JOURNAL OF VIROLOGY, Apr. 1996, p. 2378–2386 Vol. 70, No. 4
0022-538X/96/$04.00+0

BK Virus Large T Antigen: Interactions with the Retinoblastoma Family of Tumor Suppressor Proteins and Effects on Cellular Growth Control

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Received 24 October 1995/Accepted 22 December 1995

BK virus (BKV) is a polyomavirus which infects a large percentage of the human population. It is a potent transforming agent and is tumorigenic in rodents. BKV DNA has also been found in human brain, pancreatic islet, and urinary tract tumors, implicating this virus in neoplastic processes. BKV T antigen (TAg) is highly homologous to simian virus 40 TAg, particularly in regions required for mitogenic stimulation and binding to tumor suppressor proteins. The experiments presented in this report show that BKV TAg can bind the tumor suppressor protein p53. BKV TAg also has the ability to bind to members of the retinoblastoma (pRb) family of tumor suppressor proteins both in vivo and in vitro. However, these interactions are detected only when large amounts of total protein are used, because the levels of BKV TAg normally produced from viral promoter-enhancer elements are too low to bind a significant amount of the pRb family proteins in the cell. The low levels of BKV TAg produced by the viral promoter elements are sufficient to affect the levels and the phosphorylation patterns of these proteins and to induce serum-independent growth in these cells. Additional events, however, are required for full transformation. These data further support the notion that BKV TAg can affect cellular growth control mechanisms and may in fact be involved in neoplastic processes.

BK virus (BKV) is a member of the polyomavirus family that was originally isolated from the urine of a renal transplant patient (32). From 70 to 80% of the human population is infected with BKV in early childhood (17, 31, 64). BKV persists in the kidneys, where it remains in a latent state after the initial infection. However, upon immunosuppression of the host, BKV can be reactivated and is thought to be associated with hemorrhagic cystitis (1). BKV DNA has also been found in various human tumors including brain, pancreatic islet, and, most recently, urinary tract tumors, implicating the virus in tumorigenesis (11, 59, 77).

BKV shares a great deal of homology (69 to 75%) with other members of the polyomavirus family, including simian virus 40 (SV40) and JC virus (JCV) (28, 68, 72), although these viruses differ greatly in their host ranges and transforming efficiencies (16, 72). SV40 is the prototype member of the family and the most well characterized. SV40 lytically infects monkey cells and has been shown to be a potent transforming agent in rodents in vivo and in rodent cells in vitro (21, 54). JCV and BKV are both human viruses and are also tumorigenic in rodents. JCV has a more limited transforming ability than BKV, particularly in rodent cells (5, 12, 29, 42, 69, 74, 79, 82, 83). In addition, BKV has the ability to induce focus formation in human embryonic kidney cells when expressed alone and to induce full transformation when expressed in the presence of an activated *ras* oncogene (66, 70, 76). The widespread presence of known transforming viruses in the human population and the particular associations of these viruses with various human tumors underscore the importance of a greater understanding of the molecular basis of their transforming potential.

Transformation by BKV, like that by SV40, has been shown to require the viral large T-antigen (TAg) protein (62, 66; reviewed in references 27 and 68). The TAg proteins of these two viruses share more than 75% amino acid homology (72). SV40 TAg has been shown to exert its role in transformation for the most part by binding to and presumably inactivating cellular tumor suppressor proteins including p53, pRb, p107, and p130. p53 binds near the carboxy terminus of SV40 TAg, a region previously shown to be important for mitogenic stimulation and transformation (67). p53 functions to regulate the G_1-to-S phase transition, particularly in response to DNA damage (47, 49, 55). By binding p53, therefore, SV40 TAg is able to override a key cell cycle checkpoint. pRb, the product of the retinoblastoma susceptibility gene, and its related family members, p107 and p130, all bind to a common region of TAg termed the CR2 domain on the basis of its homology to the adenovirus E1A conserved region 2 (10, 14, 20, 24, 35, 53, 57, 86). This domain encompasses amino acids 101 to 114 of SV40 TAg and includes an LXCXE motif shown by mutational analysis to be absolutely required for binding these pRb family members (14, 19, 24, 26, 60, 86).

Several lines of evidence point to the importance of the retinoblastoma family of proteins in cell cycle regulation. First, overexpression of pRb, p107, or p130 can induce growth arrest (34, 61, 80, 86, 87). Second, DNA tumor virus oncoproteins such as SV40 TAg, human papillomavirus E7, and adenovirus E1A specifically bind the pRb family of proteins (3, 7, 22, 53, 85). Viral DNA replication during infection by these viruses requires stimulation of cellular DNA synthesis. These viruses specifically bind cell cycle regulatory proteins such as pRb, p107, and p130 to subvert normal control of cell growth and allow rapid progression into the DNA synthesis phase of the cell cycle. Third, the gene encoding pRb is mutated in a variety of human cancers (40, 52). Mutations in the p107 and p130 genes have not been associated with human cancers; a possible redundancy of function of these two proteins has been suggested as an explanation for the lack of such mutations in these

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genes (84). Additionally, the interactions recently described between members of the retinoblastoma family and members of the cyclin/cdk complex families provide even stronger evidence for a role for pRb, p107, and p130 in cell cycle control (4, 18, 23, 35, 39, 41, 43, 48, 53, 58). The specific mechanism(s) for cell cycle control by the pRb family of proteins is not known but appears to involve upstream regulation by cyclin/cdk complexes and downstream functional regulation of the E2F family of transcription factors (2, 73; reviewed in reference 63). pRb, the prototype member of the family, has been studied extensively and can be used as a model for the role of the pRb family of proteins in cell cycle control. Early in G_1, pRb is in a hypophosphorylated form and is bound to members of the E2F transcription factor family (8). pRb has been shown to inhibit E2F-dependent transcriptional activation when complexed with E2F (2, 37, 38, 45, 63, 71). After undergoing phosphorylation, which occurs in a cell cycle-dependent manner and is regulated by cyclin/cdk complexes, pRb releases E2F, leaving the transcription factor free to activate genes required for entry into S phase (4, 13, 25, 39, 41; reviewed in reference 84).

SV40 TAg binds only to the hypophosphorylated or functionally active form of pRb, increasing the amount of excess free E2F independent of the stage of the cell cycle (14, 57). By binding to and activating pRb and its related family members, SV40 TAg is able to induce cellular DNA synthesis, thus providing the machinery required for viral DNA synthesis in the permissive host. Through this and other mechanisms, TAg is also able to induce cellular proliferation and transformation in the nonpermissive host cell, in which normal viral replication is blocked. Given the importance of interactions between SV40 TAg and these tumor suppressor proteins, we wanted to determine if such interactions were also occurring with the BKV large TAg. Although BKV TAg-pRb complexes have been detected with in vitro-translated pRb (19), we wished to examine the interaction in vivo and extend the analysis to the other retinoblastoma protein family members.

Our results indicate that BKV TAg is able to bind all three members of the retinoblastoma family of proteins both in vivo and in vitro. At physiological levels of BKV TAg expression, however, most of these three proteins in the cell are not complexed with BKV TAg. Nonetheless, the same amount of BKV TAg is able to bind most of the p53 in the cell. We also found that at the levels normally produced by the virus, BKV TAg is able to induce serum-independent growth but unable to fully transform cells. These results imply that BKV TAg is affecting cellular growth control through direct interactions with critical regulatory proteins but that an additional event(s), probably including increased expression of BKV TAg, is required for full transformation.

MATERIALS AND METHODS

Cell culture. All cell lines were maintained in Dulbecco's modified Eagle's Medium (GIBCO), supplemented with 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% fetal bovine serum, at 37°C in a 5% CO_2 incubator. BSC-1 and CV-1 are African green monkey kidney cells. COS-1 cells are CV-1 cells expressing the early region of SV40 (33). BSC-1, CV-1, and COS-1 cells were obtained from the American Type Culture Collection.

Plasmids. pBK-Neo was constructed by cloning the entire early region of BKV (Dun) (72) as a *Pvu*II fragment (3.2 kb) into the *Eco*RI site of pSV2neo (75). Plasmids encoding GST-pRb and GST-p107 were both kindly provided by J. Nevins. GST-pRb contains nucleotides encoding amino acids 379 to 928 of pRb cloned into the pGEX2T vector (46). GST-p107 contains nucleotides encoding amino acids 255 to 1068 of p107 cloned into the pGEX2TK vector (23).

Transfections. The BSC-BKT cell line was established by transfection of BSC-1 cells with pBK-Neo by the calcium phosphate method as previously described (44). DNA (20 μg) was added to subconfluent cells in 100-mm-diameter dishes (10 μg of test plasmid, 10 μg of pGEM3zf−). At 24 h posttransfection, cells were fed with regular growth medium, and at 48 h posttransfection, the cells were split 1:5 into dishes for drug selection in regular growth medium plus 500 μg of G418 (GIBCO) per ml. Foci were picked and screened for clonal cell lines stably expressing BKV TAg.

Soft agar assays. Cells were tested for the ability to form colonies in soft agar, as previously described (6), in 60-mm dishes with a 7-ml base layer of 0.5% agarose (Boehringer low EEO) and a 1.5-ml overlayer of 0.33% agarose containing 5,000 or 25,000 cells. Dishes were scored for colony formation after 26 days at 37°C.

Growth curves. Cells (2×10^4) were seeded into 2-cm^2 wells of a 24-well plate containing medium plus 10% fetal bovine serum. At 24 h after plating, the medium was changed to 0.1 or 10% as indicated. Cells from duplicate wells were counted every other day for 10 days. Medium was replaced on alternate days.

Metabolic labeling and cellular lysate preparation. Cells were labeled at 70 to 80% confluence in 10-cm^2 dishes at 37°C. For ^{32}P labeling, dishes were washed once with phosphate-buffered saline (PBS) and then incubated overnight at 37°C in 2.5 ml of labeling medium (Dulbecco's modified Eagle's medium lacking phosphate plus 10% fetal bovine serum) plus 500 μCi of $^{32}P_i$ (9,000 Ci/mmol; NEN). After being labeled, the cells were washed twice with ice-cold PBS and then incubated in 1 ml of ice-cold lysis buffer (50 mM Tris [pH 7.5], 250 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 5 μg of phenylmethylsulfonyl fluoride per ml, 5 μg of aprotinin per ml, 5 μg of leupeptin per ml, 50 mM sodium fluoride, 0.1 mM sodium orthovanadate) for 30 min on ice. Lysates were transferred to microcentrifuge tubes, and cell debris was cleared by centrifugation for 5 min at 4°C. Supernatants not used immediately were stored at −80°C.

Unlabeled lysates were prepared from subconfluent cells by washing twice with PBS and then incubating for 30 min in 1 ml of either lysis buffer or E1A lysis buffer (ELB; 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.0], 250 mM NaCl, 0.1% Nonidet P-40, 5 μg of phenylmethylsulfonyl fluoride per ml, 5 μg of aprotinin per ml, 5 μg of leupeptin per ml, 50 mM sodium fluoride, 0.2 mM sodium orthovanadate). Lysates were transferred to microcentrifuge tubes, and cell debris was cleared by centrifugation for 5 min at 4°C.

Antibodies. Antibodies used were monoclonal antibodies PAb 416 and PAb 430 (anti-TAg monoclonal antibodies [36]), Ab-1 (anti-p53 monoclonal antibody [Oncogene Science]), Rb Ab-1 and Rb Ab-4 (anti-pRb monoclonal antibodies [Oncogene Science]), C-18 (anti-p107 polyclonal antibody [Santa Cruz Biotechnology]), and C-20 (anti-p130 polyclonal antibody [Santa Cruz Biotechnology]).

Immunoprecipitations. For ^{32}P-labeled samples, lysates were precleared with 250 μl of 3% protein A-Sepharose beads (Pharmacia) in lysis buffer and then incubated with primary antibody for 60 min on ice. Unlabeled lysates were incubated with primary antibody without preclearing. All samples were then incubated with 125 μl of 3% protein A-Sepharose beads in lysis buffer with rocking at 4°C for 60 to 90 min. Immune complexes bound to the beads were washed three times with lysis buffer and released into gel-loading buffer (2% sodium dodecyl sulfate [SDS], 60 mM Tris [pH 6.8], 10% glycerol, 0.1% bromophenol blue, 292 mM β-mercaptoethanol) by boiling for 3 min. Samples were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) with 8% polyacrylamide (50). Gels containing unlabeled samples were subjected to immunoblotting as described below. Gels containing ^{32}P-labeled samples were dried and exposed to film.

Immunoblotting. Following SDS-PAGE, proteins were transferred to nitrocellulose in 50 mM Tris–40 mM glycine–0.04% SDS–20% methanol, adjusted to pH 8.31 with acetic acid. Transfer took place for 90 min at 63 V and 16°C. After transfer, the filter was placed in a blocking solution of 5% nonfat dried milk in PBS-T (PBS plus 0.1% Tween 20) for 1 h at room temperature with rocking. The filter was then incubated for 90 min at room temperature with rocking in blocking solution containing the primary antibody, washed three times in PBS-T, and incubated with the secondary antibody (horseradish peroxidase linked [Amersham]) for 1 h at room temperature with rocking. The filter was washed three times in PBS-T and then developed with enhanced chemiluminescence reagents (Amersham). For reprobing, filters were first stripped in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris [pH 6.7]) at 50°C for 30 min and then probed as described above.

In vitro binding assays. GST-pRb and GST-p107 were produced in *Escherichia coli* BL21, while glutathione *S*-transferase (GST) was produced in strain DH5α. Bacteria were grown overnight at 37°C, diluted 1:10, and grown for 2 h. Expression was induced by adding 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and growing for an additional 2 h. Bacteria were pelleted by centrifuging at 2,600 × *g* for 10 min at room temperature and resuspended in 1 ml of PBS. Bacteria were sonicated in microcentrifuge tubes, and sonicates were cleared of debris by centrifuging for 5 min at 4°C. Sonicates were incubated with glutathione-Sepharose 4B (Pharmacia) for 30 min with rocking and then washed three times in PBS and twice in ELB. Indicated amounts of cellular lysates were then added to the purified fusion protein and diluted to 0.5 ml in ELB. Samples were rocked at 4°C for 90 min, washed three times in ELB, and released in gel-loading buffer by boiling for 3 min. The samples were resolved by SDS-PAGE (8% polyacrylamide) and analyzed by immunoblotting as described above.

RESULTS

SV40 TAg interacts with and functionally inactivates tumor suppressor proteins including p53, pRb, and the pRb family members p107 and p130 (7, 14, 20, 24, 35, 53, 86). Inactivation of these growth control proteins is important for viral replication and transformation. The presence of an intact LXCXE motif in the amino terminus of SV40 TAg is required for binding to the pRb family of proteins (14, 19, 24, 35, 60). BKV TAg not only has high overall homology to SV40 TAg but also contains an LXCXE motif (72). Previous binding studies with extracts from mouse cells transfected with BKV and in vitro-translated pRb have shown that BKV TAg can interact with pRb in vitro (19). Given the prevalence of BKV in the human population and the high degree of homology between SV40 TAg and BKV TAg, particularly in the pRb-binding domain, our initial aim was to determine if BKV TAg had the ability to bind members of the pRb family of proteins in vivo. These experiments should help us to understand what effect a known transforming virus present in the human population may have on cell proliferation and cell cycle regulation.

Our requirement for an easily transfectable, nontransformed kidney cell line with wild-type pRb and p53 led us to use the monkey kidney fibroblast cell line BSC-1. For our studies, we made a stably transfected cell line (BSC-BKT) containing the early region of BKV encoding both large TAg and small t antigen. To begin to further understand the interactions of BKV TAg with cell cycle regulatory proteins, metabolic labeling and immunoprecipitation experiments were performed. Using ^{32}P-labeled extracts, we found that BKV TAg forms complexes with the tumor suppressor protein p53. BKV TAg binds most of the phosphorylated p53 present in the cell, as can be determined by comparing the amount of p53 in the anti-BKV TAg immunoprecipitate with the amount of p53 present in the anti-p53 immunoprecipitate (Fig. 1). A similar conclusion was reached by Bollag et al. using ^{35}S labeling (5). However, a small amount of p53 remains in the supernatant after immunoprecipitation of BKV TAg (data not shown), indicating that some p53 remains free, as is the case with SV40 TAg (15).

To analyze interactions with the retinoblastoma family proteins in vivo, we performed immunoprecipitations with equivalent amounts of total protein from each cell line followed by immunoblotting to determine if complexes were present in cell lysates. Under these conditions, we were able to detect complexes of pRb and SV40 TAg. However, using equivalent amounts of total lysate from BSC-BKT cells, we were unable to detect complexes of pRb and BKV TAg, a result which was obtained repeatedly under a variety of lysis and immunoprecipitation conditions (data not shown).

Probing parallel blots with antibodies to TAg revealed a large difference in the amounts of BKV TAg and SV40 TAg present in equivalent amounts of total cellular protein (for example, see Fig. 3B, lanes 1 and 3). Thus, the inability to detect pRb in the BKV TAg immunoprecipitations could be due to the relatively low levels of BKV TAg. This difference in TAg levels has been reported previously and is attributed to the lower expression and stability of BKV TAg (5). To determine the approximate difference in the amount of TAg protein, we performed immunoblotting experiments for TAg in whole-cell lysate samples and found that the level of SV40 TAg in COS-1 cells was 50 to 100 times higher than the level of BKV TAg in BSC-BKT cells. With this difference in TAg expression in mind, we repeated the experiments with equivalent amounts of TAg protein instead of equivalent amounts of total cellular protein (Fig. 2). Again, pRb was detected in an anti-SV40 TAg immunoprecipitate (Fig. 2A, lane 6). With 50 times more total protein (7,500 μg) from BSC-BKT cells to account for the difference in TAg levels, pRb was detected in the anti-BKV TAg immunoprecipitate (Fig. 2A, lane 9). We also found the converse to be true: with 7,500 μg of BSC-BKT lysate, we could detect BKV TAg in an anti-pRb immunoprecipitate (Fig. 2B, lane 5) at levels approximately equivalent to those of the SV40 TAg detected in an anti-pRb immunoprecipitation of 150 μg of COS-1 total protein (Fig. 2B, lane 3). Thus, both TAg proteins are equally capable of binding pRb.

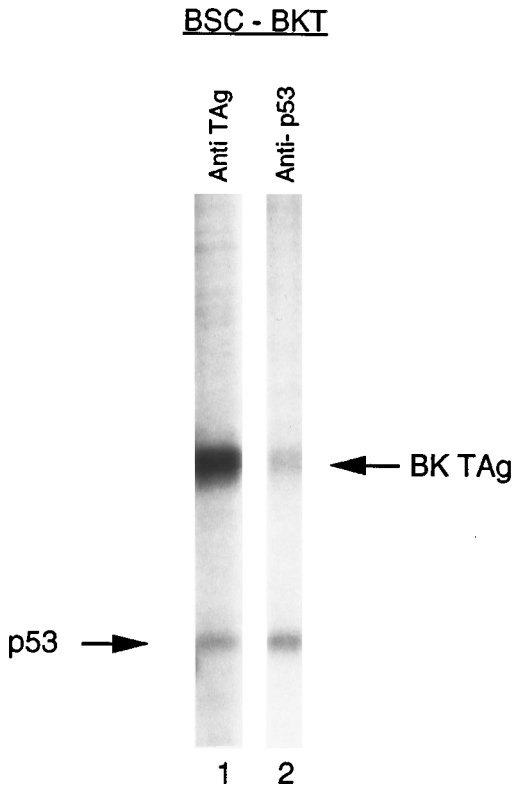

FIG. 1. Analysis of BKV TAg-p53 complexes. ^{32}P-labeled proteins from BSC-BKT cells were precipitated with the indicated antibodies and separated by SDS-PAGE. Both lanes are the same exposure of the same gel.

We next wished to examine interactions between BKV TAg and the other members of the retinoblastoma protein family. With 50 times more total protein from BSC-BKT cells to account for the difference in TAg expression, we found that as with pRb, BKV TAg could form complexes with p107 (Fig. 3A). Similarly, immunoprecipitation of p107 followed by immunoblotting for TAg showed that BKV TAg was present in the p107 immunoprecipitates when 50 times more total protein from BSC-BKT cells were used (Fig. 3B). We were limited in our analysis of BKV TAg interactions with p130 by the lack of anti-p130 antibodies available for use in immunoprecipitations. However, we were able to detect complexes by immunoprecipitating for TAg and immunoblotting for p130 (Fig. 4). Again, we found that BKV TAg-p130 complexes were detectable only when 50 times more total protein was used from BSC-BKT cells than from COS-1 cells (compare lanes 5 and 7).

In these studies, we also noted that the total level of pRb, p107, and p130 was decreased in the cell lines expressing TAg compared with the parental cell lines and that most of the remaining pRb, p107, and p130 was of a faster-migrating form (Fig. 2A, whole-cell lysates; Fig. 3C; and Fig. 4, whole-cell

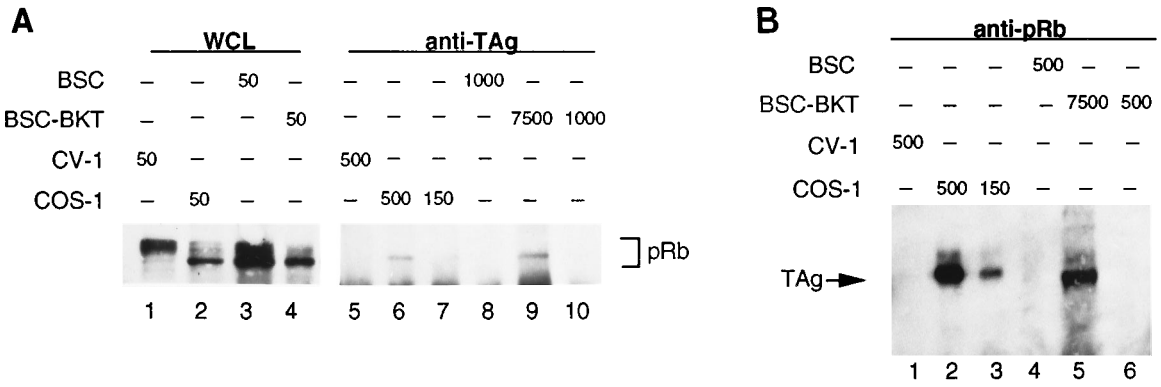

FIG. 2. Analysis of BKV TAg-pRb complexes. (A) Whole-cell lysates (WCL) or lysates immunoprecipitated with monoclonal antibody PAb 430 (anti-TAg) were separated by SDS-PAGE, transferred to nitrocellulose, and probed with Rb Ab-1. (B) Lysates immunoprecipitated with Rb Ab-1 (anti-pRb) were separated by SDS-PAGE, transferred to nitrocellulose, and probed with PAb 416. The corresponding whole-cell lysate samples can be seen in Fig. 3B, which shows portions of the same gel. For each panel, the numbers above the lanes indicate the amount of lysate used (micrograms).

lysates). This change cannot be attributed to a difference in the percentage of cells in the G_1 phase of the cell cycle, because flow cytometry analysis of cells harvested at the same time that the lysates were prepared indicates that there is no apparent difference between the cell cycle distribution of the TAg-expressing cells and the parental cells (data not shown).

To confirm our in vivo results and to analyze interactions of BKV TAg with human retinoblastoma family proteins, we used GST fusion proteins of human pRb and p107 to assess complex formation in vitro (Fig. 5). To normalize for the amount of TAg in the lysates, we used 100 times more total protein from BSC-BKT cells than from COS-1 cells. Using a purified GST-pRb fusion protein encoding the carboxy-terminal pocket domain of human pRb required for interactions with the TAg and E1A proteins (amino acids 379 to 928), we were able to detect equivalent complex formation between GST-pRb and both SV40 and BKV TAgs (lanes 5 and 6). These data are in agreement with previous data for BKV and SV40 TAg binding to in vitro-translated pRb (19). Each TAg is also able to bind equivalent amounts of a GST-p107 fusion protein encoding the carboxy-terminal pocket region of the human p107 protein (amino acids 255 to 1068) (lanes 8 and 9). Neither TAg interacts with the GST protein alone, indicating that TAg complex formation is specific (lanes 11 and 12). When these experiments were performed without normalization for TAg levels (i.e., with equivalent amounts of total protein), the interaction between BKV TAg and pRb or p107 was barely detectable (data not shown). These results support our in vivo findings that the BKV TAg must be present in amounts approximately equivalent to those of SV40 TAg to bind equal amounts of these cellular proteins, and they also indicate that there is no detectable difference between monkey and human pRb and p107 with respect to their interactions with BKV TAg.

The comparative binding studies presented here have shown that BKV TAg has the ability to bind the tumor suppressor proteins p53, pRb, p107, and p130. Normal physiological levels of BKV TAg expressed in the cell are sufficient to bind p53 but are too low to bind significant amounts of the pRb family of tumor suppressor proteins present in the cell. Thus, we were interested in examining the growth effects of BKV TAg in these cells to determine if it, like SV40 TAg, is able to drive uncontrolled proliferation. To examine this, we first set up growth assays in which equal numbers of cells of each TAg-expressing cell line and its matching parental cell line were seeded in medium containing 10 or 0.1% serum and counted every other day (Fig. 6). The COS-1 cells grew under both

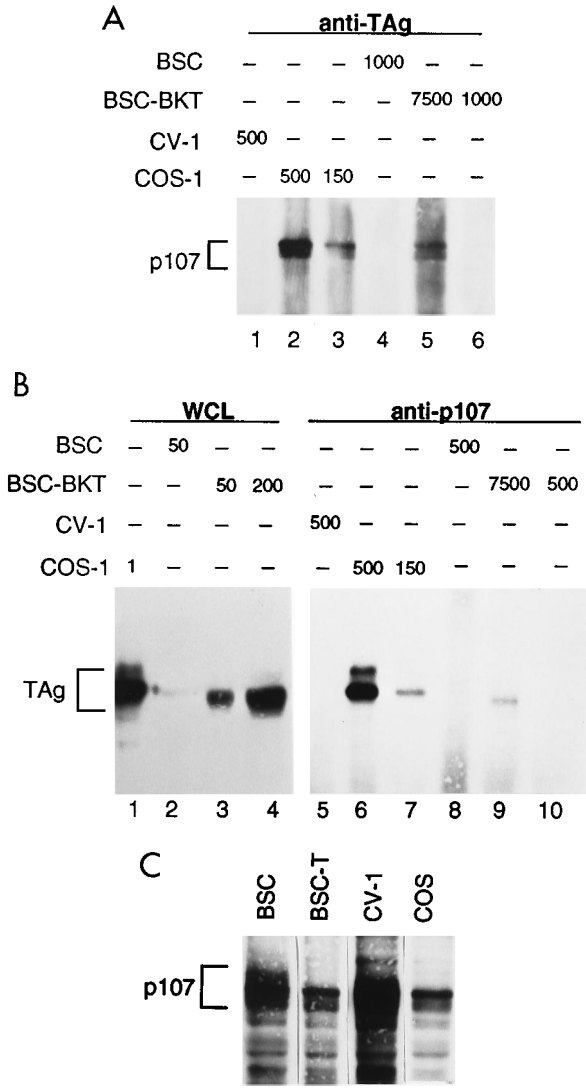

FIG. 3. Analysis of BKV TAg-p107 complexes. (A) The nitrocellulose filter for Fig. 2A was reprobed with C-18. (B) Whole-cell lysates (WCL) or lysates precipitated with C-18 were separated by SDS-PAGE, transferred to nitrocellulose, and probed with monoclonal antibody PAb 416. The faint band in lane 2 is due to spillover from lane 1. The numbers above the lanes indicate the amount of lysate used (in micrograms). (C) Whole-cell lysates (50 μg each) from each cell line were probed with C-18. BSC-T indicates BSC-BKT cells.

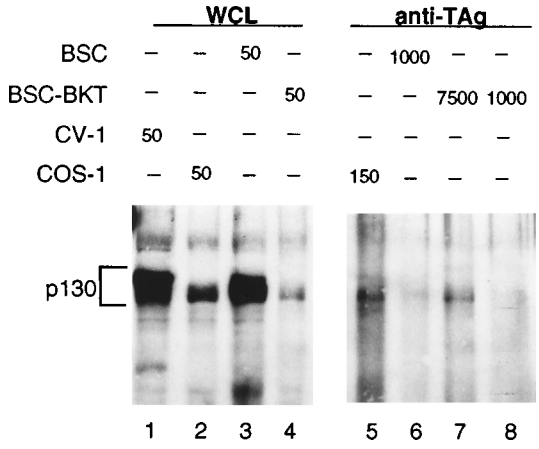

FIG. 4. Analysis of BKV TAg-p130 complexes. The nitrocellulose filter used for Fig. 2A was reprobed with C-20. Numbers above the lanes indicate the amount of lysate used (in micrograms).

conditions. The BSC-BKT cells were also able to grow in both 10 and 0.1% serum, although the peak amount of growth in 0.1% serum lagged slightly behind that of COS-1 cells. In contrast, the BSC-1 and CV-1 parental cells were able to grow only in 10% serum. These results indicate that BKV TAg as well as SV40 TAg is able to induce serum-independent growth. Finally, to determine if BKV TAg was able to fully transform cells and allow for anchorage-independent growth, we performed soft agar assays on both the TAg-expressing cell lines and their parental cell lines (Fig. 7; Table 1). Only the COS-1 cells showed colony formation in soft agar, while neither the parental cell lines (CV-1 and BSC-1) nor the BSC-BKT cells displayed the fully transformed phenotype. These results indicate that at these levels of expression, the BKV TAg does not have the same ability as SV40 TAg to fully transform cells, as measured by growth in soft agar.

DISCUSSION

The experiments described in this paper address the potential role of BKV TAg in cellular transformation, specifically by examining its interactions with the tumor suppressor proteins p53, pRb, p107, and p130. BKV is present in a majority of the human population and has been associated with various human tumors. Its role as a potent transforming agent in rodent cells and the high degree of homology between BKV TAg and SV40 TAg, which is known to bind key cell cycle regulatory proteins, led us to these studies.

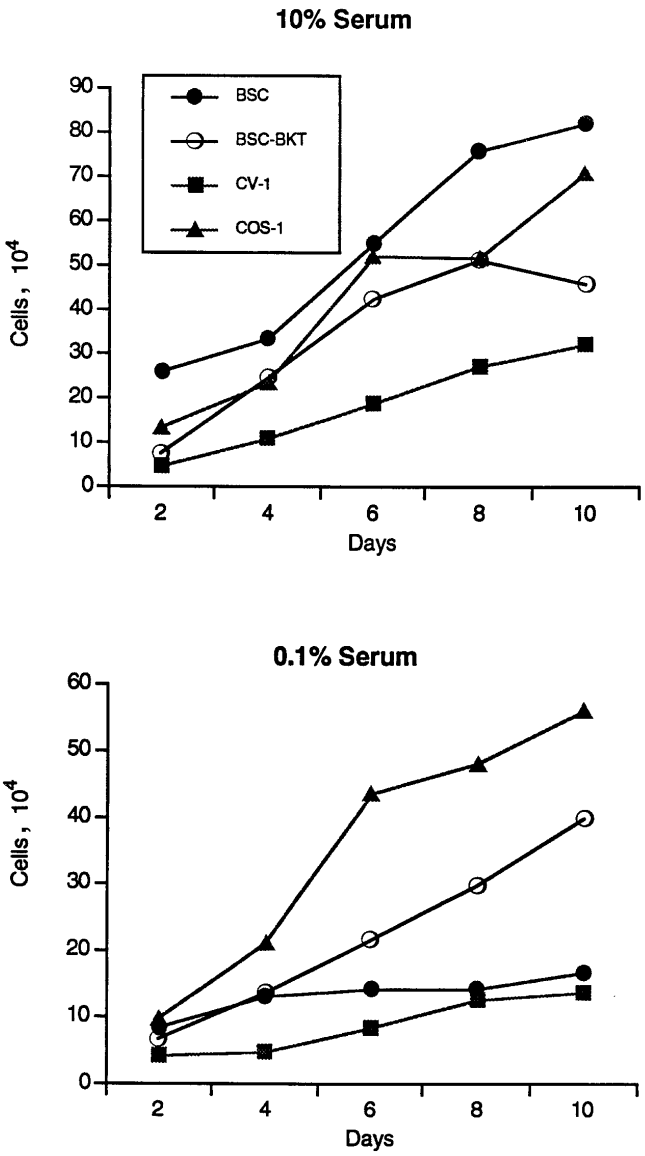

FIG. 6. Growth in serum-independent medium. For each cell line, 2×10^4 cells were grown in medium containing 10 or 0.1% serum. Cells from duplicate wells were counted every other day, and the mean number is shown.

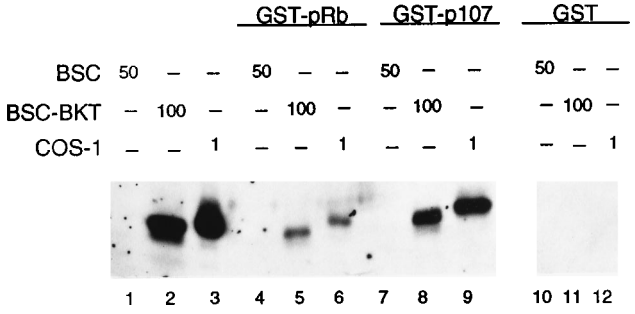

FIG. 5. Analysis of in vitro complex formation. GST-pRb, GST-p107, and GST were purified as described in Materials and Methods. Indicated amounts of cell lysates were added to equivalent amounts of each glutathione-Sepharose-bound fusion protein. Bound complexes were released, separated by SDS-PAGE, transferred to nitrocellulose, and probed with monoclonal antibody PAb 416. Lanes 1 to 3 contain whole-cell lysate samples for each cell line. Numbers above the lanes indicate the amount of lysate used (in micrograms).

Our results show that BKV TAg can bind most of the p53 present in the cell, as has been reported previously (5). This implies that BKV TAg, at normal, physiologic levels can potentially interfere with p53-mediated pathways. The two major relevant functions of p53 are induction of G_1 arrest and apoptosis in response to DNA damage, both of which are critical to the ability of a cell to respond to external and internal stimuli and prevent accumulation of potentially deleterious mutations (9, 47, 49, 51, 55, 56). The fact that this virus may be inactivating a key cell cycle regulator again emphasizes the importance of gaining a better understanding of it.

BKV TAg also has the ability to bind to the retinoblastoma family of proteins, pRb, p107, and p130, both in vivo and in

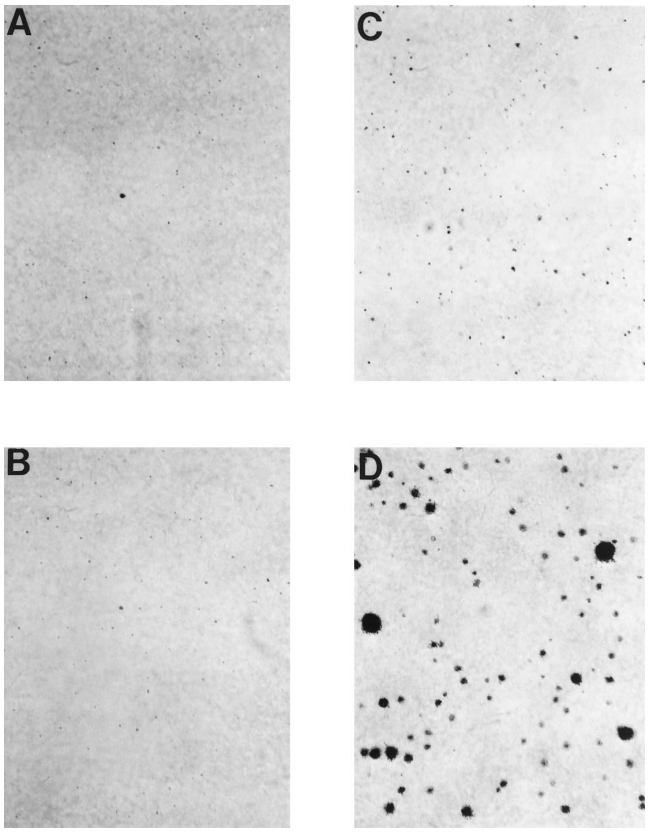

FIG. 7. Anchorage-independent growth. Shown are photomicrographs of BSC-1 (A), BSC-BKT (B), CV-1 (C), and COS-1 (D) cells plated in soft agar.

vitro. All three of these proteins have previously been shown to bind SV40 TAg (14, 20, 24, 35, 57, 86). The major difference here is that detection of BKV TAg interactions with pRb, p107, and p130 requires the use of large amounts of cellular lysates to normalize the TAg levels from each virus. Only when we used 50 to 100 times the amount of lysate used for SV40 TAg could we detect binding of BKV TAg to any of the pRb family members. We found this to be true in both our in vivo studies with monkey cell lysates and our in vitro studies with GST fusion proteins of the human retinoblastoma family members, suggesting that the interactions between BKV TAg and both monkey and human pRb family members are the same. Previous studies indicate that BKV TAg is normally expressed at low levels in a variety of host cells, including human embryonic kidney cells (5, 62, 66, 74, 76). In addition, BKV DNA levels in normal tissue surrounding urinary tract tumors are lower than in the tumors themselves (59). Theoretically, increased levels of BKV TAg in human kidney cells would lead to viral replication and cell lysis or, potentially, transformation. Since BKV can remain latent in kidney tissue without having apparent cytopathic effects or transformation, we would predict that the low levels we obtain in monkey kidney cells are in fact reflective of a persistent infection of human kidneys.

The fact that there is little BKV TAg in the cells expressing the BKV early region implies that only a very small fraction of pRb, p107, and p130 is bound and inactivated. We do find that in spite of the difference in TAg expression, both SV40 TAg and BKV TAg seem to be affecting the overall mobility patterns of pRb, p107, and p130. In the presence of either TAg a greater percentage of these proteins is in the underphosphorylated, faster-migrating forms, indicating that although BKV TAg is present at a much lower level, it is having some similar effects on the cell to those of SV40 TAg. Our results differ from those of Ludlow et al. (57), which indicate that SV40 TAg does not affect phosphorylation patterns of pRb; we cannot explain this discrepancy. Nonetheless, for BKV TAg, we would argue that these effects are indirect and not mediated through direct binding. We find it interesting that both TAg proteins are inducing expression of the growth-suppressive forms of the pRb family of proteins yet simultaneously inducing proliferation and growth. For SV40 TAg, this can be explained by its ability to bind most if not all of the growth-suppressive forms of these proteins and inactivate them in this way. However, we have shown that binding of these proteins is not extensive in the presence of low levels of BKV TAg. Therefore, unless this low level of inactivation is sufficient, BKV TAg must be affecting proliferation by other mechanisms.

The difference in TAg expression may be due to lower levels of expression from the BKV promoter and enhancer elements or to decreased stability of the BKV TAg protein compared with SV40 TAg (5, 16). In fact, the greatest sequence divergence between the two viruses is found in the early-region promoter and enhancer elements (40% homology), possibly accounting for the difference in expression levels (16, 72). The question, then, is whether the interactions found with the normal, low-level expression of BKV TAg are in fact physiologically significant. To address this question, we performed studies on the growth-stimulatory abilities of the BKV TAg. Our results show that BKV TAg can induce serum-independent growth equivalent to that induced by SV40 TAg. In contrast, BKV TAg cannot induce anchorage-independent growth whereas SV40 TAg can. These results imply that BKV TAg is altering normal growth response signals by allowing for growth in low-serum media. One possible explanation for this result is that the TAg-p53 interaction is sufficient to induce serum-independent growth. If BKV and SV40 TAgs are truly functionally homologous, this finding is not in agreement with the results of Thompson et al. for SV40 TAg, which showed that a mutant SV40 TAg encoding an intact p53-binding domain was not sufficient to induce serum-independent growth (78). A mutational analysis will be required to determine which BKV TAg domain(s) is involved in serum-independent growth. It appears, however, that higher levels of interaction with the pRb family members may be required for full transformation. This could be accomplished by increased expression of BKV TAg. In support of this model, it has previously been shown that integration of early-region sequences of the virus allows for the difference between growth in low-serum media and full transformation in BKV-infected human embryonic kidney cells

TABLE 1. Colony formation by BKV and SV40 TAgs in soft agar

Cell line	No. of cells plated	No. of agar colonies[a]
CV-1	5,000	1, 1
	25,000	0, 0
COS-1	5,000	87, 84
	25,000	256, 216
BSC	5,000	2, 0
	25,000	0, 0
BSC-BKT	5,000	1, 0
	25,000	0, 3

[a] Colonies per dish. Data are from duplicate dishes.

(70). This integration event could result in higher expression of BKV TAg by using nearby cellular promoter-enhancer elements. Alternatively, mutations and alterations in the viral promoter-enhancer elements could lead to increased transforming activity (83). Additionally, mutations in the JCV enhancer are commonly found in viruses isolated from tissue from patients with progressive multifocal leukoencephalopathy associated with JCV infection, as opposed to nonclinical isolates (30). Full transformation occurring as a result of increased expression of BKV TAg is in agreement with the results of Trowbridge and Frisque showing that for full transformation by JCV, a threshold level of TAg expression must be reached (79). They showed that a construct containing the JCV TAg under the control of SV40 promoter-enhancer elements expressed higher levels of TAg and had more potent transforming ability. Support for this model also comes from the increased levels of BKV TAg RNA in the tumor tissues of transgenic mice compared with normal tissue samples (12, 74). It will be interesting to see if BKV TAg levels are increased in human tumors, as would be predicted by the model. Indeed, recent results from Monini et al. have shown that while BKV early-region DNA in neoplastic tissues from urinary tract tumors can be detected by Southern blot hybridization, detection in normal, surrounding tissue can be achieved only by a high-sensitivity PCR technique (59). Further study of the BKV TAg mRNA and protein levels in these cells is required to determine if increased DNA levels correlate with increased TAg expression.

Another possible model for the role of BKV TAg in oncogenesis would involve a first step in which BKV TAg binds to or otherwise inactivates tumor suppressor proteins and a second step involving activation of cellular oncogenes. This is supported by previous studies showing that human embryonic kidney cells persistently infected with BKV exhibited a semitransformed phenotype. In the presence of a second oncogene, activated Ha-*ras*, full transformation results (66, 70, 76). We would note that if BKV TAg is inhibiting p53 function, this could predispose the cell to a second hit by preventing DNA repair before entry into S phase. We would like to determine if the DNA damage response mediated by p53 is altered in the presence of BKV TAg.

Studies with SV40 TAg have shown that complex formation with tumor suppressor proteins results in their functional inactivation (7, 14, 20, 24, 35, 53, 86). The studies presented here have shown that complex formation of these proteins with BKV TAg is occurring at a low level; therefore, an assessment of functional inactivation is required. For example, it will be interesting to determine if the levels of free E2F are increased in the presence of low levels of BKV TAg, even though levels of hypophosphorylated pRb/p107/p130 are increased. The ability of BKV TAg to induce serum-independent growth indicates that the growth suppression function of these proteins might be impaired to some extent.

Given the prevalence of BKV in the human population and the high homology to another known pathogenic human virus, JCV, we believe that a better understanding of the functions of BKV TAg and the potentially pathogenic effects this protein may be having in the human host is imperative. During infection by JCV, the virus also remains in a latent form until it is activated in the immunosuppressed host, where it can then cause progressive multifocal leukoencephalopathy (65). The same activation event is probably occurring for BKV in the immunocompromised host (81). The increase in the immunocompromised population as a result of increased human immunodeficiency virus infections and organ transplants suggests that BKV may now affect an even larger population. Our results that BKV TAg can readily bind to p53, can bind to the retinoblastoma family of tumor suppressor proteins as well as affect their expression levels, and can induce serum-independent growth demonstrate the need for a greater understanding of the functional significance of these interactions as they relate to cellular proliferation and growth control in the human host.

ACKNOWLEDGMENTS

We thank the members of our laboratory for useful discussions and comments about this work; Joe Nevins for the GST fusion protein constructs; and Ed Harlow for hybridomas.

This work was supported by American Cancer Society grant VM-11A. K.F.H. was supported in part by NIH training grant 5-T32-GM07315 and the Nancy Newton-Loeb Foundation.

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