

Sirolimus and Other Mechanistic Target of Rapamycin Inhibitors Directly Activate Latent Pathogenic Human Polyomavirus Replication

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Background. Human polyomaviruses can reactivate in transplant patients, causing nephropathy, progressive multifocal leukoencephalopathy, Merkel cell carcinoma, pruritic, rash or trichodysplasia spinulosa. Sirolimus and related mechanistic target of rapamycin (mTOR) inhibitors are transplant immunosuppressants. It is unknown if they directly reactivate polyomavirus replication from latency beyond their general effects on immunosuppression.

Methods. In vitro expression and turnover of large T (LT) proteins from BK virus, JC virus (JCV), Merkel cell polyomavirus (MCV), human polyomavirus 7 (HPyV7), and trichodysplasia spinulosa polyomavirus (TSV) after drug treatment were determined by immunoblotting, proximity ligation, replicon DNA replication, and whole virus immunofluorescence assays.

Results. mTOR inhibition increased LT protein expression for all 5 pathogenic polyomaviruses tested. This correlated with LT stabilization, decrease in the S-phase kinase-associated protein 2 (Skp2) E3 ligase targeting these LT proteins for degradation, and increase in virus replication for JCV, MCV, TSV, and HPyV7. Treatment with sirolimus, but not the calcineurin inhibitor tacrolimus, at levels routinely achieved in patients, resulted in a dose-dependent increase in viral DNA replication for BKV, MCV, and HPyV7.

Conclusions. mTOR inhibitors, at therapeutic levels, directly activate polyomavirus replication through a Skp2-dependent mechanism, revealing a proteostatic latency mechanism common to polyomaviruses. Modifying existing drug regimens for transplant patients with polyomavirus-associated diseases may reduce symptomatic polyomavirus replication while maintaining allograft-sparing immunosuppression.

Keywords. human polyomavirus; large T antigen; latency; Skp2; mTOR inhibition; sirolimus; tacrolimus; MCPyV; BKV; proteostasis.

There are at least 11 human polyomaviruses (HPyVs), 5 of which cause established diseases, especially among immunosuppressed transplant recipients. HPyVs are small, double-stranded circular DNA viruses [1]. Each of these viruses is near-ubiquitous with most HPyVs infecting > 50% of adults as lifelong infections [2, 3]. Little is known about the mechanism for how these viruses remain latent in their hosts and reactivate from latency.

The 5 pathogenic HPyVs (BK virus [BKV], JC virus [JCV], Merkel cell polyomavirus [MCV], trichodysplasia spinulosa

polyomavirus [TSV], and human polyomavirus 7 [HPyV7]) cause disease through uncontrolled replication [4, 5]. BKV, JCV, HPyV7, and TSV infections are associated with kidney nephropathy, progressive multifocal encephalopathy, hyperkeratosis, and trichodysplasia spinulosa, respectively, in transplant recipients [6]. MCV, causing about approximately 80% of Merkel cell carcinomas, leads to cancer if the virus genome fragments and integrates into the host cell during virus replication [7, 8]. Immune surveillance plays a key role in controlling polyomavirus burden since polyomaviral replication is markedly increased in immunosuppressed transplant patients prior to onset of clinical disease [9–11]. Immune surveillance control is also evident for people with multiple sclerosis treated with the immunomodulator natalizumab, who become uniquely at risk for progressive multifocal leukoencephalopathy due to JCV replication [12].

It is not known whether drug therapies enhance polyomavirus replication beyond general effects on immunosuppression. Both mechanistic target of rapamycin (mTOR) and calcineurin inhibitors are widely used as immunosuppressants following organ transplantation [13, 14]. Therapeutic serum levels for long-term graft survival for sirolimus (rapamycin) and tacrolimus

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(FK506) range from 5 to 10 ng/mL (4.4–22 nM) and 5 to 20 ng/mL (6–24 nM), respectively [15, 16]. Merkel cell carcinoma risk increases 1.9-fold among transplant patients on mTOR inhibitors whereas this risk is significantly reduced for those on tacrolimus therapies [17]. In contrast, risk for BK nephritis has not been associated with mTOR inhibitor therapy [18].

Little was known until recently about how polyomaviruses evade immune responses to become lifelong, latent infections. Viral latency is an immune evasion strategy allowing long-term persistence with reduced viral antigen expression that might provoke sterilizing immunity. For herpesviruses and retroviruses, viral latency depends on repression of transcriptional activators that control messenger RNA transcription needed to generate proteins required for viral replication.

MCV latency relies on a different strategy. Like all polyomaviruses, MCV has a single DNA replication protein called large T antigen (LT) [19]. Accumulation of LT in the infected cell results in LT helicase multimerization on the viral DNA origin to initiate replication. The LT protein, however, also encodes specific phospho-peptide motifs recognized by cellular Skp-Cullin-Fbox (SCF) E3 ligases that continuously degrade LT, reducing its accumulation to levels below that required for functional assembly on the viral DNA [20]. Since polyomavirus reactivation is regulated by protein levels rather than RNA transcription, we refer to this as protein-mediated or proteostatic latency. Typically, MCV defaults to an immune evasive latency program after infecting host cells, which may contribute to its lifelong, low-level, persistent infection. If, however, specific cellular SCF pathways are turned off, as occurs during nutrient deprivation or inflammation, MCV LT protein levels will rise so that virus replication is initiated, allowing escape to a new host. In addition to LT proteostasis, MCV encodes microRNAs that feedback to inhibit LT expression [21, 22] and LT protein itself is a potent autorepressor of its own promoter [20]. These strategies reveal that this virus has evolved multiple, tightly controlled mechanisms to suppress LT protein expression until needed for activated virus replication and transmission.

Several cellular SCF E3 ligases recognition motifs on MCV LT, including for Skp2 (S-phase kinase-associated protein 2), Fbw7 (F-box/WD repeat-containing protein 7), and β -TrCP (β -transducin repeat-containing protein), have been mapped to MCV LT phosphorylation sites [20]. Notably, phosphoinositol-3-kinase (PI3K) Akt-mTOR inhibitors promote MCV replication by inhibiting the Skp2 E3 ligase, which targets phospho-serine 220 on the MCV LT molecule [20]. It is not known if other HPyVs are affected similarly.

Our study shows that mTOR inhibitors increase expression of LT proteins for all of the pathogenic human polyomaviruses (BKV, JCV, MCV, HPyV7, and TSV). Sirolimus (rapamycin), a representative mTOR inhibitor used in posttransplantation regimens, was highly activated in a dose-dependent fashion at therapeutic levels for increasing BKV, MCV, and HPyV7 DNA replication, whereas tacrolimus (FK506), a calcineurin inhibitor,

was not. This correlated to loss of Skp2, which interacts with all 5 HPyV LT proteins. All polyomaviruses examined except BKV showed significant increased virus production during mTOR but not calcineurin inhibition, providing evidence for a new mechanism for polyomaviral latency based on viral replication protein proteostasis.

MATERIALS AND METHODS

Cell Culture

The 293 cells (ATCC CRL-1573) were maintained in Dulbecco's modified Eagle's medium (Cellgro, number 10-013) supplemented with 10% fetal bovine serum (Seradigm). Transfections were performed using Lipofectamine 2000 (Life Technologies) following the manufacturer's instruction.

Plasmid Construction

The MCV LT codon-optimized expression construct used in this study was described elsewhere [19, 20]. Complementary DNA (cDNA) for HPyV7 and TSV LT were generated by reverse transcriptase following RNA extraction of genomic LT-transfected 293 cells. cDNAs were reamplified with primers T7 and BGH reverse and ligated into pCR2.1/TOPO. JCV LT cDNA was obtained by amplifying and annealing exons 1 and 2 with primers JC-F/HPyV2 exon1_over and JC-R/HPyV2 exon2_over. BKV cDNA was synthesized and cloned in pUC57 by GenScript. cDNAs were then subcloned in pcDNA6.delSV40ori using EcoRV, SalI/SacII, EcoRV, and EcoRV/SacII sites, for BKV, JCV, HPyV7, and TSV, respectively. pcDNA6.delSV40ori was obtained by deleting the SV40 ori sequence from pcDNA6 (Life Technologies) by site-directed mutagenesis (Quikchange Lightning, Agilent number 210515) with primers delSV40ori-F and delSV40ori-R. All DNA constructs used in this study were sequence-confirmed. MCV ori is described elsewhere [23]. The complete noncoding control region (NCCR) of BKV (nt 1-387; GenBank: V01108.1), and HPyV7 (nt 1-385; GenBank: HM011566.1) were amplified and cloned into pCR2.1 (Life Technologies) within EcoRV/XhoI sites using BKV.NCCR.EcoRV-F, BKV.NCCR.XhoI-R, HPyV7.NCCR.EcoRV-F, and HPyV7.NCCR.XhoI-R. Primer sequences used for amplification and plasmid list are found in [Supplementary Tables 1 and 2](#).

Chemical Treatment

The following drugs were used in this study: PP242 (Selleckchem; S2218), sirolimus (Selleckchem; S1039), torin 1 (MilliporeSigma; 475991), cycloheximide (CHX) (MilliporeSigma; 239763), tacrolimus (FK506; MilliporeSigma, F4679). Treatment with different compounds was carried out for 18 hours unless stated otherwise in the figure legends.

Immunoblotting

For immunoblotting, cells were lysed in buffer (1% sodium dodecyl sulfate [SDS], 10 mM Tris pH 8.0, and 1 mM

ethylenediaminetetraacetic acid) containing protease inhibitors (MilliporeSigma; number 5892953001). Primary antibodies were incubated overnight at 4°C. Antibodies to LT (CM2B4 [19], PAb416 [kind gift of Ed Harlow], Xt7 [kind gift of Dr Chris Buck]), α -tubulin (12G10, DSHB), Akt (pan; C67E7, Cell Signaling), Phospho-Akt (Ser473) (number 9271, Cell Signaling), Skp2 (D3G5, Cell Signaling), and p27 Kip1 (D69C12, Cell Signaling) were used for specific protein detection. Quantitative western blots were carried out using infrared IRDye 800CW goat antimouse, IRDye 800CW goat antirabbit antibody, IRDye 680RD antimouse (H + L), or IRDye 680 antirabbit (H + L). Protein levels were quantitated and normalized to α -tubulin, using an Odyssey (LI-COR) or a ChemiDoc MP (Bio-Rad) imaging systems.

Lentiviral Infection for Short Hairpin RNA Knockdown

Small hairpin RNAs (shRNAs) targeting Skp2 (TRCN0000007534) was cloned into lentiviral vector pLKO.1 neo (Addgene number 10878) and random sequence scrambled shRNA [20] was used as control. Lentivirus production was carried out as described in [20]. Lentivirus infection was performed in the presence of 8 μ g/mL polybrene. Cells were lysed in 1% SDS lysis buffer 48 hours after transduction.

Origin Replication Assay

The MCV replication origin assay was performed as described elsewhere [20]. In brief, a plasmid containing the minimal MCV ori (ori97, 12.5 ng) described in [23] was transfected in 293 cells with MCV LT-expressing plasmid (6.25 ng). Inhibitor treatments were carried out 24 hours posttransfection, for 18 hours prior to harvesting cells. Episomal DNA was extracted using the Hirt method. Extracted DNA was digested with DpnI prior to quantitative polymerase chain reaction (qPCR) analysis. A similar method was performed to assess origin replication for BKV and HPyV7 using 25 ng and 12.5 ng of LT-expressing plasmid for BKV and HPyV7, respectively.

HPyV Molecular Clone Replication Assay

The 293 cells were transfected with BKV (GenBank; V01108.1), JCV (GenBank: J02226.1), MCV (GenBank: JF813003.1), HPyV7 (GenBank: HM011566.1), and TSV (GenBank: GU989205.1) genomic clones for 24 hours and were treated either with dimethyl sulfoxide (DMSO; .1%), PP242 (1 μ M), or tacrolimus (1 μ M). Cells at 85%–90% confluence were harvested 18 hours posttreatment for genomic DNA extraction and qPCR and immunofluorescence analysis [20].

Quantitative PCR

For origin replication assay, DpnI-resistant ori plasmid was subjected to qPCR using primers pCR.Repli.S and pCR.Repli.AS which amplifies a 101-bp sequence in pCR2.1. For molecular clone replication assay, DNA was subjected to qPCR

using primers amplifying BKV (qPCR.BKV.F1, qPCR.BKV.R1), JCV (qPCR.JCV.F1, qPCR.JCV.R1), MCV (MCV350.1605-1585, MCV350.1505-1528), HPyV7 (HPyV7.qPCR-F, HPyV7.qPCR-R), and TSV (qPCR.TSV.F1, qPCR.TSV.R1). Quantification of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (gapdh-F, gapdh-R) was performed for normalization. qPCR reactions were performed using a QuantStudio 3 real-time PCR system with PowerUp SYBR Green master mix (ThermoFisher Scientific). Amplification reactions of all target genes were performed following the manufacturer's recommendation. Results were expressed as viral copy numbers per cell equivalent from values plotted against standards for viral genes and GAPDH. Primer sequences used for amplification are shown in [Supplementary Table 1](#).

Immunofluorescence

Immunofluorescence protocol was previously described in [20] with modifications as follows. Cells on glass slides were fixed with 4% paraformaldehyde for 20 minutes and permeabilized with 0.1% Triton-X in phosphate-buffered saline (PBS) for 10 minutes prior to blocking with 5% bovine serum albumin in PBS–0.5% Tween 20. Cells were then incubated with CM2B4 (MCV LT antibody, 1:1000), PAb416 (BKV, JCV, and HPyV7 LT antibody, 1:1000) or XT7 (TSV antibody, 1:200) overnight at 4°C. Incubation with secondary antibody Alexa Fluor 568–conjugated antimouse (1:2000, Life Technologies) was done for 1 hour at room temperature. Stained cells were mounted with Vectashield containing 4',6-diamidino-2-phenylindole (DAPI; D3571, 0.5 μ g/mL, Invitrogen). Images were acquired with a charge-coupled device camera (U-CMAD3, Olympus) on an epifluorescent microscope (AX70 Provis, Olympus).

Proximity Ligation Assay

Proximity ligation assays (PLAs) were performed using Duolink In Situ Red Starter kit (MilliporeSigma). In brief, cells were grown on poly-L-lysine (MilliporeSigma, P4832) pretreated coverslips prior to transfection. Fixation and permeabilization were carried out as above. Cells were then treated with blocking solution for 60 minutes at 37°C inside a humidity chamber. Samples were incubated with the same primary antibodies and dilutions used for immunoblotting for 3 hours at 4°C. Washing and detection steps including ligation, amplification, and DAPI staining were carried out according to the manufacturer's instructions. LT expression was assessed following incubation with secondary antibody Alexa Fluor 488–conjugated anti-mouse (1:500, Life Technologies) for 1 hour at 37°C. Images were captured using fluorescence and confocal microscopy (Olympus). Image processing was performed using Image J. Positive PLA signal (average number of red dots per cell) for a total number of 50 cells was also determined using Image J. Statistical significance was tested in a 1-way analysis of variance (Prism 7).

RESULTS

mTOR Inhibition Increases LT Protein Expression for 5 Pathogenic Polyomaviruses

MCV LT protein expression is increased by inhibition of the PI3K-Akt-mTOR pathway [20] but it is unknown if other HPyV LTs are affected similarly. cDNA for BKV, JCV, MCV, HPyV7, and TSV LT proteins was expressed in 293 cells and treated for 18 hours with the mTOR active-site inhibitor PP242, a preclinical TORKinib [24]. mTOR inhibition significantly increased expression of BKV, JCV, MCV, HPyV7, and TSV LT proteins 2- to 5-fold compared to the DMSO control (Figure 1A and 1B). Loss of Akt S⁴⁷³ phosphorylation, a target of mTORC2 [25], was used as a measure for mTOR inhibition. Similarly, Skp2 protein levels also declined with mTOR inhibition.

To determine if mTOR inhibition increased LT translation or inhibited LT protein turnover, CHX immunoblotting was performed. CHX blocks new protein synthesis allowing determination of protein turnover rates. As seen in Figure 2A and 2B, mTOR inhibition led to significantly decreased BKV, MCV, and HPyV7 LT turnover (increased stabilization). Differential JCV and TSV LT stability after PP242 treatment did not reach significance when averaged over 3 replicates, but viral proteins were consistently elevated by PP242 treatment (Figure 2A).

Skp2 E3 Ligase Regulates HPyV LT Stability

MCV LT is a substrate of Skp2 [20], but it is unknown if the other HPyV LTs are also targeted for degradation by the SCF^{Skp2} complex. To determine if Skp2 binds to each LT protein, in situ PLAs for interaction of Skp2 with each LT was performed (Figure 3A). Positive signals for Skp2-LT interaction were present for all 5 different LT proteins, including MCV LT, but not empty vector (no LT) or irrelevant immunoglobulin G antibody negative controls. It was previously shown by co-immunoprecipitation experiments that Ser220 in MCV LT is important for its interaction with Skp2 [20]. This was confirmed by Skp2 PLA with a mutant MCV LT in which S220 was mutated to a nonphosphorylatable alanine (LT^{S220A}). To quantify this interaction, the average of PLA red dots for 50 cells was calculated. Decreased PLA signal for Skp2-LT interaction was observed for MCV LT^{S220A} compared to MCV LT wild-type with an average of 4 and 9 PLA dots per cell, respectively.

We next compared PP242 and tacrolimus each at 1 μ M for 18 hours, a calcineurin inhibitor, for MCV and HPyV7, performed twice (Figure 3C). PLA dot numbers were determined from 50 cells each time and condition. For MCV, PP242 reduced Skp2-MCV LT interaction (average 5 dots per cell) compared to DMSO (9 dots per cell) or tacrolimus (7 dots per cell, Figure 3C). For HPyV7, a smaller PLA signal reduction occurred after PP242 treatment (4 dots per cell) compared to DMSO (8 dots per cell) and tacrolimus (7 dots per cell, Figure 3C). Reduced PLA signal was not observed for BKV when cells were treated with either PP242 or tacrolimus (7 dots each) compared to DMSO (8 dots

per cell). These results are consistent with mTOR inhibition, and less so calcineurin inhibition, reducing LT targeting by Skp2 for MCV and HPyV7.

To formally demonstrate Skp2 targeting HPyV LTs, Skp2 was knocked down by short-hairpin RNA interference-mediated gene silencing (shRNA), achieving > 80% reduction of Skp2 protein expression in cells by 48 hours (Figure 3D). Levels of p27^{Kip1}, a known substrate for Skp2-mediated degradation [26], served as a positive control. Skp2 knockdown was associated with a 1.7- to 4-fold increased LT protein expression for all of the polyomavirus replication proteins tested, including BKV, which showed the lowest level of LT induction after Skp2 knockdown (Figure 3D).

mTOR Inhibition Increases BKV, MCV, and HPyV7 DNA Replication

To determine effects of mTOR inhibition on polyomavirus replication in a dose-dependent fashion, we generated replicon replication assays [23] for 3 pathogenic HPyVs (BKV, MCV, and HPyV7). Each viral replication origin (NCCR) was cloned into a neutral plasmid (pCR2.1), and cotransfected with the corresponding viral LT protein plasmid into 293 cells. Viral DNA replication was measured by qPCR after BamHI and DpnI digestion to eliminate bacterially replicated plasmid DNA. HPyV LT and Skp2 levels were assessed by immunoblotting (Supplementary Figure 1). Half maximal effective concentration (EC₅₀) values were determined from the maximum level of DNA replication for each virus plasmid.

Tacrolimus was not effective in increasing DNA replication for any of the 3 viruses (Figure 4A), although minimal activation of MCV DNA replication was seen at the highest levels tested (10 μ M). These levels are 3–4 logs higher than standard therapeutic tacrolimus ranges (6–24 nM or 5–20 μ g/L) [16]. In contrast, sirolimus markedly increased origin replication in a dose-dependent manner for all 3 viruses, with EC₅₀ values of 6 nM, 4 nM, and 3 nM for BKV, MCV, and HPyV7, respectively. These EC₅₀ values are within therapeutic ranges for sirolimus maintenance immunosuppression in patients (4.4–13 nM or 4–12 μ g/L) [27]. Both PP242 and torin 1, another preclinical TORKinib [24], also induced DNA replication of all 3 HPyVs in a dose-dependent fashion (EC₅₀ values of 72 nM, 305 nM and 301 nM for PP242 [for BKV, MCV and HPyV7, respectively] and of 8 nM, 27 nM and 159 nM for torin 1 [for BKV, MCV and HPyV7, respectively]).

mTOR Inhibition but Not Calcineurin Inhibition Activates Whole Virus Replication of JCV, MCV, HPyV7, and TSV

To extend these findings to whole virus replication, 293 cells were transfected with BKV, JCV, MCV, HPyV7, and TSV molecular clones and treated with PP242 (1 μ M) or tacrolimus (1 μ M). PP242 treatment, but not tacrolimus treatment, increased viral DNA replication for JCV, MCV, HPyV7, and TSV up to 3-fold (Figure 4B). This correlated with increased

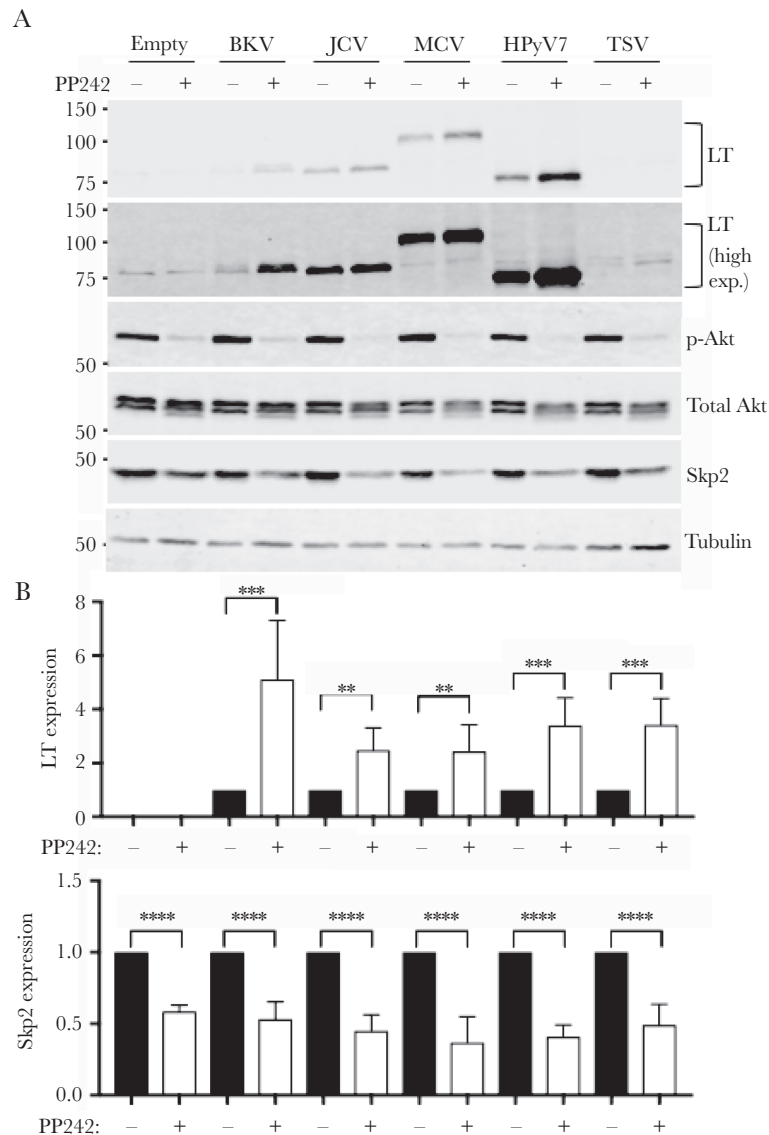


Figure 1. Mechanistic target of rapamycin (mTOR) inhibition increases BK virus (BKV), JC virus (JCV), Merkel cell polyomavirus (MCV), human polyomavirus 7 (HPyV7), and trichodysplasia spinulosa polyomavirus (TSV) large T (LT) protein levels. *A*, HPyV LT steady-state levels for BKV, JCV, MCV, HPyV7, and TSV were increased with an mTOR inhibitor (PP242, 5 μ M) treatment in 293 cells. LT proteins were detected using a cocktail of PAb416 (BKV, JCV, HPyV7), CM2B4 (MCV), and Xt7 (TSV). *B*, Quantitative analysis of LT and Skp2 expression levels (mean \pm standard error of the mean, $n = 4$). Relative fold change in LT and Skp2 expression levels (PP242, 5 μ M) is calculated and normalized to control (0.1% dimethyl sulfoxide). ** $P \leq .01$; *** $P \leq .001$; **** $P \leq .0001$.

numbers of cells staining for LT proteins by immunofluorescence (Figure 4C). In contrast, we did not find significant PP242- or tacrolimus-dependent changes in viral BKV DNA replication or LT expression, although we could not exclude technical issues with recircularization of the BKV genome during cloning that may have interfered with measuring BKV replication.

DISCUSSION

We find that, in addition to their effects on immunosuppression, sirolimus and other mTOR inhibitors directly activate pathogenic polyomavirus replication by inhibiting the SCF E3 ligase Skp2. This results in accumulation of LT proteins, which

activate polyomavirus replication, a finding we described for MCV [20] but now extend to the other HPyVs as well.

Of note, mTOR inhibitors have not been found to be significantly associated with BKV replication or BKV-related disease among transplant patients [28–30], which may be consistent with a lack of whole BKV replication after mTOR inhibition (Figure 4)—despite clear evidence for increased BKV LT stabilization and activation in replicon assays. For other polyomavirus diseases in transplant patients, particularly JCV-, TSV-, and HPyV7-related diseases, reevaluation of the immunosuppression therapy may be helpful in disease control.

Polyomaviruses maintain viral latency by multiple, evolutionarily driven pathways to suppress viral replication after infection

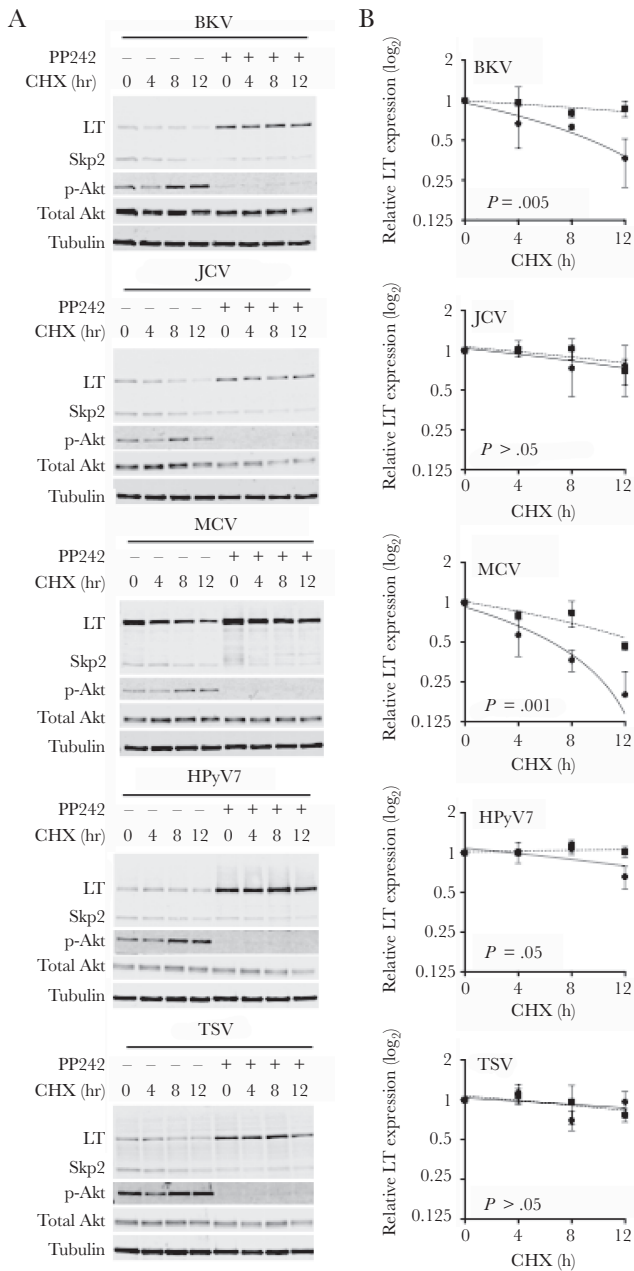


Figure 2. Mechanistic target of rapamycin inhibition increases BK virus, Merkel cell polyomavirus, and human polyomavirus 7 large T (LT) steady-state levels in a cycloheximide (CHX) chase course. *A*, LT-transfected 293 cells were treated with either 0.1% dimethyl sulfoxide (DMSO) or 5 μ M PP242 for a minimum of 16 hours prior to CHX treatment (0.1 μ g/ μ L). Cells were harvested at time T0, T4, T8, and T12 following CHX treatment. *B*, Quantitative analysis of LT levels (log₂) is calculated and normalized to non-cycloheximide-treated samples at time 0 (mean \pm standard error of the mean, n = 3). Differences between slopes from DMSO and PP242-treated cells was tested following nonlinear regression analysis (Prism 7). Abbreviations: BKV, BK virus; CHX, cycloheximide; HPyV7, human polyomavirus 7; JCV, JC virus; LT, large T; MCV, Merkel cell polyomavirus; TSV, trichodysplasia spinulosa polyomavirus.

to prevent activating a sterilizing host immune response. This has the paradoxical effect of limiting laboratory growth of native wild-type polyomavirus strains so that highly active mutant strains are often recovered in vitro [31]. Inpatient evolution also occurs

in immunosuppressed persons, most notably by duplication and rearrangement of the BKV NCCR, which is not found in a naturally transmissible BKV strains in healthy humans having an intact immune system [32]. The biological “bug” of poor polyomavirus growth in vitro turns out to be an evolutionary feature in vivo. We show that chemical treatment generally results in a 2- to 4-fold change both in replicon systems and with intact virus replication. Although modest, this change is statistically significant and appears to shift these viruses from a nonreplicating state (latency) to a replicating state (lytic replication; see model in Figure 5).

Proteostatic latency relies on continuous degradation of LT protein by cellular E3 ligases such as SCF^{Skp2} but it is not limited to SCF^{Skp2} only. It is likely, as seen for MCV, that other E3 ligases, such as β -TrCP and Fbw7, also contribute to polyomavirus replication control [20] and may be affected by mTOR signaling. Also, MCV LT serine 220 is probably only one of several SCF^{Skp2} recognition sites on MCV LT since the LT^{S220A} mutation reduced but did not eliminate Skp2 MCV LT interaction (Figure 3B). Proteostatic latency might help explain autoinhibitory features in other persistent viruses, including RNA viruses [33]. E3 ligases degrade their substrates so bulk immunoprecipitation requires overexpression, often with artificial tags, and can generate misleading results when interaction is not found. We and others find that PLA is more sensitive and has higher specificity for detecting in situ E3 ligase-substrate interactions [34–36].

Several important limitations should be considered in evaluating our data. We tested relatively few drugs and experiments were performed in 293 cells, a well-established approach for quantitative polyomavirus replication but that may not reflect tissue specific mTOR-polyomavirus relationships. Our results were not uniform for all viruses across all experimental conditions. For example, we did not find significant differences in LT protein turnover after mTOR inhibition (Figure 2B) for JCV and TSV after mTOR inhibition. This was due to experimental variation for these 2 viruses that showed similar but not significant trends as BKV, MCV, and HPyV7. We also did not find evidence for mTOR-dependent whole BK virus replication despite subgenomic assays, suggesting that this virus does not behave similarly to other polyomaviruses. We do not know if this is because BKV is less responsive to mTOR inhibition, possibly due to competing inhibitory promoter effects [37], or if this is due to a technical issue in recircularizing BKV whole genome clones.

Newer cancer therapies based on small molecule PI3K-Akt-mTOR pathway inhibitors are likely to activate polyomavirus replication but the clinical significance of this effect cannot be predicted. If new drug-related polyomavirus syndromes emerge, as occurred with natalizumab and JCV [12], these findings may help guide prevention or treatment.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to

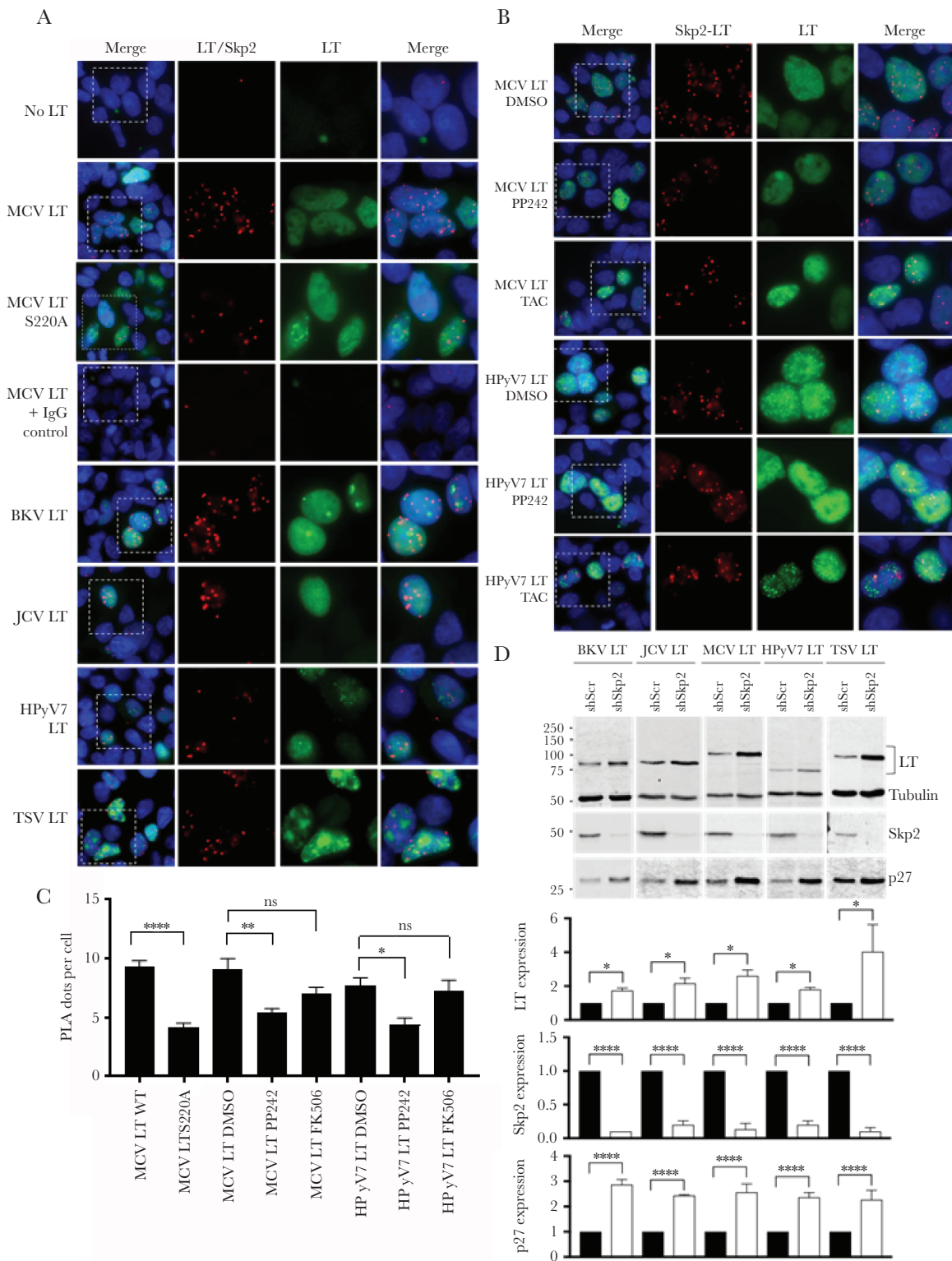


Figure 3. Skp2 and human polyomavirus (HPyV) large T (LT) interaction. *A*, Proximity ligation assays (PLAs) were performed using mouse CM2B4 (Merkel cell polyomavirus [MCV]), PAb416 (BK virus [BKV], JC virus [JCV], and HPyV7) or Xt7 (trichodysplasia spinulosa polyomavirus [TSV]) and rabbit Skp2 antibodies. Images were captured by confocal microscope (Olympus, 60 \times). Mouse immunoglobulin G (IgG) and empty vector (no LT) was used to assess specificity of the assay. *B*, Mechanistic target of rapamycin inhibition reduced Skp2-LT interaction. Cells were treated with 0.1% dimethyl sulfoxide (DMSO), 1 μ M PP242, or 1 μ M tacrolimus (TAC) for 18 hours prior to PLA. *C*, Quantification of Skp2-LT PLA dots. Positive PLA signal (average number of red dots per cell) for 50 cells was measured using ImageJ. Statistical significance was tested in a 1-way analysis of variance (Prism 7). *D*, SCF-Skp2 E3 ubiquitin ligase knockdown increases BKV, JCV, MCV, HPyV7, and TSV LT levels. HPyV LT expression levels for BKV, JCV, MCV, HPyV7, and TSV in Skp2 knockdown 293 cells. LT proteins were detected using a cocktail of PAb416 (BKV, JCV, HPyV7), CM2B4 (MCV), and Xt7 (TSV). Quantitative analysis of LT, Skp2, and p27 expression in Skp2 knockdown 293 cells. Relative fold-change expression levels are calculated and normalized to scramble (shScr) (mean \pm standard error of the mean, $n = 4$). Statistical significance was tested in multiple t test analysis (Prism 7). * $P \leq .05$; ** $P \leq .01$; **** $P \leq .0001$; ns, not significant.

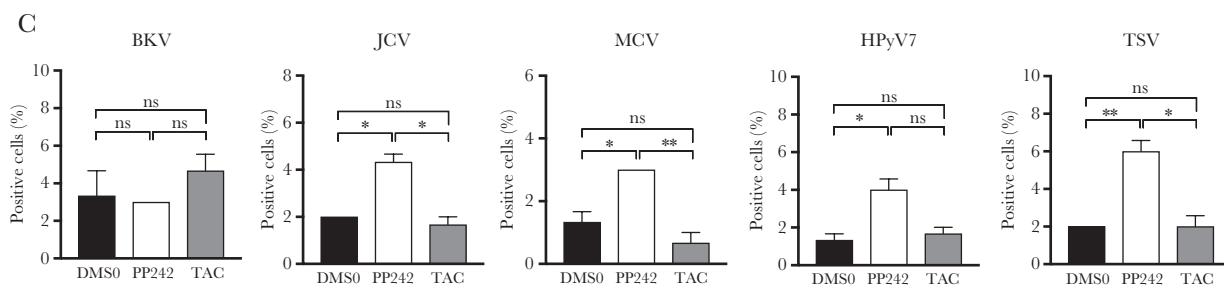
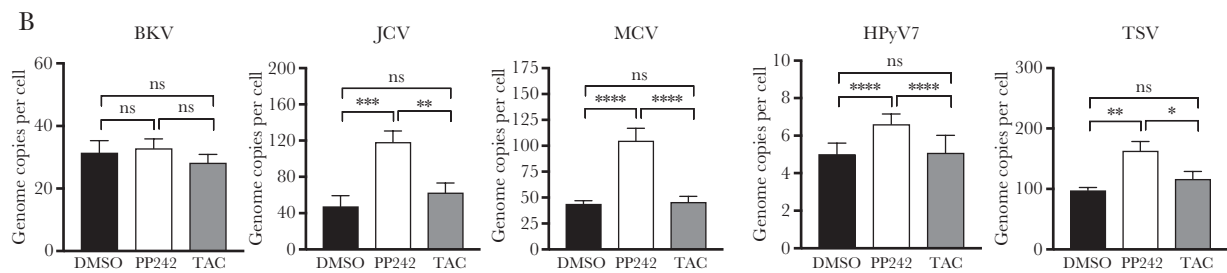
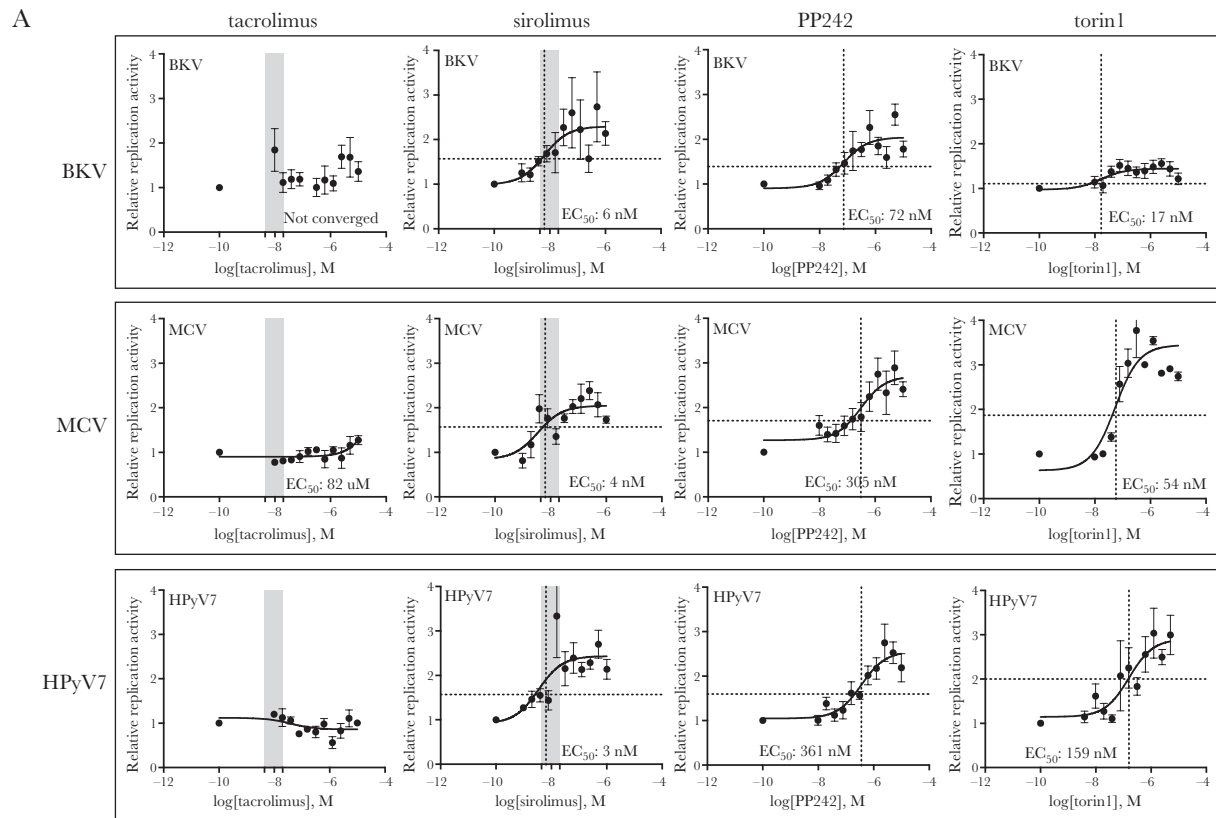


Figure 4. Mechanistic target of rapamycin (mTOR) inhibition activates polyomavirus replication. **A**, BK virus (BKV), Merkel cell polyomavirus (MCV), and human polyomavirus 7 (HPyV7) DNA replication in an HPyV origin replication assay. The 293 cells were transfected with plasmids encoding BKV, MCV, or HPyV7 large T (LT) proteins with an origin-containing plasmid from respective HPyV. Cells were treated with 2-fold serial dilutions starting at 10 μ M tacrolimus (TAC), 1 μ M sirolimus, 10 μ M PP242, and 5 μ M torin 1. Half maximal effective concentration (EC_{50}) values were assessed from dose-responsive curves for each inhibitor. Replication activities are reported as percentage of 0.1% dimethyl sulfoxide (DMSO), which was set at 100% ($n = 4$). Statistical significance was tested in a 1-way analysis of variance (ANOVA; Tukey multiple comparison) analysis (Prism 7). **B**, mTOR inhibition increases HPyV production. The 293 cells were transfected with molecular clones from BKV, JC virus (JCV), MCV, HPyV7, and trichodysplasia spinulosa polyomavirus (TSV). Transfected cells were treated with 0.1% DMSO, 1 μ M PP242, or 1 μ M tacrolimus for 18 hours. Genome replication was assessed by quantitative polymerase chain reaction and values are reported as genome copies per cell equivalent. **C**, Quantification of percentage of LT-expressing cells in molecular clone replication assay. HPyV LT protein was detected by immunofluorescence using PAb416 (BKV, JCV, HPyV7), CM2B4 (MCV), and X7 (TSV) antibodies. Statistical significance was tested in a 1-way ANOVA (Tukey multiple comparison; Prism 7). * $P \leq .05$; ** $P \leq .01$; *** $P \leq .001$; **** $P \leq .0001$; ns, not significant.

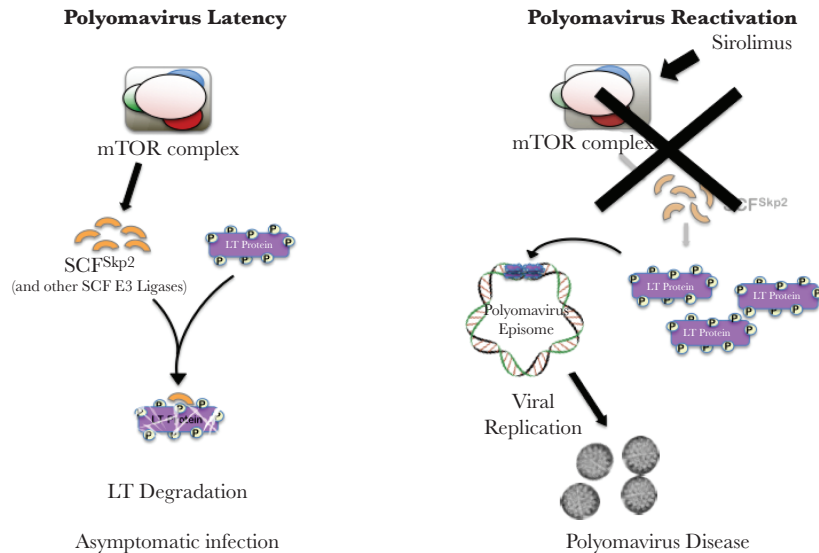


Figure 5. Model for human polyomavirus (HPyV) reactivation in transplant recipients on mechanistic target of rapamycin (mTOR) inhibitor therapy. HPyV large T (LT) proteins are continuously degraded by different E3 ligases, including Skp2. mTOR inhibition negatively regulates Skp2 levels, which results in increased HPyV LT stability and viral replication.

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Notes

We also find a report (Mechali F, Hsu CY, Castro A, Lorca T, Bonne-Andrea C. Bovine papillomavirus replicative helicase E1 is a target of the ubiquitin ligase APC. *J Virol* 2004; 78:2615-9) that describes a similar proteostatic latency mechanism for bovine papillomavirus.

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Potential conflicts of interest. All authors: No reported conflicts of interest.

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