA) plasmid was generated by amplifying the EMsb59-64 (genomic) SV40 TAg plasmid (42) with the following primers: 5′-GCCGAATTCACCATGGATAAAGTTTTAAACAGAGAG-3′ and 5′-TGCTAGCATTC

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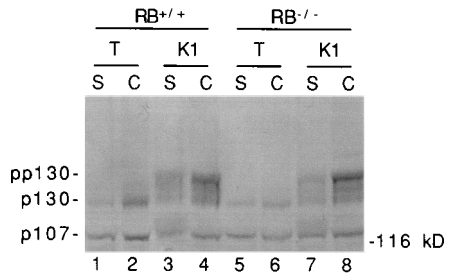

FIG. 1. Analysis of p130 and p107 expression. Lysates of MEFs derived from $RB^{+/+}$ or $RB^{-/-}$ embryos, expressing either wild-type TAg (T) or the K1 mutation of TAg, as indicated, were resolved in an SDS–6% polyacrylamide gel. p130 and p107 were detected by Western blot analysis with the antibody Z83, which recognizes both proteins. S, subconfluent cells; C, confluent cells.

CATAGGTTGGAATCTCAGTTGCATCCCAGAAGCC-3′. The 270-bp amplification product was cut with *Eco*RI and *Pfl*MI and subcloned into pSG5-T cut with the same enzymes. pSG5-T_{83-708} was created by digesting pZIP-T_{83-708} (44) with *Bam*HI and cloning the resulting fragment into the *Bam*HI site of pSG5 (Stratagene).

Immunoprecipitations and Western blots (immunoblots). Cells were washed with phosphate-buffered saline and lysed in EBC buffer (50 mM Tris-HCl [pH 8.0], 120 mM NaCl, 0.5% Nonidet P-40, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml, 0.1 mM phenylmethylsulfonyl fluoride, 4 mM sodium fluoride, 0.1 mM sodium orthovanadate). Extracts were then cleared by centrifugation at 12,000 × *g* for 10 min, and protein concentrations were determined by the Bradford assay (Bio-Rad). Cell lysates were incubated with the relevant antibodies for at least 1 h at 4°C. Immune complexes were collected on protein A-Sepharose beads, washed with NET-N (20 mM Tris-HCl [pH 8], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40), boiled in sodium dodecyl sulfate (SDS)-containing buffer, and separated in SDS-polyacrylamide gels. Proteins were transferred onto polyvinylidene difluoride membranes (Polyscreen; New England Nuclear) in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% [vol/vol] methanol, 0.01% SDS) (60). Membranes were blocked in TBS (10 mM Tris-HCl [pH 8.0], 150 mM NaCl) with 5% bovine serum albumin (BSA; United States Biochemical) prior to incubation with the appropriate antibody. Detection of the immune complexes was performed with an alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (Boehringer Mannheim), after which a colorimetric assay with nitroblue tetrazolium-BCIP (5-bromo-4-chloro-3-indolylphosphate toluidinium; Bio-Rad) was performed.

Antibodies. Fifty microliters of a 1:1 mixture of tissue culture supernatant of the mouse monoclonal antibodies PAb419 (27) and PAb101 (ATCC) was used for all immunoprecipitations of TAg and the various mutants of TAg. A 1:20 dilution of the same antibodies in TBS–5% BSA was used for Western blots. The rabbit polyclonal antibody C-20 (Santa Cruz Biotechnologies) was used for immunoprecipitations of p130. The rabbit polyclonal antibody C-18 (Santa Cruz Biotechnologies) was used for immunoprecipitations of p107. For Western blots of p130 and p107, the antibody Z83 (provided by P. Whyte) or HG41 was used. HG41 was generated by immunizing RBF/Dnj mice with a baculovirus-produced p130 protein and fusing the splenocytes of positive animals to NS1 cells. Hemagglutinin (HA) epitope-tagged proteins were immunoprecipitated and/or analyzed by Western blotting with the mouse monoclonal antibody 12CA5, as specified in the figure legends. Cyclin A was immunoprecipitated with the mouse monoclonal antibody C-160 (24). Cyclin E was immunoprecipitated with the rabbit polyclonal antibody C-19 and subjected to Western blotting with the mouse monoclonal antibody HE12 (both from Santa Cruz Biotechnologies). E1A was immunoprecipitated and analyzed by Western blotting with the mouse monoclonal antibody M73.

Phosphatase treatment. Proteins were immunoprecipitated and collected on protein A-Sepharose beads as described above. The beads were washed with NET-N and then with 1× phosphatase buffer (as provided). The beads were then incubated with 500 U of lambda phosphatase (New England Biolabs) in a volume of 50 μl for 30 min at 37°C. The beads were then washed in NET-N again and boiled in SDS-containing buffer.

RESULTS

p130 is un(der)phosphorylated in TAg-expressing cells. Lysates of MEFs immortalized with either TAg or the K1 mutant of TAg were analyzed for the expression of p130 and p107 by Western blotting with an antibody that recognizes both proteins (39, 68). The K1 mutant of TAg (E107K) fails to bind to pRb, p107, and p130 (12, 19, 68). Wild-type TAg and the K1 mutant were expressed at similar levels in each of these cell lines (not shown). In the cell lines expressing K1, p130 and p107 appeared as broad bands in subconfluent cultures (Fig. 1, lanes 3 and 7), while in confluent cultures p107 migrated as a narrow band (Fig. 1, lanes 4 and 8). This gel migration change reflects hyperphosphorylation of p107 in growing, but not quiescent, cells (3). Cell lines expressing wild-type TAg, on the other hand, contained only narrow bands of p130 and p107 that comigrated with the corresponding fastest-migrating species from the K1-expressing cells (Fig. 1, lanes 1, 2, 5, and 6). The mobility change observed in TAg-expressing cells was not affected by the RB status of the cells, since a similar result was obtained with the corresponding lines derived from $RB^{+/+}$ or $RB^{-/-}$ MEFs (Fig. 1, compare lanes 1 to 4 with lanes 5 to 8).

Since p130 and p107 are phosphoproteins, we suspected that the mobility change observed in TAg-expressing cells was due to a change in phosphorylation state. To test this directly in the case of p107, lysates from subconfluent $RB^{+/+}$ and $RB^{-/-}$ MEFs expressing TAg or K1 were immunoprecipitated with an antibody specific for p107 (Fig. 2, left panel). Western blotting for p107 revealed a difference in the mobility of p107 when precipitated from cells expressing wild-type TAg compared with cells expressing the K1 mutation, consistent with the results shown in Fig. 1. Treating the p107 immunoprecipitated from K1-expressing cells with phosphatase resulted in the loss of the upper band (Fig. 2, lanes 6 and 7). The remaining species comigrated with the p107 found in wild-type TAg-expressing cells. Similarly, when the p130 immunoprecipitated from K1-expressing cells was treated with phosphatase, the signal collapsed into a single band that comigrated with the form found in wild-type TAg-expressing cells (Fig. 3A, lanes 4 to 6). These results suggested that TAg reduced the amount of phosphorylated p107 and p130.

To determine whether the effect of TAg on p130 phosphorylation was specific for MEFs, we compared a monkey cell line that expresses TAg, COS-1, with a monkey cell line that does not express TAg, CV1P. The p130 immunoprecipitated from COS-1 cells migrated differently than that from CV1P cells (Fig. 3A, lanes 1 and 2). When p130 precipitated from CV1P cells was treated with phosphatase, its gel mobility changed to match that of p130 from COS-1 cells (Fig. 3A, compare lanes 1 and 3). The faster-migrating bands observed in Fig. 3A may represent various species of p107, since the anti-p130 antibody used for immunoprecipitation cross-reacts to some extent with p107.

It has been previously reported that pRb phosphorylation is

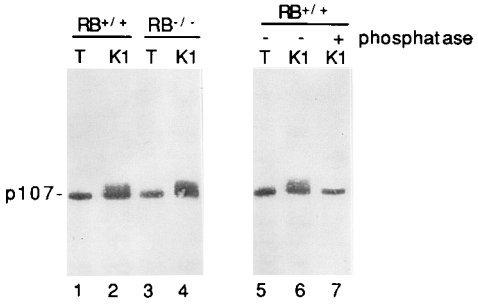

FIG. 2. Analysis of p107 by immunoprecipitation and phosphatase treatment. Cell lysates of $RB^{+/+}$ and $RB^{-/-}$ MEFs expressing wild-type TAg (T) or the K1 mutation, as indicated, were immunoprecipitated with the anti-p107 polyclonal antibody C-18 (Santa Cruz). In lane 7, the immunoprecipitated material was treated with lambda phosphatase as described in Materials and Methods. Immune complexes were resolved in an SDS–5% polyacrylamide gel. p107 was detected by Western blot analysis with the antibody HG41. HG41 reacts with both p107 and p130.

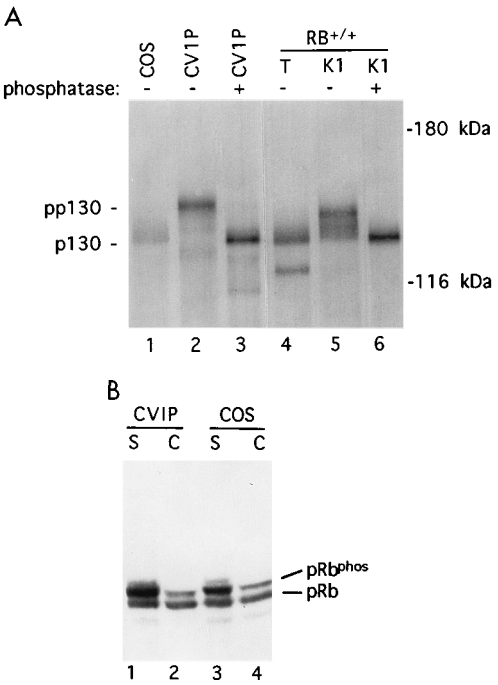

FIG. 3. Analysis of p130 and pRb expression in TAg-expressing cells. (A) Lysates of COS cells, CV1P cells, and $RB^{+/+}$ MEFs expressing wild-type TAg (T) or the K1 mutation of TAg, as indicated, were immunoprecipitated with the anti-p130 antibody C-20 (Santa Cruz). In lanes 3 and 6, the immunoprecipitated material was treated with lambda phosphatase as described in Materials and Methods. Immune complexes were resolved in an SDS–5% polyacrylamide gel. p130 was detected by Western blot analysis with the antibody HG41. Bands migrating faster than p130 (lanes 2, 3, and 4) may represent various species of p107, which is also recognized by this antibody. (B) Lysates of CV1P cells (lanes 1 and 2) or COS cells (lanes 3 and 4) were resolved in an SDS–5% polyacrylamide gel. pRb was detected by Western blot analysis with the antibody XZ77. S, subconfluent cells; C, confluent cells.

not affected by TAg (40, 41). We have confirmed this observation for COS-1 and CV1P cells (Fig. 3B). In the subconfluent cells, the proportion of phosphorylated pRb was greater than in confluent cells, consistent with the known dependence of pRb phosphorylation on the cell cycle (13). Notably, the overall phosphorylation patterns of the two cell lines are similar, independent of TAg expression. The phosphorylation patterns of pRb in the MEFs immortalized by TAg and K1 also appeared identical (68). Thus, TAg alters the phosphorylation state of p130 and p107 while apparently leaving the cell cycle-dependent phosphorylation of pRb intact.

The effect of TAg on p130 phosphorylation requires the LXCXE domain and N-terminal sequences. From experiments comparing TAg with the K1 mutant of TAg (Fig. 1 to 3), it was apparent that an intact LXCXE domain was required to affect the phosphorylation state of p130. We used a transient expression assay in a human osteosarcoma cell line, U-2 OS, to confirm this observation and to determine whether any other regions of TAg were also required.

U-2 OS cells were transfected with a plasmid encoding a cDNA for human p130 tagged with the HA epitope to distinguish it from the endogenous protein (61). The transiently expressed p130 could be immunoprecipitated with an antibody against HA and analyzed by Western blotting with an antibody against p130. The transfected p130, like the endogenous protein, migrated as a series of bands in an SDS-polyacrylamide gel (Fig. 4A, lane 1). When the anti-HA-immunoprecipitated protein was treated with phosphatase, p130 appeared as a single band (Fig. 4A, lane 2). The phosphorylation state of p130 could now be determined in the presence of various mutants of TAg, and the abilities of TAg mutants to associate with p130 could be tested by coprecipitation.

When TAg was coexpressed with p130, p130 appeared relatively underphosphorylated (Fig. 4A, compare lanes 1 and 3). Cotransfection of K1 had no effect on the phosphorylation state of p130 (Fig. 4A, lane 4). The phosphatase treatment of p130 coexpressed with K1 again resulted in a faster-migrating p130 species with a mobility similar to that of p130 from TAg-expressing cells (Fig. 4A, lane 5). The results obtained with TAg and K1 in this transient expression assay in human U-2 OS cells were fully consistent with the data from mouse and monkey stable cell lines. Thus, loss of the hyperphosphorylated

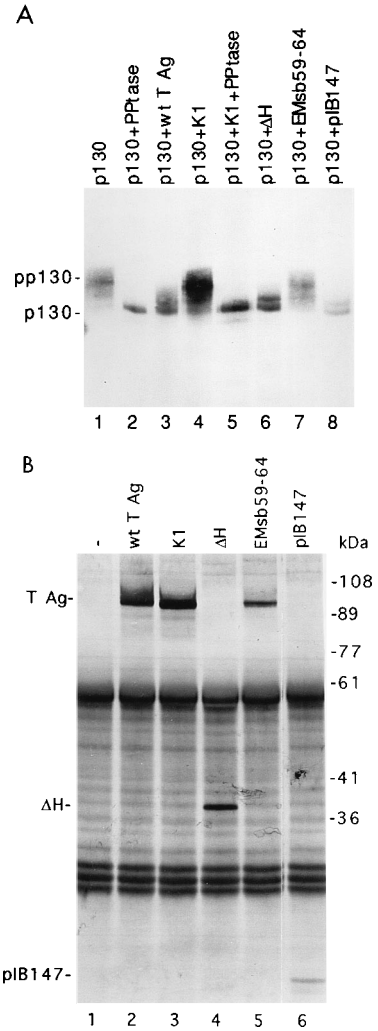

FIG. 4. Transient expression of p130 and TAg in U-2 OS cells. U-2 OS cells were transfected with HA-tagged p130 and the indicated TAg plasmids (described in the text). (A) Lysates of transfected cells were immunoprecipitated with the antibody 12CA5 against the HA tag. In lanes 2 and 5, the immunoprecipitated material was treated with lambda phosphatase as described in Materials and Methods. Lanes 2 and 5 differ from lanes 1 and 4, respectively, only by the phosphatase treatment. Immune complexes were resolved in an SDS–5% polyacrylamide gel. p130 was detected by Western blotting with the antibody HG41. (B) The same lysates as those shown in panel A were immunoprecipitated with a mixture of the antibodies PAb 419 and PAb 101 against TAg. Immune complexes were resolved in an SDS–10% polyacrylamide gel. TAg was detected by Western blotting with the antibodies PAb 419 and PAb 101. PPtase, phosphatase; wt, wild type.

species of p130 is correlated with the presence of TAg in several different systems.

We proceeded to test several mutants of TAg by cotransfection with p130 into U-2 OS cells and analyzed their effects on p130 phosphorylation by immunoprecipitation with anti-HA antibody and then by Western blotting. The construct ΔH, which expresses only the N-terminal 270 residues of TAg, was at least as efficient as wild-type TAg in altering the phosphorylation state of p130 (Fig. 4A, lane 6). A similar result was also obtained with pIB147, expressing just the N-terminal 147 residues of TAg (Fig. 4A, lane 8). Hence, the N-terminal 147 residues of TAg are sufficient to decrease the level of p130 phosphorylation. The expression of TAg and the various mutants of TAg was controlled by immunoprecipitation and Western blotting with antibodies specific for TAg (Fig. 4B).

Interestingly, the mutant EMsb59-64 did not appear to affect p130 phosphorylation (Fig. 4A, lane 7, and Fig. 5A, lane 6). This mutation replaces residues 59 to 64 of TAg with the amino acid sequence NAAIRS (deleting residues 59 to 64 without substitution led to an unstable protein [42]). In contrast, EMdl65-70, with a deletion of the residues 65 to 70, affected p130 phosphorylation in a way similar to that of wild-type TAg (Fig. 5A, top panel, lane 7).

Since the EMsb59-64 and EMdl65-70 mutants of TAg each contain an intact LXCXE domain, they were expected to be able to bind to p130. We tested this directly by coexpressing each mutant with HA-tagged p130 and assaying for coimmunoprecipitation. When p130 was precipitated with an antibody against HA, wild-type TAg was coprecipitated (Fig. 5A, middle panel, lane 3), but the two LXCXE mutants, K1 and PVU-1, were not coprecipitated (Fig. 5A, middle panel, lanes 4 and 5). The EMsb59-64 mutant of TAg was also coprecipitated with p130 (Fig. 5A, middle panel, lane 6), as was the EMdl65-70 mutant (Fig. 5A, middle panel, lane 7). The expression of each TAg construct is shown in Fig. 5A, bottom panel. In the converse experiment (Fig. 5B), p130 could be coprecipitated with wild-type TAg (lane 3) but not with the K1 and PVU-1 mutants (lanes 4 to 5). Coprecipitation of p130 was observed with the mutants EMsb59-64 (Fig. 5B, lane 6) and EMdl65-70 (lane 7) as well as ΔH (lane 8) and pIB147 (lane 9).

Although the N-terminal TAg mutants EMsb59-64 and EMdl65-70 were expressed at similar levels and associated efficiently with p130, only EMdl65-70 had a wild-type effect on p130 phosphorylation. Therefore, EMsb59-64 clearly dissociates the ability of TAg to bind to p130 from its ability to affect p130 phosphorylation. The ability of the mutant EMsb59-64 to bind to p130 but not affect its phosphorylation suggested that at least some residues within the first exon of TAg (residues 1 to 82) were required to alter the phosphorylation state of p130. We tested the ability of TAg with the entire first exon deleted (T_{83-708}) to associate with p130 and affect its phosphorylation. As shown in Fig. 6, T_{83-708} (lane 5) behaved identically to EMsb59-64 (lane 6). In the top panel of Fig. 6, wild-type TAg (lane 3) and ΔH (lane 7) could decrease the level of phosphorylated p130, but T_{83-708} (lane 5) and EMsb59-64 (lane 6) left the hyperphosphorylated species of p130 intact. As before, association between TAg and p130 was assayed by coprecipitation. T_{83-708} could be coprecipitated with p130, as shown in the bottom panel of Fig. 6 (lane 5).

The RB family of proteins is also targeted by the E1A and E7 oncoproteins. Since it has been reported that TAg may have some similarity to E1A and E7 in the N terminus (16), we wanted to determine whether either of these proteins affected p130 phosphorylation. To investigate this, we cotransfected plasmids expressing p130 with either E1A or E7 into U-2 OS cells. Both E7 and E1A were expressed at high levels and could

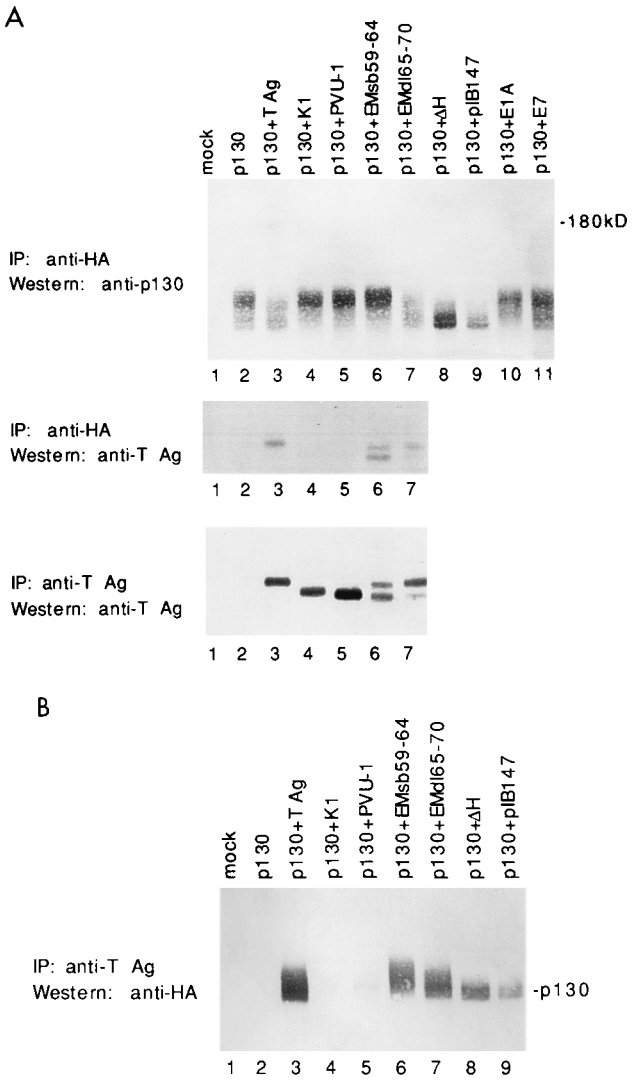

FIG. 5. Coprecipitation of transfected p130 and TAg. U-2 OS cells were transfected as described in the legend to Fig. 4 and immunoprecipitated with the anti-HA antibody 12CA5 (A, top and middle panels). Immune complexes were resolved in an SDS–5% polyacrylamide gel. p130 was detected by Western blot analysis with the antibody HG41 (top panel). Coprecipitating TAg was detected by Western blot analysis with a mixture of the antibodies PAb 419 and PAb 101 (middle panel). TAg was also immunoprecipitated directly from the same lysates (bottom panel) with a mixture of the antibodies PAb 419 and PAb 101, resolved on a 5% polyacrylamide gel, and detected by Western blot analysis with PAb 419 and PAb 101. (B) In a similar experiment, U-2 OS cells were transfected with the indicated plasmids and immunoprecipitated with a mixture of PAb 419 and PAb 101. Coprecipitating p130 was detected by Western blot analysis with the antibody 12CA5 against the HA tag. IP, immunoprecipitation.

efficiently coprecipitate p130 (data not shown). However, the phosphorylation state of p130 did not appear to be affected by the coexpression of E1A or E7 (Fig. 5A, top panel, lanes 10 and 11). Furthermore, 293 cells, which stably express E1A, contain extensively phosphorylated p130 (58a). Thus, E1A and E7 appear to be able to bind p130 but not to affect its phosphorylation.

The altered phosphorylation state of p130 in the presence of TAg is not due to the disruption of cyclin A or E binding. p130 and p107 have been reported to associate with cyclin A-cdk2 and cyclin E-cdk2 via the spacer domain, a sequence conserved between p130 and p107 but not shared with pRb (18, 21, 22,

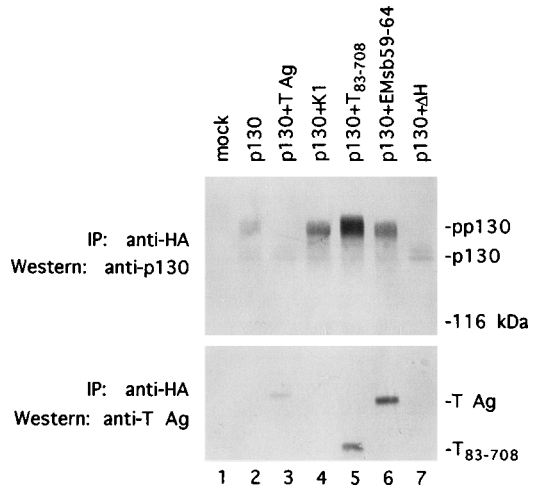

FIG. 6. Coprecipitation of p130 and N-terminal mutants of TAg. The indicated constructs were transfected into U-2 OS cells. Lysates of transfected cells were immunoprecipitated with the anti-HA antibody 12CA5. Immune complexes were resolved in an SDS–5% polyacrylamide gel. p130 was detected by Western blot analysis with the antibody 12CA5 (top panel). Coprecipitating TAg was detected by Western blotting with a mixture of the antibodies PAb 419 and PAb 101. The TAg mutation, ΔH, could not be resolved on a 5% polyacrylamide gel. IP, immunoprecipitation.

39). Furthermore, in vitro kinase reactions of cyclins A and E immunoprecipitated from cell lysates resulted in phosphorylation of p107 and p130 (39). In our experiments, p130 efficiently associated with cyclins E and A in vivo (Fig. 7A, lane 2, and Fig. 7B, lane 2). Since TAg and cyclins E and A bind to different regions of p130, the presence of TAg would not necessarily be expected to disrupt the association between p130 and the cyclins. However, the effect of TAg on p130 phosphorylation could result from the loss of cyclin-cdk binding because of steric hindrance.

To test this hypothesis, we precipitated lysates from cells cotransfected with p130 and TAg with antibodies against cyclin E or A. Consistent with the observations of others (34), we detected multiple species of cyclin E. We were able to coprecipitate p130 using antibodies to either cyclin E (Fig. 7A, top panel) or cyclin A (Fig. 7B, top panel) in the presence of transfected TAg. TAg, but not K1, was also coprecipitated with the cyclins (Fig. 7A and B, middle panels). Furthermore, an immunoprecipitation against HA coprecipitated both TAg (but not K1) and cyclin E (data not shown). This demonstrates that p130, TAg, and cyclin E are part of the same molecular complex. Therefore, the ability of TAg to affect the phosphorylation of p130 does not appear to be due to disruption by TAg of cyclin A or E binding to p130.

DISCUSSION

In this report we have demonstrated that the expression of TAg results in the appearance of altered phosphorylation states of p130 and p107. Specifically, there is a decrease in the level of the hyperphosphorylated species. This effect requires the LXCXE of TAg (residues 103 to 107), which mediates the association between TAg and pRb, p107, and p130. In addition, sequences within the amino-terminal 82 residues of TAg are required. The mutation or deletion of this region resulted in loss of the ability of TAg to alter the phosphorylation state of p130, and yet these mutants of TAg retained the ability to associate efficiently with p130. TAg sequences C terminal to residue 147 were found to be completely dispensable for the effect of TAg on p130 phosphorylation.

We considered the possibility that SV40 small t antigen (small t) could participate in large TAg's effect on p130 phosphorylation. Small t contains the first 82 amino acids of large TAg, and the unique region of small t can interact with a cellular phosphatase, PP2A (50). However, our large TAg, K1, and PVU-1 constructs do not express small t; hence, the effect of large TAg on p130 phosphorylation is not dependent on small t, either alone or in association with a phosphatase.

While the wild-type TAg, K1, and PVU1 constructs used in these experiments express the cDNA version of TAg, the other mutants of TAg used in the transient transfection assay express TAg from viral genomic DNA that also encodes small t. We have shown herein that small t is not necessary for the effect of TAg on p130 phosphorylation. However, the N-terminal mutants of genomic TAg are predicted to also express a mutant small t, as the reading frames of small t and large TAg overlap

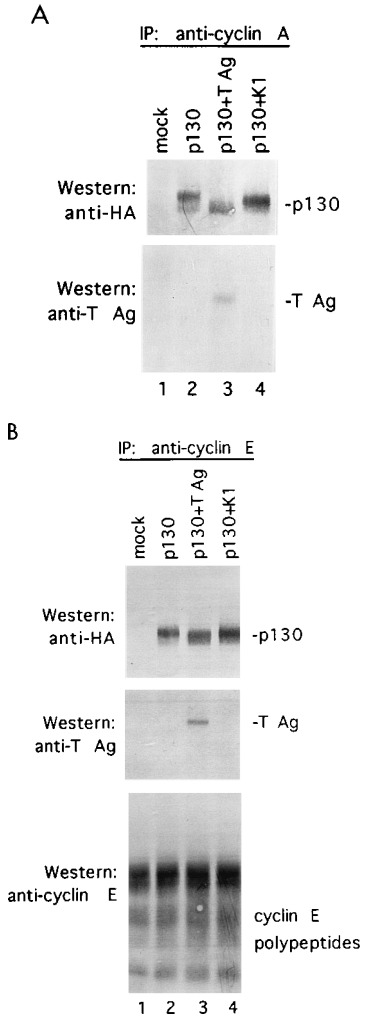

FIG. 7. Coprecipitation of p130 and cyclins E and A in the presence of TAg. U-2 OS cells were transfected with the indicated constructs. Transfected cells were immunoprecipitated with the antibody C-19 (Santa Cruz) against cyclin E (A) or with the antibody C-160 against cyclin A (B). Immune complexes were resolved by SDS-polyacrylamide gel electrophoresis on a 7.5% gel. Cyclin E was detected by Western blotting with the antibody HE12 (Santa Cruz). Coprecipitating TAg was detected by Western blotting with a mixture of the antibodies PAb 419 and PAb 101. Coprecipitating p130 was detected by Western blotting with the antibody 12CA5. IP, immunoprecipitation.

in this region. In order to be certain that small t did not in any way influence our results with the N-terminal mutants of large TAg, we generated and tested a cDNA version of the EMsb59-64 mutant. The cDNA version of this mutant (used in the experiment shown in Fig. 6) behaves in a manner identical to that of the genomic version (used in the experiment shown in Fig. 5): it associates with p130 but does not affect its phosphorylation. This demonstrates that the expression of small t did not affect the result obtained with this N-terminal mutant. This is further confirmed by the results obtained with $T_{83\text{-}708}$, a mutant of TAg missing the first exon (the first 82 amino acids; hence, the region of overlap between large TAg and small t is deleted). This N-terminal deletion mutant of TAg, like the EMsb59-64 mutant, did not affect p130 phosphorylation but retained the ability to associate with p130.

We have not detected any changes in pRb phosphorylation in the presence of TAg (Fig. 3B and reference 68). This suggests that the interaction of pRb with TAg is biochemically different from the interaction of p130 and p107 with TAg. In the case of pRb, the un(der)phosphorylated form of the protein is thought to be the active form and is the only form targeted by TAg. Hyperphosphorylated pRb has no known activity and is not able to associate with TAg (40, 41).

Several mechanisms could be proposed to account for the effect of TAg on p130 phosphorylation. TAg could cause the loss of the hyperphosphorylated species of p130 by the inhibition of a kinase, activation of a phosphatase, or protein degradation, possibly by preferential degradation of the hyperphosphorylated species. In many cases, steady-state levels of p130 appeared to be lower in the presence of TAg, but the effect was most pronounced on the hyperphosphorylated species. The effect of TAg on p130 could be directly mediated by the N terminus of TAg or by an associated protein.

It is possible that p130 and p107, like pRb (14, 20), can be substrates for cyclin D-cdk4. Cells transformed by TAg and by a variety of other means exhibit changes in cyclin D-associated proteins. In normal diploid cells, cyclin D is present in a complex with cdk4, proliferating cell nuclear antigen (PCNA), and p21. In certain cells transformed by SV40 large TAg, the cyclin D-cdk4-p21-PCNA complex is missing and is replaced by a cdk4-p16 complex (66). We have not detected any rearrangement of cdk4 subunits in the MEFs expressing TAg or K1 (68a). It has been suggested that p16 may become induced because TAg causes the cell to become functionally pRb deficient and that the cell responds by attempting to block pRb phosphorylation by inhibiting the RB kinase, cdk4, by overexpressing p16 (54). This hypothesis is not consistent with the observation that pRb is phosphorylated normally in cells transformed by TAg. The un(der)phosphorylated states of p130 and p107 in cells expressing TAg also cannot be fully explained by the induction of p16, since the effect is observed in U-2 OS cells which are p16 deficient (11a).

Alternatively, TAg could block the access of kinases that would normally phosphorylate p130 and p107. There is not as yet a consensus on which kinases are responsible for p107 and p130 phosphorylation. However, it is known that p107 and p130, but not pRb, stably associate with cyclin A-cdk2 and cyclin E-cdk2 in vivo and become phosphorylated by in vitro kinase reactions following immunoprecipitation with antibodies to cyclin A or E (39). The inhibition of phosphorylation by the associated cyclin A- or E-cdk2 was an attractive possibility since it would account for the observation that p130 and p107 phosphorylation was affected by TAg, whereas pRb phosphorylation was not. This prompted us to test whether the association with TAg might sterically interfere with the ability of cyclin E or A to bind to p130. Our results clearly show that this is not the case. However, we cannot formally rule out the possibility that the activity of the associated kinase may be affected by the presence of TAg.

What might the functional consequence be of altering the phosphorylation state of p107 or p130? p130 and p107, like pRb, are phosphorylated in a cell cycle-dependent manner in the G_1 phase (3, 43, 58a). It is not known whether the various phosphorylated species of p107 and p130 have clearly definable functions. However, hyperphosphorylated p130 is not completely inert, since it can associate with cyclins E and A (Fig. 7 and data not shown).

The N terminus of large TAg has been shown to make a contribution to transformation separately from the LXCXE (RB-binding) and p53-binding domains (38, 52, 58, 59, 67, 69). Interestingly, the EMsb59-64 mutant used in this study has been shown to be severely defective in transforming activity, even though it is expressed as a stable protein (42). It is unclear how the amino terminus of TAg contributes to transformation or to what extent, if any, the effect on p130 and p107 phosphorylation contributes to transformation by TAg. The N terminus of TAg has also been implicated in binding to heat shock protein 73 (53), but the significance of this association is unknown.

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