Enterocyte Proliferation and Intestinal Hyperplasia Induced by Simian Virus 40 T Antigen Require a Functional J Domain[▽]

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Transgenic mice expressing the simian virus 40 large T antigen (TAg) in enterocytes develop intestinal hyperplasia that progresses to dysplasia with age. This induction requires TAg action on the retinoblastoma (Rb) family of tumor suppressors and is independent of the p53 pathway. In cell culture systems, the inactivation of Rb proteins requires both a J domain in TAg that interacts with hsc70 and an LXCXE motif that directs association with Rb proteins. Together these elements are sufficient to release E2Fs from their association with Rb family members. We have generated transgenic mice that express a J domain mutant (D44N) in villus enterocytes. In contrast to wild-type TAg, the D44N mutant is unable to induce enterocyte proliferation. Histological and morphological examination revealed that mice expressing the J domain mutant have normal intestines without loss of growth control. Unlike mice expressing wild-type TAg, mice expressing D44N do not reduce the protein levels of p130 and are also unable to dissociate p130-E2F DNA binding complexes. Furthermore, mice expressing D44N in a null p130 background are still unable to develop hyperplasia. These studies demonstrate that the ectopic proliferation of enterocytes by TAg requires a functional J domain and suggest that the J domain is necessary to inactivate all three pRb family members.

The DNA tumor virus simian virus 40 (SV40) encodes a dominant-acting oncoprotein, large T antigen (TAg). This protein is sufficient to induce transformation in multiple mammalian cell lines and to induce neoplasia in numerous tissues when expressed ectopically in transgenic mice (1). TAg functions by interacting with key cellular regulatory proteins; for instance, its LXCXE motif is responsible for binding to the retinoblastoma (Rb) family of tumor suppressors (6). In addition, the carboxy-terminal region of TAg binds, stabilizes, and inactivates the tumor suppressor p53 (3, 16, 25).

The first 70 amino acids of TAg have sequence identity with the J domain of the DnaJ class of molecular chaperones (15). The J domain of TAg binds to hsc70, the major DnaK homologue present in mammalian cells, and stimulates its ATPase activity (34). This interaction results in the release of unfolded peptides from the substrate-binding domain of hsc70 (29). The inactivation of Rb proteins by TAg requires a functional J domain to interact with hsc70 and to release E2Fs from their binding to Rb family members. This release results in the upregulation of E2F transactivation activity and subsequent progression of cells into the S phase.

The residues histidine-proline-aspartate (HPD) are absolutely conserved within the J domain of all known, active DnaJ homologues (28). Substitution mutations in any of these residues render the J domain less able to activate the ATPase activity of hsc70. Studies in cell culture show that the TAg J domain plays a role in transformation. The J domain of TAg cooperates with the Rb binding motif (LXCXE) to inactivate the growth-suppressive functions of p130, p107, and pRb. In cell culture, J-domain mutants are defective for altering p130

and p107 phosphorylation and are unable to degrade p130; however, these mutants do not affect the phosphorylation state of pRb (31). Single-amino-acid-substitution mutants in the J domain, such as H42Q, are unable to disrupt Rb family-E2F DNA binding complexes in mouse embryo fibroblasts (MEFs) (40). Another single-amino-acid-substitution J-domain mutant, D44N, is unable to bind hsc70 and does not disrupt p130-E2F4 complexes (32). On the other hand, D44N induces focus formation and anchorage-independent growth, although at a somewhat reduced frequency compared to wild-type TAg (TAg^{wt}) (9, 24, 31). The TAg J domain confers a growth advantage to normal MEFs but is dispensable in the case of MEFs lacking both p130 and p107 (31). These data indicate that p107 and p130 have overlapping growth-suppressing activities whose inactivation is mediated by the J domain of TAg.

The mouse intestinal epithelium is organized into numerous finger-like projections, the villi, and the structures responsible for their renewal, the crypts of Lieberkuhn. The intestinal epithelium contains four types of differentiated cells: enterocytes and goblet, enteroendocrine, and Paneth cells. These cells are derived from a small number of multipotent stem cells that reside near the base of each crypt. These stem cells give rise to a zone of proliferating cells, which differentiate as they migrate towards the luminal surface, with the exception of the Paneth cells, which migrate towards the base of the crypt. As a result, villi are mainly composed of enterocytes with some goblet and enteroendocrine cells. The differentiated cells at the top of villi are then extruded into the intestinal lumen, while Paneth cells are thought to be engulfed by macrophages. Intestinal villi and crypts can be readily separated from extraneous tissue, thus allowing the isolation of proteins or nucleic acids from cell populations greatly enriched for nonproliferating, terminally differentiated cells (villi) or from their proliferating, multipotent progenitors (crypts) (23, 38). Overall, the stratification of proliferation and differentiation along the crypt-villus axis

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makes the intestine an attractive model system for examining the regulation of mediators of the cell cycle.

Expression of full-length TAG in murine enterocytes results in intestinal hyperplasia that progresses to dysplasia by 4 to 6 months of age (12, 18). The hyperplasia requires TAG action on the Rb family of tumor suppressors, since a mutant TAG that cannot bind Rb proteins exhibits a normal intestine with growth-arrested enterocytes (2). While many studies have shown the effects of full-length TAG as a transgene in different tissues, a limited number of studies have been done on the expression of different TAG mutants (27, 36). Observations in cell culture strongly suggest that the J domain of TAG is required to overcome the p130- and/or p107-mediated repression of E2F-dependent transcription and thus is required for the transformation induced by TAG (39). To determine the J-domain requirements in a normal tissue model, we have generated transgenic mice using the intestinal fatty acid binding protein promoter to direct expression of the D44N TAG mutant (TAG^{D44N}) to villus enterocytes. Our results indicate that an intact J domain is required to induce intestinal proliferation mediated by TAG and suggest that different tissues require different signals to undergo tumorigenic proliferation.

MATERIALS AND METHODS

Production and maintenance of transgenic mice. A BamHI/XhoI fragment (2.7 kb) from pRSVbneoT (29), encoding full-length TAG, was ligated to the 3.9-kb BclI/XhoI vector portion of pIFABP.GFP (M. T. Sáenz Robles et al., submitted for publication). The resulting plasmid (pIFABP.T) was the target for site-directed mutagenesis (QuickChange kit; Stratagene) to introduce the D44N mutation into TAG^{wt}. The resulting construct (pIFABP.D44N) was confirmed by sequence analysis and digested with SacII/MluI to render a 4.2-kb fragment containing the IFABP promoter, the coding sequences for SV40 large TAG mutant D44N, and the SV40 polyadenylation signals. Pronuclear microinjection of this fragment resulted in transgenic mice (Fabpi-SV40 TAG^{D44N}) expressing TAG^{D44N} and small t antigen in the enterocytes of the small intestine. Three different transgenic lines were established (A, C, and G), all of which showed similar phenotypes. Unless noted, TAG^{D44N} animals used in this report were from pedigree C. Transgenic mice expressing SV40 TAG^{wt} under the control of the rat intestinal fatty acid binding protein promoter (pedigree 103) were obtained from Jeff Gordon (18). For simplification, we refer to the mice as TAG^{wt} (Fabpi-SV40 TAG^{wt}) and TAG^{D44N} (Fabpi-SV40 TAG^{D44N}). Murine viral pathogens (hepatitis, minute, lymphocytic, choriomeningitis, ectromelia, polyoma, Sendai, pneumonia, and mouse adenoviruses and enteric bacterial pathogens) and parasites were absent from the mouse colony. All lines were maintained by crosses to nontransgenic FVB mice. The genotype of each mouse was determined by PCR amplification as described by Sáenz Robles et al. (submitted).

Immunohistochemistry. Analysis of the proliferative status by incorporation of bromodeoxyuridine (BrdU) has been described by Sáenz Robles et al. (submitted). Detection of TAG proteins was performed in 5- μ m-thick histological sections using appropriate dilutions of the anti-TAG antibody PAb419 (11) as the primary antibody and biotinylated anti-mouse-Fab as the secondary antibody, followed by a streptavidin-peroxidase ARK kit plus development of the peroxidase reaction with diaminobenzidine substrate (DAKO), according to the manufacturer's instructions.

Immunoblot analysis. Material enriched in intestinal villi was prepared and analyzed by Western blotting as described elsewhere (23). Appropriate dilutions of the following primary antibodies were used: TAG mouse monoclonal PAb416 (11); anti-pRb-mouse immunoglobulin G (IgG) (G-3245) from BD-Pharmingen; and anti-p130-rabbit IgG (C-20) (sc-317), anti-p107 rabbit IgG (C-18) (sc-318), anti-E2F1 rabbit IgG (C-20) (sc-193), anti-E2F2 mouse IgG1 (TFE25) (sc-9967), and anti-E2F3 rabbit IgG (C-18) (sc-878) from Santa Cruz Biotechnologies. Goat anti-mouse A2554 and goat anti-rabbit A0545 (Sigma) were used as secondary antibodies.

RT-PCR analysis. For reverse transcription-PCR (RT-PCR), whole intestine or intestinal fractions were collected, lysed, and homogenized in buffer containing guanidine isothiocyanate, an RNase inhibitor, and the total RNA was extracted using the RNeasy kit (QIAGEN). One microgram of RNA was reverse

transcribed into cDNA using the Superscript First-Strand synthesis kit (Gibco BRL). The cDNAs were amplified with PCR using primers specific for alcohol dehydrogenase (ADH) (5'-TGCACCACCACTGCTTAG and 5'-GATGCAG GGATGATGTTT), E2F1 (5'-TTGCTGTCTGTTTGTCTAGCC and 5'-CG GAGATTTTACACCTTTCCCTG), E2F2 (5'-TTCGCTTTACACGCAG ACCG and 5'-AATGAACCTTCTGGTCAGGAGCC), E2F3a (5'-AGCCTCT ACACCACGCCACAAG and 5'-ATCCAGGACCCATCAGGAGAC), RB (5'-TCACACAACCAGCAGTGC and 5'-CTATCCGAGCGCTCTGTTC), p107 (5'-TAGATATCTTTCAAATCCATATGAAGACC and 5'-ATTGTAC ACCACGACTCC), p130 (5'-TTGGGACTCTGTCTCGGTGTCTAAG and 5'-A ATGCGTCATGCTCCAGAACACCAG), and TS (5'-TCAGTTCTATGTGGTG AATGGGG and 5'-TGGGAAAGGTCTTGGTTCTCGC). Primers for b-myb and cyclin E1 were previously described (37).

To ensure that these reactions were within the linear range of the assay, we optimized the number of cycles required to obtain nonsaturated signals. Exponential amplifications of PCR products were obtained as follows: 2 min at 94°C; a series of 25 cycles at 94°C for 30 s, variable annealing temperatures for 30 s, and 72°C for 30 s; and a final extension step of 5 min at 72°C. The annealing temperatures and product sizes were 60°C and 420 bp for E2F1, 60°C and 289 bp for E2F2, 60°C and 309 bp for E2F3a, 56°C and 270 bp for RB, 57°C and 499 bp for p130, 56°C with a 2-min extension and 2,250 bp for p107, 55.5°C and 235 bp for TS, 58°C and 350 bp for b-myb, and 55°C and 608 bp for cyclin E1. The products were resolved on 2% agarose gels and stained with GelStar (Bio-Whittaker Molecular Applications).

EMSA. For the electrophoretic mobility shift assay (EMSA), equal amounts of top and bottom strands of an oligonucleotide containing an E2F binding site (5'-ATTTAAGTTTCGCGCCCTTCTCAA-3') were annealed and 28 pmol of the double-stranded oligonucleotide was then end labeled with [γ -³²P]ATP as described previously, with modifications (33). To purify the end-labeled oligonucleotide from unlabeled [γ -³²P]ATP, the oligonucleotide was subjected to centrifugation through Centri-spin TM-20 columns as per the manufacturer's protocol (Princeton Separations). Thirty micrograms of protein extracts with or without antibodies was diluted in gel shift buffer (10 mM HEPES KOH [pH 7.9], 20 mM KCl, 3 mM MgCl₂, 0.5 mM EGTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.05% NP-40), plus an additional mixture of 100 mM dithiothreitol, 1.5 mg/ml bovine serum albumin, 50 μ g/ml salmon sperm DNA, and 10% glycerol and incubated with 2 μ g of labeled probe for 30 min at room temperature. Antibodies used for the supershift assay were pRb mouse IgG (G-3245) (14001A) from BD-Pharmingen and anti-p130 rabbit IgG (C-20) (sc-317), anti-p107-rabbit IgG (C-18) (sc-318), anti-E2F1 mouse IgG2a (KH95) (sc-251) or anti-E2F1 rabbit IgG (C-20) (sc-193), anti-E2F2 mouse IgG1 (TFE25) (sc-9967) or anti-E2F2 rabbit IgG (C-20) (sc-633), anti-E2F3a rabbit IgG (N-20) (sc-879), anti-E2F3 rabbit IgG (C-18) (sc-878), anti-E2F4 rabbit IgG (A-20) (sc-1082), and anti-E2F5 rabbit IgG (H-ill) (sc-I 699) from Santa Cruz Biotechnologies, Inc. The p130 antibody shows residual cross-reactivity with the p107 protein, therefore partially shifting p107-containing complexes. The samples were then subjected to gel electrophoresis through a nondenaturing polyacrylamide gel (4% bis-acrylamide, 0.25 $\times$ Tris-borate-EDTA, 0.1% ammonium persulfate, 0.001% TEMED [*N,N,N',N'*-tetramethylethylenediamine]) in 0.25 $\times$ Tris-borate-EDTA running buffer at 4°C for approximately 4 to 6 h. The gel was then dried for approximately 2 h and exposed to film for various lengths of time at -80°C.

RESULTS

Transgenic mice expressing the J-domain mutant D44N in enterocytes show a normal intestinal phenotype. Mice expressing the TAG^{wt} in the intestinal enterocytes develop hyperplasia by 6 weeks after birth and progress to dysplasia with age (4 to 6 months) (12, 17, 18, 23). Additionally, these mice show a significant increase in the size of intestinal crypts (23). However, mice expressing a single-amino-acid-substitution J-domain mutant TAG (TAG^{D44N}) in enterocytes possess normal intestines and do not show any signs of hyperplasia (Fig. 1A).

Expression of TAG^{wt} under the control of the IFABP promoter was previously shown to be restricted to villus enterocytes (18). We confirmed the expression patterns of TAG in TAG^{wt} and TAG^{D44N} mice by immunostaining. Paraffin sections

FIG. 1. (A) Expression of both TAG^{wt} or TAG^{D44N} is restricted to villus enterocytes. Intestinal tissues were embedded in paraffin, and 5- μ m sections were stained with an anti-TAG antibody. The brown stain reflects the presence of TAG in nuclei. (B) Expression levels of TAG in TAG^{wt} and TAG^{D44N} villus samples. Protein extracts from villi of nontransgenic, TAG^{wt}, and TAG^{D44N} transgenic mice were subjected to immunoblots for TAG. Protein levels of β -tubulin were used as loading controls.

stained with anti-TAG antibody revealed that expression of either TAG^{wt} or TAG^{D44N} was restricted to villus enterocytes (Fig. 1A). Western blot analysis confirmed that similar amounts of wild-type and mutant proteins were expressed in the intestines of the transgenic mice (Fig. 1B). To assess the proliferative status of transgenic intestines, histological sections were stained with anti-BrdU. In agreement with the morphological phenotype observed, we found numerous BrdU-positive cells in villus enterocytes of mice expressing TAG^{wt} (Fig. 2B), while none were present in the mice expressing TAG^{D44N} (Fig. 2C). No signs of ectopic proliferation were found in mice expressing TAG^{D44N}, and proliferation was restricted to the crypts as in nontransgenic mice (Fig. 2, compare panels A and C). We have analyzed three independent TAG^{D44N} transgenic lines and observed the same phenotype described above.

As previously reported, TAG^{wt} mice show a significant in-

crease in the total length of the small intestine, at some times doubling the length of the control littermates (23). In contrast, the intestines of TAG^{D44N} mice do not show a significant difference in intestinal length in comparison to nontransgenic littermates (Fig. 3) in either male or female specimens.

Expression of TAG^{D44N} results in increased pRb and p107 protein levels in villus enterocytes. To assess the steady-state levels of Rb family proteins (p130, p107, and pRb), we used intestinal extracts enriched for villus cells from TAG^{wt} and TAG^{D44N} mice or from their nontransgenic littermates (Fig. 4A). TAG^{D44N} mice were able to upregulate p107 protein expression to the same degree as mice expressing TAG^{wt}. As expected, extracts from mice expressing TAG^{wt} show reduced p130 protein levels in comparison to nontransgenic littermates. However, in agreement with observations in cell culture experiments (31), TAG^{D44N} mice were unable to trigger a reduction in the steady-state levels of p130. Interestingly, a profound

FIG. 2. Ectopic proliferation of enterocytes requires a functional J domain. Proliferation in TAG^{D44N} mice is restricted to the crypts as in nontransgenic mice. Intestinal sections stained with anti-BrdU show numerous BrdU-positive cells in villus enterocytes of mice expressing TAG^{wt}, but none was present in the mice expressing TAG^{D44N} or nontransgenic mice.

upregulation of pRb protein levels was found in TAG^{D44N} mice compared to nontransgenic and TAG^{wt} mice.

To determine whether the changes in protein levels were paralleled by a change in the steady-state levels of the corresponding messenger, we performed semiquantitative RT-PCR analysis (Fig. 4B). As previously reported, TAG^{wt} mice were able to upregulate p107 but exhibited no change in the pRb and p130 mRNA levels in comparison to nontransgenic littermates (Sáenz-Robles et al., submitted). Though TAG^{D44N} mice show increased levels of p107 and pRb proteins, we did not find any changes in their mRNA levels and conclude that the increased levels of these proteins probably occur posttranscriptionally.

TAG^{D44N} affects activator E2F protein levels differentially. Extracts from TAG^{wt} mice show upregulation of all activator E2F (E2F1, E2F2, and E2F3a) protein levels (Sáenz Robles et al., submitted), which in turn activates S-phase genes and induces cell proliferation. To determine the effect on steady-state levels of activator E2Fs in TAG^{D44N} mice, we performed Western blot analysis using intestinal extracts enriched for villus cells (Fig. 5A). We found a modest but consistent up-regulation of E2F2 and E2F3a in TAG^{D44N} intestines in comparison to nontransgenic intestines. In contrast, no change in E2F1 protein levels in TAG^{D44N} mice compared to nontransgenic mice was observed.

To determine whether these changes were reflected in the

FIG. 3. Expression of TAG^{D44N} does not increase intestinal length in mice. The total length of the small intestine in adult male or female mice was measured, and the average length of intestine in each group (control, TAG^{wt}, and TAG^{D44N}) is represented. The error bars indicate the standard deviation in the group. Intestines expressing TAG^{D44N} show similar length to those in control mice.

FIG. 4. TAg^{D44N} expression in villus enterocytes alters the pRb protein levels but does not affect their transcript levels. (A) Steady-state protein levels of Rb-family members in nontransgenic, TAg^{wt} , and TAg^{D44N} villus samples. Whole-cell extracts from villi of nontransgenic, TAg^{wt} , and TAg^{D44N} transgenic mice as well as knockout (-/-) intestinal controls were subjected to immunoblots for p130, p107, and pRb. Protein levels for β -tubulin were used as loading controls. (B) Steady-state transcript levels of Rb family members in nontransgenic, TAg^{wt} , and TAg^{D44N} villus samples. cDNAs were reverse transcribed from equal amounts of total RNA extracts and subjected to PCR using specific primers. Transcript levels of alcohol dehydrogenase 5 (Adh5) were used as loading controls.

messenger for the E2Fs, we performed semiquantitative RT-PCR (Fig. 5B). As expected, TAg^{wt} mice were able to upregulate E2F1, E2F2, and E2F3a mRNA levels. However, no change in mRNA levels was observed in TAg^{D44N} intestines. Thus, the increased protein levels of E2F2 and E2F3a probably occur posttranscriptionally.

TAg^{D44N} is unable to disrupt p130-E2F complexes. Rb proteins exert their effects in part by binding to and regulating the E2F family of transcription factors. To examine the effects of TAg^{D44N} expression on Rb-E2F DNA binding complexes we performed a series of EMSA experiments. A radiolabeled oligonucleotide probe with consensus E2F binding sites was incubated with extracts from nontransgenic, TAg^{wt} , and TAg^{D44N} growth-arrested enterocytes (villi), and the products were resolved on a nondenaturing gel. Antibody supershift

experiments were performed to determine which Rb-protein constituted those Rb-E2F DNA binding complexes. We did not observe any differences in DNA binding complexes between nontransgenic and TAg^{D44N} mice, a fact consistent with the normal morphological phenotype observed (Fig. 6). We found three major bands in both nontransgenic and TAg^{D44N} mice. The slowest-migrating band represents a p130-E2F complex. Two faster-migrating bands do not contain Rb proteins and represent E2F4-DNA and E2F5-DNA complexes (data not shown). Additionally, we found a minor slowly migrating band in nontransgenic, TAg^{wt} , and TAg^{D44N} mice that represents a p107-E2F complex. In contrast, no p130-E2F complexes were detected in enterocytes expressing TAg^{wt} . As reported previously, TAg^{wt} induces the formation of Rb-free E2F2-DNA and E2F3a-DNA binding complexes (Sáenz Rob-

FIG. 5. Expression of TAg^{D44N} in villus enterocytes affects activator E2F protein levels differentially without altering their transcript levels. (A) Protein extracts from villi of nontransgenic, TAg^{wt} , and TAg^{D44N} mice were subjected to immunoblot analysis. Nuclear extracts from S-phase-synchronized normal MEFs were used as a positive control for E2F1. Whole-cell extracts from 293 cells overexpressing E2F2 were used as a positive control for E2F2, and whole-cell extracts from mammary gland tumor were used as a positive control for E2F3a. (B) RNA from villi of nontransgenic, TAg^{wt} , and TAg^{D44N} mice was subjected to RT-PCR analysis using specific primers. Transcript levels of Adh5 were used as loading controls.

FIG. 6. An intact J domain is required to disrupt p130-E2F DNA binding complexes in villus enterocytes. Gel shift analysis with a radiolabeled nucleotide probe containing a consensus E2F binding site was performed on protein extracts isolated from villi of nontransgenic, TAg^{wt}, and TAg^{D44N} mice. The composition of each migrating complex was determined by supershift analysis with specific antibodies. α p130, anti-p130; α p107, anti-p107.

les et al., submitted), which are absent in both nontransgenic and TAg^{D44N} mice. Furthermore, supershifts with antibodies against pRb, E2F2, and E2F3a failed to detect any pRb-E2F2 or pRb-E2F3a DNA binding complexes in TAg^{D44N} intestines (data not shown). Thus, despite the modest upregulation in E2F2 and E2F3a protein levels in TAg^{D44N} mice, there is no evidence that the DNA binding activity of these transcription factors is increased.

TAg^{D44N} is defective for the regulation of E2F target genes. To further confirm that there is no ectopic E2F activity present in TAg^{D44N} mice, we analyzed the expression of some of the genes regulated by E2Fs (7, 13, 30). We used semiquantitative RT-PCR (Fig. 7) and in some cases (cyclin E1 and thymidylate synthase [data not shown]) real-time PCR to assess the mRNA levels of b-myb, cyclin E1, and thymidylate synthase. In contrast to TAg^{wt} mice, samples from TAg^{D44N} intestines did not show any upregulation of these genes (Fig. 7). These results agree with our previous EMSA experiments, suggesting that no ectopic E2F activity is present in TAg^{D44N} mice.

TAg^{D44N} is unable to induce hyperplasia in the p130^{-/-} background. Unlike TAg^{wt}, the expression of TAg^{D44N} in enterocytes did not downregulate p130 protein levels and was unable to dissociate p130-E2F complexes. To determine if inactivation of p130 by TAg is sufficient to induce hyperplasia, we crossed TAg^{D44N} mice into the p130^{-/-} background. The proliferative status of these transgenic intestines was assessed in intestinal sections stained with anti-BrdU. No signs of ectopic proliferation were found in TAg^{D44N}; p130^{-/-} mice, and proliferation was restricted to the crypts, as found in nontransgenic mice (data not shown). To determine the steady-state levels of Rb family members and activator E2Fs in TAg^{D44N};

FIG. 7. TAg^{D44N} is unable to upregulate E2F target genes in intestinal enterocytes. Steady-state transcript levels of E2F target genes were determined in nontransgenic, TAg^{wt}, and TAg^{D44N} villus samples. cDNAs were reverse transcribed from equal amounts of total RNA extracts and subjected to PCR using specific primers for b-myb, cyclin E, and thymidylate synthase (TS). Transcript levels of Adh5 were used as loading controls.

p130^{-/-} mice, we performed Western blot analysis using intestinal extracts. We found that the absence of p130 had no effect on the ability of TAg^{D44N} to regulate the protein levels of Rb and E2F family members (Fig. 8A and B).

DISCUSSION

The J domain of TAg is required to induce enterocyte proliferation and intestinal hyperplasia. Cell culture studies using various mutants of SV40 TAg suggest that the J domain contributes to transformation. For instance, an in-frame deletion mutant of TAg missing part of the J domain (*d11135*) is defective for the induction of dense foci despite being able to bind Rb proteins and p53 (26, 29). In contrast, amino-acid-substitution mutants of the J domain, such as D44N, are able to induce focus formation and anchorage-independent growth in cultured cells, although at a somewhat reduced frequency compared to TAg^{wt} (9, 24, 31). These differences in the observed phenotypes raise the question of why some J-domain alleles are defective for focus formation while others are not. One possibility is that the interaction of the J domain with hsc70 is allele specific. For instance, perhaps a particular J-domain mutant can stimulate hsc70 enough to achieve partial action on all or some of its substrates, leading to a subset of phenotypes observed with TAg^{wt} (8, 35). Another possibility is that the SV40 J domain binds cellular proteins in addition to hsc70. In this scenario, some mutant alleles would disrupt TAg interaction with specific subsets of the unknown cellular targets, thus losing the phenotypes resulting from this interaction while maintaining interaction with hsc70 or vice versa. Presumably, alleles resulting from the total loss of the J domain, such as deletions, might result in the loss of both binding functions.

Different J-domain mutants show different phenotypes in cell culture and thus present a confusing picture about the role of the J domain in transformation. Therefore, we decided to test the requirement for the J domain in a native tissue, namely

FIG. 8. Loss of p130 does not affect the steady-state levels of pRb and E2F family members in TAG^{D44N}; p130^{-/-} mice. Whole-cell extracts from middle intestines of nontransgenic, TAG^{wt}, TAG^{D44N}, and TAG^{D44N}; p130^{-/-} transgenic mice as well as knockout (KO) (-/-) intestinal controls were subjected to immunoblots for p130, p107, and pRb (A) and E2F1, E2F2, and E2F3a (B). Protein levels of β -tubulin were used as loading controls.

the small intestine. The TAG J-domain mutant D44N is capable of inducing foci and anchorage-independent growth in cell culture, but, in contrast, we have found that its expression in villus enterocytes does not induce ectopic cell proliferation. The intestines of TAG^{D44N} mice retained normal morphological features in spite of the high levels of transgene expression in several transgenic lines tested. Based on these observations, we conclude that TAG requires a functional J domain to induce enterocyte proliferation and intestinal hyperplasia.

The J domain of TAG is required to inactivate p130. Several cell culture studies have shown that the J domain of TAG cooperates with the Rb binding motif (LXCXE) to inactivate the growth-suppressive functions of p130, p107, and pRb. Unlike TAG^{wt}, J-domain mutants do not alter the phosphorylation of p130 and p107 and are unable to degrade p130 (31). The TAG J domain confers a growth advantage to normal MEFs, but it is dispensable in the case of MEFs lacking both p130 and p107. This indicates that p107 and p130 have overlapping growth-suppressing activities whose inactivation is mediated by the J domain of TAG. In vivo and in vitro studies have shown that the TAG^{D44N} protein is unable to bind hsc70 and does not disrupt p130-E2F4 complexes (32). All of these observations strongly suggest that the J domain of TAG is required to overcome p130- and/or p107-mediated repression of E2F-dependent transcription and thus contributes to transformation induced by TAG. In agreement with the cell culture studies, we found that an intact J domain is required to reduce the p130 protein levels in villus enterocytes (Fig. 4). Our gel shift analysis indicated the presence of intact p130-E2F4 complexes in TAG^{D44N} mice that were completely absent in TAG^{wt} mice (Fig. 6). These observations strongly suggest that TAG requires a functional J domain to downregulate p130.

In contrast, TAG^{wt} is able to increase both the RNA and protein levels of p107 while TAG^{D44N} upregulates only protein

levels, suggesting that a functional J domain is required for the upregulation of the RNA levels. However, the increased p107 protein levels in the TAG^{D44N} intestines might be due to decreased turnover or degradation of the protein, resulting in protein stabilization. On the other hand, TAG^{wt} does not affect either the RNA or protein levels of pRb, though expression of TAG^{D44N} results in the accumulation of pRb protein, perhaps through alterations in the turnover rate. At this point, we do not know the details of either mechanism or if TAG requires interaction with other cellular factors to modify the pRb proteins.

A functional J domain is required for the induction of activator E2Fs. Transgenic mice expressing TAG^{wt} in villus enterocytes upregulate protein and mRNA levels of all activator E2Fs (E2F1, E2F2, and E2F3a) (Sáenz Robles et al., submitted). This activation in turn induces cell proliferation and results in intestinal hyperplasia. Consistent with the normal morphology observed for TAG^{D44N} mice, we did not find upregulation of E2F1 and found only a modest increase in E2F2 and E2F3a protein levels. The protein levels of E2F2 and E2F3a in TAG^{D44N} mice were higher than those in nontransgenic mice but significantly lower than those in the TAG^{wt} mice, and therefore they might be insufficient to induce enterocyte proliferation. Furthermore, we were unable to detect Rb-free E2F2 or E2F3a activity by gel shift analysis. Additionally, the transcripts of several E2F-targeted genes were unaffected, indicating no activation of the pathway (Fig. 7). These results indicate that the E2F2 and E2F3a protein found in TAG^{D44N} enterocytes is not active transcriptionally.

Loss of p130 alone is not sufficient to induce intestinal hyperplasia. Mice with null mutations in either *p107* or *p130* are viable and normal in certain genetic backgrounds, implying that loss of p130 or p107 alone is not sufficient to induce hyperplasia (5, 22). However, mice lacking both p107 and p130

die at birth (19, 20), suggesting that p107 and p130 perform overlapping functions. It has been shown that the inactivation of pRb in conjunction with either p130 or p107 in villus enterocytes is sufficient to cause intestinal hyperplasia (10). In agreement with these observations, our results show that loss of p130 is insufficient to induce intestinal hyperplasia. At present, we cannot assess the combined effects of a J domain and pRb in induction of hyperplasia because Rb^{-/-} mice are embryonic lethal by day 13.5 (4, 14, 21). Future studies will require a conditional Rb knockout in a TAG^{D44N} background. Similarly, elucidation of the role of p107 will require analysis of TAG^{D44N} mice in a p130^{+/-}; p107^{-/-} background. These goals represent future efforts in the lab.

Animal studies using various J-domain mutants suggest that the requirement for the J domain in transformation is cell-type and context specific. Although defective in cell culture, the J domain *d11135* mutant (lacking small t expression) specifically induced the transformation of T lymphocytes in mouse. However, the same mutant was not able to transform B lymphocytes, suggesting a cell-type-specific requirement of J domain for the transformation (36). Similarly, our study also suggests the requirement for the J domain in the induction of intestinal hyperplasia. At this point, we cannot exclude the role of small t antigen J domain in the transformation process. To our knowledge, only one other study has used a J domain mutant of TAG in another transgenic system (27). However, the requirement for the J domain alone in the development of tumors was not addressed.

Several possible explanations could contribute to the differences between the phenotype observed upon expression of TAG^{D44N} in cell culture and in our transgenic system. On one hand, the cell culture does not represent real *in vivo* conditions. Additionally, the MEFs used in most of the cell culture studies are derived from a tissue different from intestine. The differences observed between both experimental systems support the notion of tissue-specific requirements for the J domain. Since TAG encodes multiple transformation-related activities, the subset of TAG functions needed and the corresponding target proteins could differ among tissue and/or cell types.

In conclusion, our studies indicate how different consequences are produced upon the same oncogenic stimulation, depending on the system used, and state the need to evaluate molecular pathways in a cell-specific context. This is also the first animal study that shows the requirement for a J domain in the induction of E2F-dependent transcription.

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