Simian Virus 40 Large T Antigen's Association with the CUL7 SCF Complex Contributes to Cellular Transformation

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Simian virus 40 large T antigen (T Ag) is capable of immortalizing and transforming rodent cells. The transforming activity of T Ag is due in large part to perturbation of the tumor suppressor proteins p53 and the retinoblastoma (pRB) family members. Inactivation of these tumor suppressors may not be sufficient for T Ag-mediated cellular transformation. It has been shown that T Ag associates with an SCF-like complex that contains a member of the cullin family of E3 ubiquitin ligases, CUL7, as well as SKP1, RBX1, and an F-box protein, FBXW8. We identified T Ag residues 69 to 83 as required for T Ag binding to the CUL7 complex. We demonstrate that $\Delta 69$ –83 T Ag, while it lost its ability to associate with CUL7, retained binding to p53 and pRB family members. In the presence of CUL7, wild-type (WT) T Ag but not $\Delta 69$ –83 T Ag was able to induce proliferation of mouse embryo fibroblasts, an indication of cellular transformation. In contrast, WT and $\Delta 69$ –83 T Ag enabled mouse embryo fibroblasts to proliferate to similarly high densities in the absence of CUL7. Our data suggest that, in addition to p53 and the pRB family members, T Ag serves to bind to and inactivate the growth-suppressing properties of CUL7. In addition, these results imply that, at least in the presence of T Ag, CUL7 may function as a tumor suppressor.

Simian virus 40 (SV40) is well characterized as a tool to study neoplastic transformation and cell cycle regulation. Two tumor antigens are encoded by SV40: large T antigen (T Ag) and small t antigen (t ag). Both are expressed from a differentially spliced common precursor mRNA, and their expression occurs soon after infection from a transcript initiating at the early promoter. T Ag is a 708-amino-acid protein that plays a major role in viral replication, in part by driving entry into the cell cycle of quiescent cells. In addition to its critical roles in establishing productive infection, T Ag is required for viral tumorigenesis. The transforming activity of T Ag is due in large part to its perturbation of the retinoblastoma (pRB) family members and p53 tumor suppressor proteins (12, 32).

The p53 protein is a key regulator of the cell cycle in response to a variety of cellular stresses, and the gene encoding p53 is mutated in the majority of human tumors. The activities of p53 are mediated largely by its ability to transcriptionally regulate genes that either induce cell cycle arrest (e.g., *Cdkn1a*) or promote apoptosis (e.g., *Bax*). Through this transcriptional regulation, p53 plays an essential role in the maintenance of genomic integrity (20). Two carboxy-terminal regions of T Ag, residues 351 to 450 and 533 to 626, are required for its direct association with this tumor suppressor (12, 15, 33). It is well known that T Ag inactivation of the tumor-suppressing function of p53 contributes significantly to the ability of T Ag to induce entry into the cell cycle and proliferation, ultimately leading to a transformed phenotype.

Another primary transforming function of T Ag is the inactivation of pRB family members. Proteins of this family inhibit E2F activity and thus prevent G₁-S entry (31). Two regions of T Ag are required for binding and inactivation of pRB and the related proteins p107/RBL1 and p130/RBL2. A mutation in either the LxCxE domain (residues 103 to 107) or the DnaJ domain (residues 42 to 47) results in an inability of T Ag to promote full cellular transformation (32). The association of T Ag with pRB results in the release of pRB from the E2F family of transcription factors (28). In turn, dissociation of pRB from E2F results in the expression of many proteins required for entry into the S phase of the cell cycle. Thus, T Ag targeting of pRB proteins allows entry into the cell cycle. This function causes cells in cultures expressing T Ag to assume a transformed phenotype.

Targeting and inactivation of p53 and pRB family members may not be sufficient for T Ag-mediated transformation (4, 17, 22). Cell transformation caused by T Ag may involve the inactivation of additional tumor suppressors. T Ag is known to associate with p300/CBP, altering the phosphorylation state of p300 and down-regulating its transactivation-inducing abilities (8). Additionally, the interaction between T Ag and CBP was shown to be dependent on p53, and this interaction promotes acetylation of T Ag (21). The role of the interaction between T Ag and p300/CBP in T Ag-mediated transformation is not yet known. Recently, a region of T Ag close to its pRB-binding domain has been shown to associate with the spindle assembly checkpoint protein BUB1 (5). A BUB1 binding mutant of T Ag with an in-frame deletion of residues 89 to 97 was shown to be less efficient than wild-type (WT) T Ag in forming foci in Rat-1 cells, suggesting that interaction of T Ag with BUB1 is required for transformation (5).

In addition, a region close to the BUB1 binding region of T Ag has been reported to bind to a protein with a molecular mass of 185 kDa (14, 29). Having identified this protein as

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CUL7, our laboratory showed that a T Ag containing a point substitution in residue 98 (F98A) or a small in-frame deletion of residues 98 and 99 (Δ 98–99) was defective in CUL7 binding. Our data also revealed that these CUL7-binding-deficient mutants of T Ag were defective for transformation. Although the CUL7-binding mutants of T Ag were transformation defective, it was not clear whether inactivation of CUL7 activity was required for T Ag-mediated transformation.

CUL7 is the seventh mammalian cullin identified to date. Cullins are the core subunits of E3 ubiquitin ligase complexes where the cullin serves as a scaffold to recruit the Ring finger protein, RBX1, and substrate specificity factors. Our laboratory reported that CUL7 specifically associates with SKP1, RBX1, and the F-box protein FBXW8 (FBX29, FBW6) (3). In addition to homology to cullins, CUL7 has domains homologous to HERC2, a HECT domain-containing protein and suggested E3 ligase, and a domain homologous to APC10, a subunit of the anaphase-promoting complex. The homology to E3 ubiquitin ligases as well as the association with RBX1, SKP1, and FBXW8 suggests that CUL7 serves to target specific proteins for ubiquitination and subsequent degradation by the proteasome.

In this study, we identified a new domain of T Ag required for binding to CUL7. Both in this study and in recent publications from our laboratory, we show that CUL7-binding-deficient mutants of T Ag have a decreased ability to promote cells to grow to high density—one measure of the transformed phenotype (2). In addition, we demonstrate that T Ag inactivation of CUL7 is required to promote full cellular transformation. T Ag transformation is dependent on inactivation of p53 and pRB family members as well as CUL7. Our data suggest that CUL7 may also behave as a tumor suppressor.

MATERIALS AND METHODS

Plasmids. The T Ag cDNA-expressing vectors pSG5-T and pSG5-E107K have been previously described (31). The Δ 69–83 T Ag was PCR amplified with pSG5-T as a template. H70A, Q71A, F72A, D73A, F74A, F74R, Δ 71–74, F77A, T79A, T81A, and E82A point substitution mutant and deletion versions of pSG5-T Ag were generated by PCR mutagenesis using the Stratagene QuikChange kit according to the manufacturer's protocol. Epitope-tagged versions of FBXW8 and CUL7 were generated by PCR (3). Epitope-tagged versions of pRB and p130 have also been previously described (10, 30). pLB(N)CX was derived from pLNCX (Clontech). The original neomycin resistance cassette was replaced with a PCR-generated Blasticidin resistance cassette. pLB(N)CX hemagglutinin (HA)-CUL7 was generated by inserting HA-CUL7 into the multiple cloning site of pLB(N)CX. pBABE-vector and pBABE-Cre were obtained from N. Bardeesy. All constructs were verified by sequencing.

Cells. All cells were cultured in complete medium containing Dulbecco's modified Eagle's medium with 10% Fetal Clone-I serum (HyClone), 100 U of penicillin per ml, and 100 μ g of streptomycin per ml. The human osteosarcoma U-2 OS, mouse fibroblast NIH 3T3, T98G, and HeLa cell lines were obtained from the American Type Culture Collection. Primary mouse embryo fibroblasts (MEFs) were prepared from 13.5-day *Trp53^{tm1Tyj/tm1Tyj}* knockout embryos, *Cul7^{tm1Jdec/tm1Jdec}* knockout, or *Cul7^{2Losp/2Losp}* embryos as previously described (3, 31). MEF cell lines stably expressing T Ag were generated by calcium phosphate transfection of pSG5-T Ag with pEpuro in a 5:1 ratio. At 48 h after transfection, cells were selected with medium containing puromycin (2 μ g/ml) and all colonies were pooled together. Cells stably expressing pLB(N)CX, pLB(N)CX HA-CUL7, pBABE-Puro, or pBABE-Puro-Cre were generated by retroviral infection followed by antibiotic selection. Transient transfections were performed with Lipofectamine Plus Reagent (Invitrogen) according to the manufacturer's protocol.

Immunoprecipitations, Western blot assays, and antibodies. Immunoprecipitations and Western blot assays were performed as previously described (21, 27). For immunoprecipitation and Western blot analysis, the following antibodies

were used: anti-T Ag, PAb 419 and PAb 430 (9); anti-CUL7, SA12 (3) and PAb 653 (Bethyl Laboratories); anti-HA, 12CA5 and HA-11 (Covance); anti-p53, DO-1 and 122 (Neomarkers); anti-RBX-1, Ab-1 (Neomarkers); anti-SKP1 (Novagen); anti-BUB1 (polyclonal serum; a gift from T. Roberts); anti-T7 (Novagen). Cross-linking of anti-T Ag antibodies to protein A-Sepharose beads was performed as previously described (21).

Proliferation assays. Either 20,000 cells per well in 24-well plates or 50,000 cells per well in 60-mm plates were seeded in triplicate and fed every other day. For samples in 24-well plates, density (as a measure of proliferation) was measured by crystal violet staining as previously described (6). For 60-mm plates, cells were trypsinized and counted in a Coulter Counter.

RESULTS

SV40 T Ag associates with the CUL7-SCF complex. The interaction of SV40 T Ag and CUL7 has been reported by our laboratory and others (2, 29). To explore the specificity and ultimately the functional consequences of this association, we derived MEFs from *Cul7^{+/+}* and *Cul7^{-/-}* embryos and stably transfected T Ag into these cells. As shown in Fig. 1A, when T Ag was immunoprecipitated, CUL7 was coprecipitated from *Cul7^{+/+}* MEFs but not from *Cul7^{-/-}* MEFs. Similarly, immunoprecipitation for CUL7 from *Cul7^{+/+}* MEFs but not from knockout MEFs coprecipitated T Ag. NIH 3T3 cells, which express lower levels of CUL7 than MEFs and do not express T Ag, served as a control. Several different antibodies for either T Ag or CUL7 coprecipitated each other in similar assays (data not shown). Demonstration of the interaction of T Ag with CUL7 in wild-type MEFs but not in *Cul7^{-/-}* MEFs supports the notion that this interaction was specific.

We have previously shown that T Ag can associate with CUL7-binding proteins, including RBX1 and the F-box protein FBXW8 (2). To determine whether T Ag also associated with SKP1 and whether the association of T Ag with these proteins was dependent on CUL7, we compared the association in *Cul7^{+/+}* and *Cul7^{-/-}* T Ag-expressing MEFs. Immunoprecipitation for T Ag revealed coprecipitation of SKP1 and RBX1 in WT MEFs but not in *Cul7^{-/-}* MEFs (Fig. 1B). This result suggests that T Ag binding to RBX1 and SKP1 was dependent on the presence of CUL7. Because a suitable antibody was lacking, a retroviral vector expressing HA-tagged FBXW8 was used to determine if T Ag binding to FBXW8 was dependent on the presence of CUL7. HA-FBXW8 was transduced into *Cul7^{+/+}* and *Cul7^{-/-}* MEFs expressing T Ag, and immunoprecipitations for T Ag were performed. The association between T Ag and HA-FBXW8 was observed in WT but not in *Cul7^{-/-}* MEFs (Fig. 1C). Given the unequal expression of T Ag and FBXW8 in the WT and knockout MEFs, three times more protein was used for T Ag immunoprecipitation from the *Cul7^{-/-}* MEFs in lane 1 than for these cells and the WT cells in lanes 2 and 3. Even when protein expression was comparable in the two cell lines, no interaction of T Ag and FBXW8 was seen in the absence of CUL7. This interaction in WT MEFs was specific, since no coprecipitation was detected in cell lines lacking expression of either HA-FBXW8 or T Ag. Thus, T Ag associates in a CUL7-dependent manner not only with SKP1 and RBX1 but also with FBXW8.

Fine mapping of the SV40 T Ag domain that interacts with CUL7. As our investigation into the role of CUL7 in T Ag transformation depends on the identification of specific mutations that disrupt the T Ag-CUL7 interaction, we set out to identify the region of T Ag necessary for that interaction.

FIG. 1. T Ag binds specifically to the CUL7 SCF complex. (A) Lysates from *Cul7*^{+/+} and *Cul7*^{-/-} MEFs stably expressing T Ag or from NIH 3T3 cells (control) were immunoprecipitated for T Ag. Immunoprecipitates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotted with a monoclonal antibody to CUL7 (top panel) or a monoclonal antibody to T Ag (bottom panel). (B) Immunoprecipitates for T Ag from *Cul7*^{+/+} and *Cul7*^{-/-} MEFs stably expressing T Ag were Western blotted for CUL7, T Ag, SKP1, and RBX1. (C) *Cul7*^{+/+} and *Cul7*^{-/-} MEFs stably expressing T Ag were transduced with a retroviral vector expressing HA-FBXW8. Immunoprecipitation for T Ag (3 mg protein in lane 1 and 1 mg in all other lanes) was followed by Western blotting for T Ag and HA on both the immunoprecipitated samples and the whole-cell lysates (WCL).

Previously, we had shown that T Ag residues F98 and N99 were required for interaction with CUL7 (2). Given the high proximity of these residues to the BUB1 and pRB binding domains on T Ag (89 to 97 and 103 to 107, respectively), we wanted to ascertain whether other domains of T Ag were also required for CUL7 binding. A report on the X-ray crystallographic

structure of the complex formed by T Ag residues 7 to 117 and the pRB pocket revealed an exposed loop that comprises residues 69 to 83 and extends between the third and fourth α -helices of the N terminus of T Ag (13). To determine whether this loop region of T Ag contributed to CUL7 binding, we generated a series of small in-frame deletions and point substitution mutations. Plasmids expressing mutant T Ag were transiently transfected with T7-tagged CUL7 into HeLa cells. Whole-cell lysates of the transfected cells were Western blotted and revealed similar expression between samples of T7-CUL7 as well as WT and mutant T Ag expression (Fig. 2A). Immunoprecipitation of WT and mutant T Ag, followed by Western blotting for p53, revealed that each of the T Ag constructs was capable of immunoprecipitating p53, suggesting that the T Ag constructs retained some structural integrity (Fig. 2A, bottom panel). Immunoprecipitation of WT and mutant T Ag, followed by Western blotting for T7-CUL7, demonstrated that, while WT T Ag was capable of associating with T7-CUL7 (Fig. 2A, lane 14), no association between Δ 69–83 T Ag and CUL7 was detectable (lane 13). Alanine scanning mutants of T Ag within this region showed partial losses of CUL7 binding, particularly for residues D73, F77, and F77 (Fig. 2A, lanes 2 to 12). Similarly, a small in-frame deletion of residues 71 to 74 (Δ 71–74), located at the tip of the loop structure, decreased the association with CUL7 (lane 6). Although partial loss of CUL7 binding to T Ag was observed with these T Ag mutants, only Δ 69–83 appeared to exhibit a complete loss of CUL7 binding.

The ability of T Ag to interact with CUL7 is independent of its ability to bind to other known tumor suppressors. The ability of T Ag to immortalize and transform MEFs is dependent on inactivation of the tumor suppressor functions of p53 and pRB family members (1, 12, 32). To investigate the specific contribution of T Ag binding to CUL7 in T Ag-mediated cell transformation, we wanted to confirm that the T Ag interaction with CUL7 was independent of binding to p53 and pRB. To determine whether CUL7 binding was dependent on the presence of p53, we stably transfected WT T Ag into *Cul7*^{+/+}, *Cul7*^{-/-}, and *Trp53*^{-/-} MEFs; prepared lysates; and performed immunoprecipitation for T Ag. As shown in Fig. 2B, immunoprecipitation for T Ag in *Trp53*^{-/-} MEFs coprecipitated CUL7. In addition, immunoprecipitation for T Ag in *Cul7*^{-/-} MEFs coprecipitated p53. Although Western blots of cell lysates revealed slightly elevated levels of p53 in *Cul7*^{-/-} MEFs compared to WT MEFs, this result was not consistently observed. These results indicate that the T Ag-CUL7 and T Ag-p53 interactions could be formed independently and in the absence of each other.

T Ag transformation is dependent on its ability to bind and inactivate pRB as well as the pRB-related proteins p130 (RBL2) and p107 (RBL1) (32). The known binding site on T Ag for pRB is the LxCxE motif (residues 103 to 107) (7). T98G cells were transiently cotransfected with WT T Ag, Δ 69–83 T Ag, pRB-binding-deficient T Ag mutant E107K, and an HA-tagged pRB construct. Immunoprecipitation for T Ag or HA-pRB followed by immunoblotting showed that WT and Δ 69–83 T Ag coprecipitated similar levels of HA-pRB (Fig. 3A, left panel). In contrast, E107K, the pRB-binding-deficient mutant, did not coprecipitate HA-pRB. Immunoprecipitation for HA-pRB revealed coprecipitation of WT and Δ 69–83 T Ag but not

FIG. 2. T Ag residues 69 to 83 are required for CUL7 binding. (A) HeLa cells were transiently transfected with T7-CUL7 and substitutions or small in-frame deletions of T Ag (lanes: 1 and 15, controls; 2, E82A; 3, T81A; 4, T79A; 5, F77A; 6, Δ71–74; 7, F74R; 8, F74A; 9, D73A; 10, F72A; 11, Q71A; 12, H70A; 13, Δ69–83; 14 and 16, WT T Ag). Whole-cell lysates (WCL) and immunoprecipitates for T Ag were Western blotted for T7, T Ag, and p53. (B) Lysates of WT, *Trp53*^{-/-}, and *Cul7*^{-/-} MEFs stably expressing WT T Ag were immunoprecipitated for T Ag. WT MEFs and U-2 OS cells were used as negative controls. Whole-cell lysates and immunoprecipitated samples were run in a sodium dodecyl sulfate-polyacrylamide gel and Western blotted for CUL7, T Ag, and p53.

of E107K (Fig. 3A, middle panel). These experiments demonstrate that the Δ69–83 mutation disrupts the association of T Ag with CUL7 but has no effect on its ability to bind to pRB.

The ability of T Ag to induce hypophosphorylation of p130

FIG. 3. Δ69–83 T Ag binds pRB, p130, and BUB1. (A) T98G cells were transiently cotransfected with HA-pRB and WT T Ag, Δ69–83 T Ag, or a pRB-binding-deficient mutant of T Ag (E107K). Whole-cell lysates (WCL) were immunoprecipitated for HA or T Ag and Western blotted for HA (top panel) and for T Ag (bottom panel). (B) T98G cells were transiently transfected with HA-p130 and WT, Δ69–83, or E107K T Ag. Whole-cell lysates were immunoprecipitated for HA or T Ag, and immunoprecipitates and whole-cell lysates were Western blotted for HA (top panel) and for T Ag (bottom panel). (C) *Cul7*^{+/+} and *Cul7*^{-/-} MEFs stably expressing WT T Ag or Δ69–83 T Ag (lanes 1 to 4), NIH 3T3 (lane 5), or *Cul7*^{-/-} MEFs (lane 6) were immunoprecipitated for BUB1 and Western blotted for BUB1 (top panel) and T Ag (bottom panel).

depends on an intact LxCxE motif as well as an intact N-terminal J domain (16, 26, 32). To further characterize the Δ69–83 T Ag mutation, we assayed its effect on the ability of T Ag to induce dephosphorylation of p130. As shown in Fig. 3B (center panel), when WT and Δ69–83 T Ag were immunoprecipitated, comparable levels of HA-p130 were coprecipitated; in contrast, the LxCxE mutant E107K did not coprecipitate HA-p130. Similarly, immunoprecipitation for HA-p130 coprecipitated WT and Δ69–83 T Ag but not E107K (Fig. 3B, left). Western blotting of whole-cell lysates revealed that both WT

and $\Delta 69-83$ T Ag reduced the phosphorylation status of p130, while E107K had no effect on p130 status compared to HA-p130 transfected alone. The capacity of $\Delta 69-83$ T Ag to associate with and dephosphorylate p130 further supports the conclusions drawn from Fig. 3A that $\Delta 69-83$ T Ag retains pRB-binding capacity and that the structure of the J domain remains intact in this mutant.

The ability of T Ag to associate with BUB1, an essential mitotic checkpoint kinase that resides at kinetochores during mitosis, was recently reported (5). The region of T Ag required for binding to BUB1 mapped to residues 89 to 97. Given the proximity of this region to the CUL7-binding site, we assessed whether CUL7 and BUB1 bind to independent or overlapping sites on T Ag. Lysates were prepared from *Cul7*^{+/+} and *Cul7*^{-/-} MEF cell lines stably expressing WT or $\Delta 69-83$ T Ag, and immunoprecipitation for BUB1 was performed. As shown in Fig. 3C, WT and $\Delta 69-83$ T Ag were coprecipitated by BUB1 in the presence or absence of CUL7. Thus, although the regions within T Ag that are required for the binding of CUL7 and the binding of BUB1 are in close proximity, these data suggest that they are separable, as a CUL7-binding mutant of T Ag ($\Delta 69-83$) retained the ability to associate with BUB1. Furthermore, the ability of T Ag to associate with BUB1 in the absence of CUL7 indicates that the interaction of T Ag with BUB1 was not dependent on CUL7.

$\Delta 69-83$ T Ag is defective in a CUL7-dependent manner for high-density growth. One measure of cellular transformation is the ability of cells to grow to a high density on plastic dishes. Previously, we showed that the T Ag mutants defective in CUL7 binding, F98A and $\Delta 98-99$, had reduced abilities to induce cell proliferation and growth to high density (2). The question remained, however, whether this loss in growth potential was specific to the loss of T Ag's ability to bind to CUL7 or whether it was due to an additional activity. To investigate this question, we generated *Cul7*^{+/+} and *Cul7*^{-/-} cell lines expressing WT T Ag or $\Delta 69-83$ T Ag. As shown in Fig. 4A, similar levels of WT and $\Delta 69-83$ T Ag were expressed in these cell lines. To assess the transformed phenotype, equal numbers of cells were plated and fed every other day, and the growth density was determined by crystal violet staining on the days indicated in Fig. 4B (6). Our results showed that WT T Ag induced proliferation and growth to similar densities in *Cul7*^{+/+} (squares) and *Cul7*^{-/-} MEFs (circles). In contrast, *Cul7*^{+/+} MEFs expressing $\Delta 69-83$ T Ag did not proliferate as well as the WT T Ag-expressing cell lines (inverted triangles). Notably, *Cul7*^{-/-} MEFs expressing $\Delta 69-83$ T Ag grew to a density similar to that observed for the WT T Ag-expressing cell lines (triangles). This result supports the concept that T Ag inactivates CUL7, since the absence of CUL7 enabled $\Delta 69-83$ T Ag to induce growth in a manner similar to WT T Ag.

T Ag inactivation of CUL7 promotes high-density cell growth. To determine if $\Delta 69-83$ T Ag was able to overcome its growth defect if *Cul7* was lost somatically, we created a conditional knockout allele of the mouse *Cul7* gene (Fig. 5A) (3). *LoxP* sites flanking *Cul7* exons 2 to 4 (2*LoxP*) did not interfere with WT CUL7 expression. Once Cre recombinase has been transduced, a defective 1*LoxP* allele is generated, resulting in a lack of CUL7 protein expression.

T Ag or $\Delta 69-83$ T Ag was stably transfected into *Cul7*^{2LoxP/2LoxP} MEFs. Western blot assays confirmed the ex-

FIG. 4. The association of T Ag with CUL7 affects cell growth. (A) Whole-cell lysates (WCL) from *Cul7*^{+/+} and *Cul7*^{-/-} MEFs stably expressing WT or $\Delta 69-83$ T Ag were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotted for CUL7 (top panel), T Ag (middle panel), and p53 (bottom panel). (B) *Cul7*^{+/+} MEFs expressing WT T Ag (squares), *Cul7*^{-/-} MEFs expressing WT T Ag (circles), *Cul7*^{+/+} MEFs expressing $\Delta 69-83$ T Ag (inverted triangles), and *Cul7*^{-/-} MEFs expressing $\Delta 69-83$ T Ag (triangles) were plated in 24-well plates. Each data point for *Cul7*^{+/+} and *Cul7*^{-/-} MEFs expressing $\Delta 69-83$ T Ag represents an average of triplicate samples from two independently derived cell lines. Each data point for *Cul7*^{+/+} and *Cul7*^{-/-} MEFs expressing WT T Ag represents an average of triplicate samples from one cell line. Error bars are one directional (below data point). Density was assayed by crystal violet and is expressed in arbitrary units.

pression of WT and $\Delta 69-83$ T Ag (Fig. 5B, bottom panel). These cell lines were then stably transduced with a retroviral vector expressing Cre recombinase (pB-Cre) or the empty vector (pB-vec) and a second retroviral vector expressing HA-CUL7 (pL-HA-CUL7) or the empty vector (pL-vec). Expression of HA-CUL7 was confirmed by Western blotting (Fig. 5B, middle panel, lanes 2, 4, 6, and 8). Efficient loss of CUL7 expression where Cre was introduced was demonstrated by Western blotting (lanes 3 and 7). Because the CUL7 antibody also recognized HA-CUL7, it was important to confirm that *Cul7* was deleted in the cell lines in which HA-CUL7 was also expressed (lanes 1 and 5). To establish that endogenous *Cul7* was deleted, PCR of primary MEFs confirmed that pB-Cre was reproducibly capable of successfully deleting all of the detectable *Cul7* allele (data not shown). Western blot assays for

FIG. 5. CUL7 expression affects the growth of MEFs expressing CUL7-binding T Ag mutants. (A) Scheme for conditional knockout of *Cul7*. *LoxP* sites (indicated with arrowheads) flank exons 2 through 4. (B) Wild-type and Δ69–83 T Ag were stably expressed in *Cul7*^{2LoxP/2LoxP} MEFs. Each cell line was infected with either pBABE-Cre (pB-Cre) or empty vector (pB-vec) and pL(N)BCX HA-CUL7 (pL-HA-CUL7) or empty vector (pL-vec). Whole-cell lysates (50 μg) were Western blotted for CUL7, HA, and T Ag. (C and D) In studies with the cell lines shown in panel B, 50,000 WT T Ag-expressing MEFs (C) or Δ69–83 T Ag-expressing MEFs (D) were plated per 60-mm dish, and cells were counted in triplicate on the indicated days. pB-vec plus pL-vec (squares), pB-Cre plus pL-vec (circles), pB-vec plus pL-HA-CUL7 (inverted triangles), and pB-Cre plus pL-HA-CUL7 (triangles) are shown.

CUL7 demonstrate expression of endogenously expressed as well as the retrovirally transduced HA-CUL7 (Fig. 5B, top panel).

To determine whether T Ag binding to CUL7 was required for cell growth to high density, the cells shown in Fig. 5B were plated in triplicate and counted for 11 days. T Ag expression in *Cul7*^{2LoxP/2LoxP} MEFs with the addition of empty vectors (pB-vec/pL-vec) induced growth to the highest density (Fig. 5C, squares). Similar to the results presented in Fig. 4B, Δ69–83 T Ag-expressing *Cul7*^{2LoxP/2LoxP} MEFs with the addition of empty vectors (pB-vec/pL-vec) grew to a lower density. Expression of Cre recombinase (pB-Cre/pL-vec) in WT T Ag-expressing cells led to a slight reduction in growth rate and density (Fig. 5C, circles) (25). In contrast, expression of Cre recombinase and the resulting loss of CUL7 expression in cells expressing Δ69–83 T Ag led to a significant increase in growth rate and density (Fig. 5D, squares and circles). This increase in growth upon somatic loss of *Cul7* in MEFs expressing Δ69–83 supports the model that T Ag inactivation of CUL7 contributes to T Ag-mediated cellular transformation.

We asked whether the expression of exogenous CUL7 could suppress the growth increase seen when *Cul7* was somatically deleted from MEFs expressing Δ69–83 T Ag. Expression of

HA-CUL7 with Cre recombinase suppressed the growth rate compared with Cre only (Fig. 5D, triangles). Moreover, there was also a growth suppression when HA-CUL7 was introduced into the *Cul7*^{2LoxP/2LoxP} MEFs expressing WT T Ag either with or without Cre recombinase (Fig. 5C, triangles and inverted triangles). This suggests that overexpression of CUL7 may serve to suppress the growth of WT T Ag-expressing cells.

DISCUSSION

SV40 T Ag provides a powerful tool to address mechanisms of cellular transformation. This viral oncogene acts as a dominant transforming protein that targets cellular proteins and thereby alters the cell's growth properties (23). In particular, the inactivation of p53 and the pRB family members is required for cell transformation. T Ag mutants that are unable to bind to and inactivate pRB and p53 are also unable to fully transform cells (12, 32). Although inactivation of pRB and p53 is essential for T Ag-mediated transformation, it may not be sufficient. Rather, additional activities of T Ag may also contribute to the fully transformed phenotype (4, 17, 22). Our laboratory demonstrated that F98A and Δ98–99 mutants of T Ag were defective for cellular transformation, suggesting that

CUL7 binding may contribute to T Ag-mediated cellular transformation (2). These results did not exclude the possibility that there were other activities beyond CUL7 binding that are relevant for transformation. Until we were able to genetically remove *Cul7* from MEFs, we could not specifically implicate CUL7 binding deficiency in the decreased ability of CUL7-binding mutants of T Ag to promote MEFs to grow to a high density (2). In this study, we demonstrate that T Ag residues 69 to 83 were required for binding to CUL7. We showed that, when this region was deleted, the ability of T Ag to promote cell proliferation and growth to high density was decreased. When *Cul7* was deleted from $\Delta 69$ –83 T Ag-expressing cells, the cells increased their proliferative capacity and grew to higher density. The implication of this finding is that CUL7 plays a role in growth suppression and that T Ag appears to inactivate this function of CUL7.

Identification of a distinct CUL7-binding site on T Ag. Ali et al. showed that a purified peptide corresponding to the N-terminal 121 residues of SV40 T Ag coprecipitated CUL7 (2). In addition, the T Ag mutants $\Delta 98$ –99 and F98A lost the ability to bind to CUL7, implying that residues 98 and 99 were essential for binding to CUL7. However, a report on the crystal structure of the complex formed by T Ag residues 7 to 117 and the pRB pocket suggested that the side chains of residues F98 and N99 might have been inaccessible, at least when T Ag was bound to pRB (13). Consequently, these mutations may have disrupted this structural motif rather than the specific amino acid side chains involved in the interaction.

The structure of the N terminus of T Ag revealed four α -helices that span residues 7 to 102. The region between the third and fourth helices forms an exposed loop. We evaluated a number of point mutants and deletions of T Ag to investigate whether mutations in the loop region result in loss of CUL7 binding. A deletion of T Ag residues 69 to 83, which make up approximately half of the loop, resulted in complete abrogation of CUL7 binding. Alanine scanning throughout this loop region, as well as a smaller in-frame deletion ($\Delta 71$ –74), showed decreased binding to CUL7. Although we have not identified T Ag's definitive binding interface for the CUL7 complex, the mutant $\Delta 69$ –83 T Ag provides us with a new tool for assessment of the targeting of CUL7 in T Ag-mediated transformation.

A mutant in an overlapping region of T Ag was originally characterized as a replication-defective mutant by Maulbecker et al. (18). These authors described the isolation of the GMSV40 virus from a human skin fibroblast line that was stably transformed with SV40. The GMSV40 virus was defective for replication and plaque formation in vivo. Although we have not tested $\Delta 69$ –83 T Ag for replication efficiency, other CUL7-binding mutants of T Ag were able to replicate in vivo (2). The possibility remains that T Ag from GMSV40 was not able to bind CUL7, but based on our previous findings, the ability to bind CUL7 was likely not required for SV40 replication in vivo (2).

The binding site for CUL7 is distinct from the binding sites for p53, pRB, and BUB1. We demonstrated that the CUL7-binding-deficient mutant of T Ag ($\Delta 69$ –83 T Ag) is capable of association with p53. In addition, we showed that T Ag can associate with CUL7 in the absence of p53 and with p53 in the absence of CUL7. We further showed that $\Delta 69$ –83 T Ag re-

tains the ability to bind to pRB and to dephosphorylate p130. Our data suggest that $\Delta 69$ –83 T Ag retains an intact and functional LxCxE and J domain (32). A recent report has shown that deletion of residues 89 to 97 reduces binding to the mitotic checkpoint protein BUB1 (5). Given the proximity between the CUL7 and BUB1 binding domains on T Ag, we investigated a possible relationship between T Ag binding to these two proteins. Both WT T Ag and $\Delta 69$ –83 T Ag associated with BUB1 in the presence or the absence of CUL7. Although these data do not rule out the possibility that CUL7 binding is required for functional inactivation of pRB, BUB1, or p53, an important conclusion is that T Ag binding to CUL7 is independent of association with these proteins. Additionally, the $\Delta 69$ –83 T Ag mutant provided us with a new tool for accessing the role of CUL7 in T Ag-mediated transformation.

A role for CUL7 inactivation by T Ag in promoting growth to high density. Wild-type T Ag promoted the growth of WT or *Cul7*^{-/-} MEFs to similarly high densities. In contrast, $\Delta 69$ –83 T Ag, which is unable to bind CUL7, induced growth to high density only in the absence of CUL7. This result was obtained using MEFs with either germ line loss of *Cul7* or by addition of CRE recombinase to MEFs with conditional knockout alleles of *Cul7* resulting in somatic loss of *Cul7*, implying that T Ag binding to CUL7 inactivates CUL7's growth-suppressing properties. We observed that expression of CRE recombinase in the conditional knockout MEFs expressing WT T Ag resulted in a reduction of growth density. This is consistent with unpublished observations from our laboratory demonstrating that expression of CRE resulted in growth suppression even in the absence of *LoxP*-containing alleles. CRE-dependent growth suppression is likely due to its reported cellular toxicity (25). Notably, when exogenous HA-CUL7 was introduced into $\Delta 69$ –83 T Ag-expressing MEFs after somatic loss of *Cul7*, these cells had a reduced proliferative capacity. Although not to the same extent, we also observed that expression of HA-CUL7 in MEFs expressing WT T Ag reduced their growth density potential as well. This result is consistent with the model that T Ag serves to inactivate CUL7; in the presence of this excess CUL7, T Ag may not be capable of inactivating all of the available CUL7.

Our laboratory has previously reported that *Cul7* loss results in embryonic lethality. In addition, MEFs isolated from *Cul7*^{-/-} embryos display a prominent growth defect in tissue culture (3). These data suggest that CUL7 has growth-promoting properties in primary MEFs, and when *Cul7* is deleted, cell growth and proliferation are suppressed. This is in contrast with the observation that T Ag appeared to inactivate CUL7, resulting in growth promotion. We would predict that inactivation of CUL7 by T Ag would lead to a similar phenotype as *Cul7*^{-/-} MEFs, but additional functions of T Ag may allow a substrate protein to promote, as opposed to suppress, cell proliferation and growth. Perhaps in the context of T Ag, inactivation of CUL7 contributes to transformation. An example of a protein that could behave in such a manner is oncogenic RAS. When oncogenic RAS is overexpressed in normal cells, it promotes cellular senescence, but in cells that express T Ag or when p53 is inactivated, RAS contributes to transformation (11, 19, 24). Since T Ag is able to inactivate the p53 checkpoint, RAS can cooperate with T Ag to promote transformation in an unchecked manner. CUL7 may act in a similar

manner; when lost in normal cells, it leads to growth suppression, but when inactivated by T Ag, it contributes to growth.

The results presented here support the notion that, in addition to p53 and the pRB family of proteins, T Ag is required to inactivate CUL7 to fully transform cells. Although a substrate of the CUL7 SCF-like complex has not been identified, we propose that T Ag may inhibit CUL7 activity by inhibiting the ubiquitination of a substrate. We have not excluded the possibility that T Ag binding to CUL7 also redirects the E3 ubiquitin ligase activity to a novel substrate. Although the mechanism is not understood, T Ag binding to CUL7 provides us with more insight into the mechanisms by which T Ag promotes cellular transformation. T Ag's ability to promote cellular transformation continues to provide us with a powerful tool for studying cell growth and regulation.

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