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Restricted Protein Phosphatase 2A Targeting by Merkel Cell Polyomavirus Small T Antigen

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ABSTRACT

Merkel cell polyomavirus (MCV) is a newly discovered human cancer virus encoding a small T (sT) oncoprotein. We performed MCV sT FLAG-affinity purification followed by mass spectroscopy (MS) analysis, which identified several protein phosphatases (PP), including PP2A A and C subunits and PP4C, as potential cellular interacting proteins. PP2A targeting is critical for the transforming properties of nonhuman polyomaviruses, such as simian virus 40 (SV40), but is not required for MCV sT-induced rodent cell transformation. We compared similarities and differences in PP2A binding between MCV and SV40 sT. While SV40 sT coimmunopurified with subunits PP2A Aα and PP2A C, MCV sT coimmunopurified with PP2A Aα, PP2A Aβ, and PP2A C. Scanning alanine mutagenesis at 29 sites across the MCV sT protein revealed that PP2A-binding domains lie on the opposite molecular surface from a previously described large T stabilization domain (LSD) loop that binds E3 ligases, such as Fbw7. MCV sT-PP2A interactions can be functionally distinguished by mutagenesis from MCV sT LSD-dependent 4E-BP1 hyperphosphorylation and viral DNA replication enhancement. MCV sT has a restricted range for PP2A B subunit substitution, inhibiting only the assembly of B56α into the phosphatase holoenzyme. In contrast, SV40 sT inhibits the assembly of B55α, B56α and B56ε into PP2A. We conclude that MCV sT is required for Merkel cell carcinoma growth, but its *in vitro* transforming activity depends on LSD interactions rather than PP2A targeting.

IMPORTANCE

Merkel cell polyomavirus is a newly discovered human cancer virus that promotes cancer, in part, through expression of its small T (sT) oncoprotein. Animal polyomavirus sT oncoproteins have been found to cause experimental tumors by blocking the activities of a group of phosphatases called protein phosphatase 2A (PP2A). Our structural analysis reveals that MCV sT also displaces the B subunit of PP2A to inhibit PP2A activity. MCV sT, however, only displaces a restricted subset of PP2A B subunits, which is insufficient to cause tumor cell formation *in vitro*. MCV sT instead transforms tumor cells through another region called the large T stabilization domain. The PP2A targeting and transforming activities lie on opposite faces of the MCV sT molecule and can be genetically separated from each other.

Approximately 30 widely expressed serine/threonine (Ser/Thr) protein phosphatases (PPases) have been identified (1, 2). Protein phosphatase 2A (PP2A) is a combinatorial family of Ser/Thr phosphatases that are highly conserved in eukaryotes. The PP2A core enzyme structure is comprised of a 36-kDa catalytic C subunit (PP2A C) bound to a 65-kDa A regulatory subunit (PP2A A). This core heterodimer can be purified, but the PP2A holoenzyme generally contains a third, regulatory B subunit that provides substrate specificity and subcellular localization (3–7). Among the many combinatorial possibilities, there are two C catalytic subunits, at least two A scaffolding subunits, and multiple B substrate recognition subunits (8), which allow for potentially hundreds of different phosphatase specificities and activities. Phosphatidylinositol 3-kinase (PI3K)/Akt, mitogen-activated protein kinase, c-Myc, and other cancer signaling pathways that impact cellular processes, including cell cycle progression and cellular tumorigenesis are regulated by specific PP2A isoforms (9). Aberrant expression and mutations in PP2A subunits have been implicated in human cancers (8, 10–16).

PP2A activity is regulated in part by posttranslational modifications of subunits (17–23). Methylation of PP2A C at Leu309 activates the holoenzyme to allow binding of the B subunit (17), while phosphorylation of PP2A C at Thr307 causes an ~90% reduction in phosphatase activity (22). Mutational analyses revealed that these two residues impact the binding of the C subunit with various different B subunits (24), suggesting that phosphatase activity may be regulated by the selective assembly of regulatory subunits with the catalytic C subunit.

A number of polyomavirus small T (sT) antigens bind to PP2A and displace specific B regulatory subunits. Polyomaviruses BK virus (BKV), JC virus (JCV), murine polyomavirus (Py) and simian virus 40 (SV40) sTs inhibit PP2A phosphatase activity via direct interactions (25). Interactions between either SV40 or Py sT and PP2A have been particularly well studied (26). SV40 sT binds to the scaffolding A and catalytic C subunits and displaces B′/B56/PR61 and B56γ regulatory B subunits, causing activation of the PI3K/Akt, Wnt, and c-Myc signaling pathways (27–29). Never-

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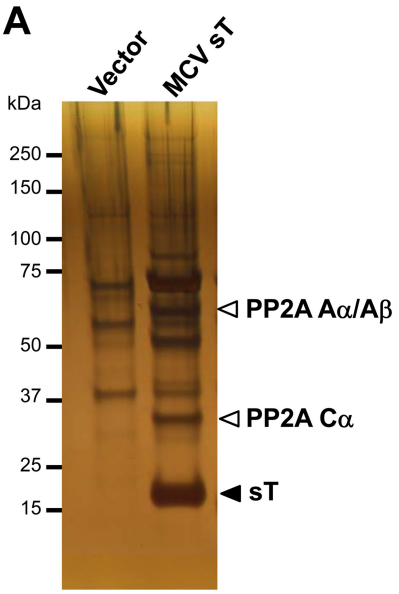
TABLE 1 Primer pairs used in this study

Gene and primer pair	Sequence (5′–3′)	
	Sense	Antisense
MCV sT		
R7A	TTGGTCCTTAACGCGAAAGAACGAGAG	CTCTCGTTCTTTCGCGTTAAGGACCAA
D44N	CTCAAACATCACCCAAATAAGGGTGGCAACCC	GGGTTGCCACCCTTATTTGGGTGATGTTTGAG
P49A	AAGGGTGGCAACGCTGTGATTATGATG	CATCATAATCACAGCGTTGCCACCCTT
S80A	GATGAGGTGGCTACCAAGTTC	GAACTTGGTAGCCACCTCATC
P84A	ACCAAGTTCGCCTGGGAGGAA	TTCCTCCCAGGCGAACTTGGT
Y88A	TGGGAGGAAGCTGGCACACTC	GAGTGTGCCAGCTTCCTCCCA
K92A	GGCACACTCGCAGATTACATG	CATGTAATCTGCGAGTGTGCC
Q96A	GATTACATGGCGAGTGGCTAC	GTAGCCACTCGCCATGTAATC
N100A	AGTGGCTACGCTGCTAGATTC	GAATCTAGCAGCGTAGCCACT
C104S	AATGCTAGATTCAGCCGCGGACCCGGG	CCCGGGTCCGCGGCTGAATCTAGCATT
C109S	GCCGCGGACCCGGGAGCATGCTGAAACAACTG	CAGTTGTTTCAGCATGCTCCCGGGTCCGCGGC
L114A	CTGAAACAAGCGAGAGACAGC	GCTGTCTCTCGCTTGTTTCAG
K118A	AGAGACAGCGCGTGCGCCTGT	ACAGGCGCACGCGCTGTCTCT
I122A	TGCGCCTGTGCCAGTTGTAAG	CTTACAACTGGCACAGGCGCA
L126A	AGTTGTAAGGCTTCCCGGCAG	CTGCCGGGAAGCCTTACAACT
H130A	CTTTCCCGGCAGGCTTGCTCCCTGAAA	TTTCAGGGAGCAAGCCTGCCGGGAAAG
K134A	TGCTCCCTGGCAACGTTGAAG	CTTCAACGTTGCCAGGGAGCA
Q138A	ACGTTGAAGGCGAAGAATTGC	GCAATTCTTCGCCTTCAACGT
L142A	CAGAAGAATTGCGCCACATGGGGAGAA	TTCTCCCCATGTGGCGCAATTCTTCTG
E146A	ACATGGGGAGCATGCTTTTGT	ACAAAAGCATGCTCCCCATGT
Y150A	TGCTTTTGTGCTCAGTGCTTC	GAAGCACTGAGCACAAAAGCA
I154A	CAGTGCTTCGCTCTGTGGTTT	AAACCACAGAGCGAAGCACTG
T162A	TTTCCGCCCGCTTGGGAGTCA	TGACTCCCAAGCGGGCGGAAA
F166A	TGGGAGTCAGCCGACTGGTGG	CCACCAGTCGGCTGACTCCCA
Q170A	GACTGGTGGGCGAAAACTCTG	CAGAGTTTTCGCCCACCAGTC
E174A	GGGCGGCGATATCACCATGGACTTGGTCCTTAACAGG	GTGAAGCAAGCAATAGTCGGTTTCTGCCAGAGTTTTCTGCCA
Y178A	GGGCGGCGATATCACCATGGACTTGGTCCTTAACAGG	GTGAAGCAAGCAAGCGTCGGTTTCTTCCAG
H182A	GGGCGGCGATATCACCATGGACTTGGTCCTTAACAGG	GGGCTCGAGTTATCAGAAGAGATGCAAGGCAAGCAAGCAATAGTCGG
MCV sT.end	GGGCGGCGATATCACCATGGACTTGGTCCTTAACAGG	GGGCCGGCTCGAGTTATCAGAAGAGATGCAAGTGAAGCAAGC
SV40 sT		
SV40sT.1	GGATCCACCATGGACAAGGTACTCAACAGAGAAGAA TCACTCCAACTGATGGACTTGC	ATGGGATGTTTCCCCACGCAGATCTCTCAAGTCCCAGCAAGTCCATCAGTTGGAGTGATT
SV40sT.2	GCGTGGGGAAACATCCCATTGATGAGAAAGGCATACTTG AAGAAGTGCAAAGAGTTCCAC	TTCTTCATCTTCTCTTCGTCTCCGCCTTTGTCCGGGTGGAACTCTTTGCACTTCTTCAAG
SV40sT.3	CGGAGACGAAGAGAAGATGAAGAAGATGAACACTCTGTACAA GAAGATGGAGGATGGTGT	AGAAGCCACCAAAGTCCGGCTGGTGAGCGTACTTCACACCATCCTCCATCTTCTTGTACA
SV40sT.4	CCGGACTTTGGTGGCTTCTGGGATGCAACCGAAGTGTTCGCC TCCAGTCTGAATCCCGGT	TGGCGCACTCAGGCCACTGCTTACAGTACATCGCATCGACACCGGGATTCAGACTGGAGG
SV40sT.5	AGTGGCCTGAGTGCGCCAAGAAGATGAGCGCAAACTGC ATCTGCCTGCTTTGCCTGCTGC	GCGGGTCCTTGCGATACAGCTTCCTGTTCTCATGTTTCATCCGCAGCAGGCAAAGCAGGC
SV40sT.6	CTGTATCGCAAGGACCCGCTGGTGTGGGTTGATTGCTAC TGCTTCGACTGCTTTCGCATG	ACAGCAGCAAAGTACCCTCGCACAAGTCGAGTCCGAACCACATGCGAAAGCAGTCGAAGC
SV40sT.7	GCGAGGGTACTTTGCTGCTGTGGTGCGACATCATTG GGCAGACCACCTATCGCGACTTGA	GCCCTGCAGGCTCGAGTCACAGCTTCAAGTCGCGATAGGTGGTCTG
SV40sT.end	CGAGCGCTGATATCGGATCCACCATGGACAAGGTACTCAACAGAGAAG	GCCCTGCAGGCTCGAGTCACAGCTTCAAGTCGCGATAGGTGGTCTG
SV40sTco.L133A	GTATCGCAAGGACCCGGCGGTGTGGGTTGATTGC	GCAATCAACCCACACCGCCGGGTCCTTGCGATAC
Fbw7.ΔF		
Fbw7.ΔF(Δ278-324)	GTGATAGAACCCCAGTTTCAATGCAAAGAAGAGGGGATTGATG	GGTTCATCAATCCCCTCTTCTTTGCATTGAAACTGGGGTTCTATCACTTG
Fbw7.end	CCGCTCGAGGTCGACATGAATCAGGAACTGCTC	GCTCTAGATCACTTCATGTCCACATCAAAGTCCAGCACCAGCAG



Identification of MCV sT binding Proteins	Mass (kDa)	Peptides (#)
Hsp90b	90	6
Hsp70 1A/1B	70	17
Hsp71	70	3
Grp78	70	24
PP2A Aα	65	7
PP2A Aβ	65	3
STK38	60	6
Tubulin β2A	52	17
Tubulin α1A	52	12
Mevalonate kinase	40	4
Hsp70	40	3
PP2A Cα	35	8
CHIP E3	35	5
PP4C	35	2
ADP/ATP translocase 3	30	4

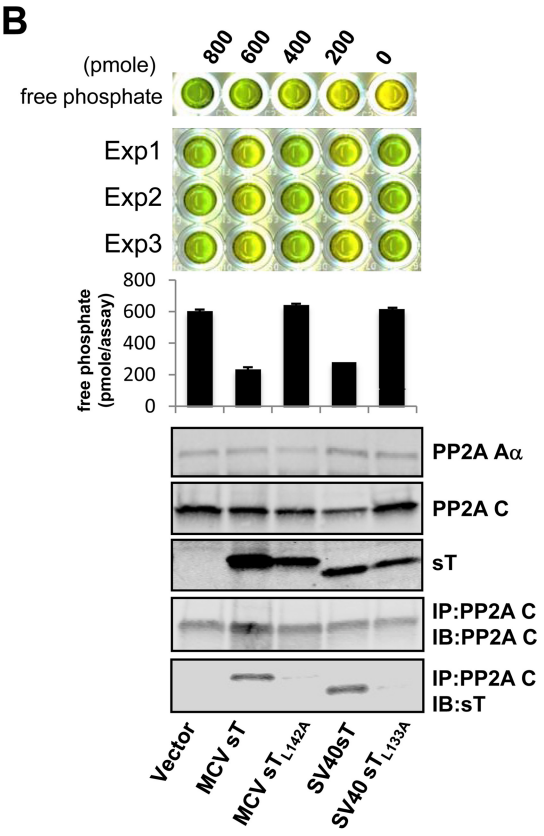


FIG 1 sT interacts with PP2A and inhibits its activity. (A) Detection of sT interaction with PP2A by FLAG-affinity purification assay and MS. N-terminally FLAG-tagged sT proteins (pCMV-tag2B.sTco) were expressed in 293 cells and immunoprecipitated. Silver staining was used to detect proteins after electrophoretic separation. Seven specific sT-binding protein bands at 90, 70, 65, 50, 40, 35, and 30 kDa identified by MS are listed. The 65- and 35-kDa proteins were identified as PP2A A (α and β) and PP2A C subunits (open arrowheads). The FLAG-tagged bait protein (MCV sT) was present at ~18 kDa (closed arrowhead). (B) sT inhibits PP2A activity *in vitro*. Small T proteins were expressed in 293 cells, from which PP2A C was immunoprecipitated and used to measure dephosphorylation activity for a peptide substrate. Both MCV and SV40 sT, but not putative PP2A binding mutant proteins, inhibited the catalytic activity of PP2A C. Expression levels of input proteins (sTs, PP2A C) before and after immunoprecipitation are shown. The data show the means ± the SE from three experiments.

theless, while SV40 sT promotes rodent cell transformation by SV40 large T, it is not transforming when expressed alone (30).

Merkel cell polyomavirus (MCV) (31) was the first human pathogen discovered by nondirected tissue transcriptome sequencing, an approach called digital transcriptome subtraction (DTS) (32), and it is the cause of most Merkel cell carcinomas (31, 33–37). Similar to other polyomaviruses, MCV encodes large T (LT) and sT early proteins that are required for MCV-positive Merkel cell carcinoma (MCC) cell survival and proliferation (38, 39). Unlike SV40 sT, however, MCV sT is a transforming oncoprotein in immortalized rodent fibroblast cells (38, 39) and has unique functions that have not been identified in other polyomavirus small T antigens. For example, MCV sT enhances LT-mediated MCV viral genome replication by stabilizing LT through sequestration of Fbw7, an E3 ligase that ubiquitinates LT for rapid turnover (40, 41). This MCV sT function was mapped to amino acids 91 to 95, defined as the large T stabilization domain (LSD) (42), and determined to be essential for rodent cell transformation (42). MCV sT is also required for the hyperphosphorylation-associated inactivation of the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), since expression of a constitutively active 4E-BP1 antagonizes the cell transformation activity of MCV sT (39), but this is independent of Fbw7 targeting (42).

MCV sT targets PP2A through direct protein-protein interactions (39), yet little is known about the molecular basis for these interactions. Unlike SV40 sT (16, 43, 44), comparable expression of MCV sT has minimal effects on PI3K/Akt signaling in 293 cells (39). Phospho-Akt protein levels are actually increased by knockdown of T antigens in an MCV-positive cell line, MKL-1 (39). Further, mutations in MCV sT that eliminate PP2A binding do not affect its transforming activity (39). Transgenic mice expressing MCV sT under a conditional bovine keratin 5 promoter develop skin hyperplasia and this phenotype requires an intact LSD but not an intact PP2A interaction domain (45). Here we show that both MCV sT and SV40 sT specifically interact with PP2A A and PP2A C subunits to inhibit the formation of the PP2A holoenzymes containing a different but overlapping array of B regulatory subunits. MCV sT inhibits the binding of B56α subunit to the PP2A A and C core enzyme, while SV40 sT inhibits the binding of B55α, B56α, and B56ε with the core heterodimer. Mechanistic diversity in regulation of phosphatase activity may cause differences in the transforming capacity of these different viral small T antigens.

MATERIALS AND METHODS

Plasmids. The pGEX-4T-PP2A Aα (46) and pMIG-PP2A Aβ (Addgene, catalog no. 15248) (47) plasmids were digested with BamHI/EcoRI and

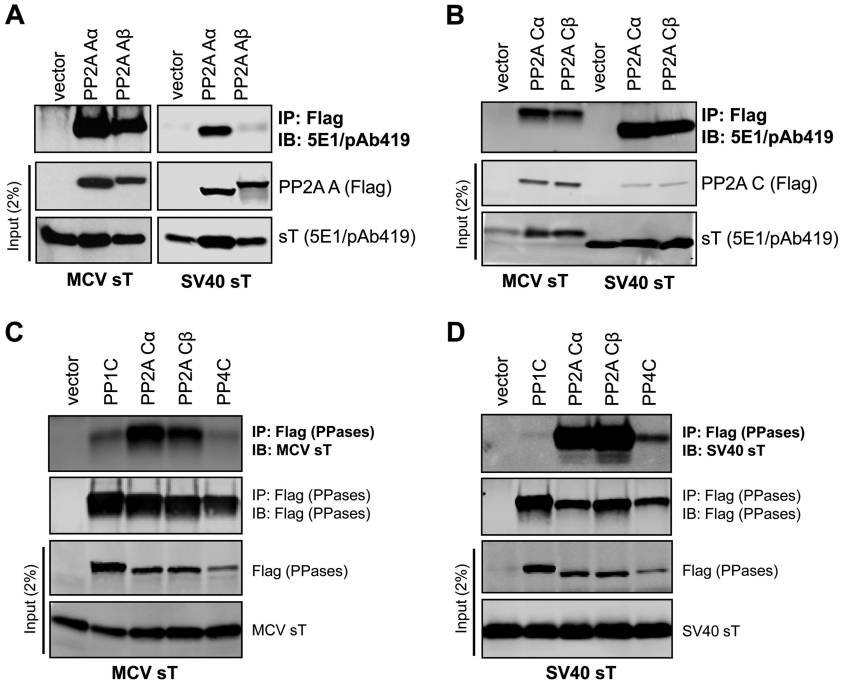


FIG 2 Comparison of PP2A targeting by MCV and SV40 sT. (A) MCV sT interacts with both PP2A Aα and Aβ. FLAG-tagged PP2A Aα and Aβ were coexpressed with either MCV sT or SV40 sT in 293 cells. PP2A Aα and Aβ proteins were immunoprecipitated with FLAG antibody. sT interaction was detected using specific antibodies CM5E1 and pAb419 for MCV and SV40 sT, respectively. MCV sT interacts with both Aα and Aβ subunits of PP2A, whereas SV40 sT binds predominantly to Aα. (B) Both MCV and SV40 sT interact with PP2A Cα and Cβ subunits. MCV or SV40 sT was coexpressed with either FLAG-tagged PP2A Cα or Cβ subunit. The PP2A C subunit was immunoprecipitated with a FLAG antibody and sT interaction was detected. Both MCV and SV40 sT interact with PP2A Cα and Cβ subunits. (C and D) PP2A is a main target protein phosphatase for both MCV and SV40 sT antigens. FLAG-tagged catalytic subunits of homologous phosphatases (PP1C, PP2A Cα, PP2A Cβ, and PP4C) were cotransfected with either MCV or SV40 sT and immunoprecipitated using FLAG-M2 agarose resin. Two isoforms (Cα and Cβ) of the PP2A catalytic subunit were detected as main interacting proteins for both MCV and SV40 sT. Weak interactions between PP1C and PP4C with both MCV and SV40 sT were also detected.

SfoI/XhoI, respectively, and ligated into BamHI/EcoRI and EcoRV/XhoI sites in pCMV-tag2B vector. B subunits of PP2A were obtained from Addgene (catalog nos. 13804, 14532, 14536, 14537, and 14535 for B55α, B56α, B56δ, B56ε, and B56γ3, respectively) (48, 49). pMIG-FLAG-B55α was digested with NheI/XhoI and inserted into pCEP-4HA vector for hemagglutinin (HA) tagging. FLAG-tagged C subunits of all protein phosphatase (PPase) constructs were kindly provided by A.-C. Gingras (50). MCV sT and SV40 sT antigen plasmids were described previously (42) (Addgene, catalog no. 40201, for MCV sT). All sT mutants and the F-box deletion mutant form of Fbw7 (pCGN-HA.Fbw7ΔF) from wild-type constructs (42) were generated using overlapping PCR mutagenesis with primer pairs (Table 1). Codon-optimized cDNAs for the SV40 sT wild type (GenBank accession number KM359729) and sT_{L133A} mutant were cloned into modified pcDNA6 vector with EcoRV and XhoI sites (39) by PCR amplification (Table 1).

Cell culture and transfection. HEK293 cell line was maintained in Dulbecco modified Eagle medium (Cellgro, catalog no. 10-013) with 10% fetal bovine serum (Sigma-Aldrich). Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Virtual docking model by interface structure similarity. The model of MCV sT structure was generated using the I-TASSER server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) using SV 40 sT structure (PDB ID 2PF4 and 2PKG) (42). The figures were generated using PyMOL. The PP2A C subunit (P67775.1), MCV sT (YP_001651047.1), and SV40 sT (YP_003708383.1) proteins were used as ligands for docking to the cocrystal structure of PP2A A and SV40 sT (PDB ID 2PF4). The docked models are based on the high similarity of the corresponding binding interfaces between MCV sT and SV40 sT, as well as the binding mode of PP2A C and PP2A A resolved in the trimeric structure of the PP2A holoenzyme (PDB ID 2IAE).

FLAG affinity purification and MS analysis. HEK293 cells transfected with either empty vector or pCMV-tag2B.sTco (N-terminal FLAG-tagged sT) were washed twice with phosphate-buffered saline and lysed in hypotonic lysis buffer (20 mM HEPES [pH 7.9], 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride [PMSF]) containing Complete protease inhibitor cocktail (Roche) at 72 h after transfection. NaCl was added to 120 mM, and the soluble cytoplasmic fraction was isolated by centrifugation at 16,000 × *g* for 20 s. FLAG-M2 agarose resin (50% slurry) was added to the cytoplasmic fraction, incubated at 4°C for 6 h, washed three times with wash buffer (20 mM Tris-HCl, 20% glycerol, 0.2 mM EDTA, 100 mM KCl, 0.5 mM PMSF), suspended with wash buffer containing 5 μg of 3×FLAG peptide (Sigma-Aldrich)/ml, and further incubated at 4°C for 30 min to elute FLAG-sT and its interacting proteins. Purified sT protein complexes were resolved by SDS-PAGE, and unique protein bands identified by silver staining (Fig. 1A) were excised from polyacrylamide gels. Mass spectrometry (MS)-based protein identification was performed at the Mass Spectrometry Core Facility at Beth Israel Deaconess Medical Center, Boston, MA.

Immunoblotting and antibodies. Cells were lysed in buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.6% SDS, 5 mM NaF) containing protease inhibitors (Roche). The lysate was resolved by SDS-PAGE and transferred to nitrocellulose membrane (Amersham). The membranes were incubated with primary antibodies for at least 2 h at room temperature and with secondary anti-mouse IgG-HRP (Amersham) or anti-rabbit IgG-HRP (Amersham) for 1 h, and signals were detected using Western Lightning Plus-ECL reagent (Perkin-Elmer). For quantitative infrared (IR) Western blot detection, IRDye 800CW goat anti-mouse, IRDye 800CW goat anti-rabbit anti-

body, or IRDye 680RD goat anti-rat (Li-Cor) was used as a secondary antibody. Signal intensities were analyzed at 700 or 800 nm by using the Odyssey IR Imaging System (Li-Cor). The following primary antibodies were used: anti-MCV sT CM5E1 (33), CM8E6 (40), and Xt7 (http://home.ccr.cancer.gov/lco/BuckLabAntibodies.htm, kindly provided by Christopher Buck); anti-SV40 sT (pAb419, sc-58665; Santa Cruz); anti-α tubulin (B-5-1-2, T5168; Sigma-Aldrich); anti-HA (16B12; Covance); anti-FLAG (M2, F-3165; Sigma-Aldrich); anti-PP2A A-alpha (6F9; Covance), anti-PP2A C-alpha (1D6; EMD Millipore); and anti-4E-BP1^{S65} (Cell Signaling).

IP analyses. Cells were lysed in immunoprecipitation (IP) buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100) freshly supplemented with protease inhibitor cocktail (Roche), 1 mM PMSF, and 1 mM benzamide. Lysates were incubated with a specific antibody at 4°C overnight and 30 μl of 50% slurry of Protein A/G Plus agarose beads (Santa Cruz) were added for a further 3 h. Bead-antibody-antigen-protein complexes were washed with IP buffer and high-salt IP washing buffer (50 mM Tris-HCl [pH 7.4], 500 mM LiCl). Beads were resuspended in 2× SDS loading buffer, and denatured proteins were resolved by SDS-PAGE and immunoblotted. FLAG-M2 agarose resin (50% slurry) was used for FLAG-tagged PPase immunoprecipitation (Fig. 2C and D).

PP2A *in vitro* protein phosphatase assay. Cellular PP2A activity was assessed *in vitro* by using a PP2A immunoprecipitation phosphatase assay kit (Upstate). Either MCV or SV40 sT wild type and PP2A binding mutants (L142A for MCV or L133A for SV40 sT) were expressed into HEK293 cells. Cells were harvested at 48 h after transfection and cell lysates (100 μg) were used for PP2A C subunit immunoprecipitation. The phosphatase activity of PP2A was measured using a universal phosphopeptide substrate (K-R-pT-I-R-R) and malachite green phosphate detection solution according to the manufacturer's instruction. Aliquots of the same extracts were analyzed by immunoblotting to determine the expression levels of sT, PP2A C subunit proteins before the immunoprecipitation was performed.

MCV origin replication assay. The MCV replication origin assay was described previously (40). Briefly, 293 cells were transfected with T antigen expression vector (LT/sT, 0.3 μg) and pMCV-Ori339(97) (0.3 μg) by Lipofectamine 2000 (Invitrogen) in 12-well plate. Episomal DNA was collected 48 h after transfection and digested DNA with BamHI and DpnI was subjected to Southern hybridization or quantitative real-time PCR (qPCR) using SYBR green. The threshold cycle (C_T) value was calculated as fold change using $2^{-\Delta CT}$ equation, where $2^{-[CT(\text{origin replication by LT+sT}) - CT(\text{origin replication by LT})]}$. The data show the means ± the standard errors (SE) from three experiments.

RESULTS

MCV sT interacts with A and C subunits of PP2A and inhibits its activity. To verify binding between MCV sT and the PP2A A and PP2A C heterodimeric core complex, FLAG affinity purification (Fig. 1A) was performed with the cytosolic fraction of MCV sT or empty vector control expressing HEK293 cells. MCV sT-binding protein bands at 90, 70, 65, 50, 40, 35, and 30 kDa (Fig. 1A) were excised for MS protein identification. The 65- and 35-kDa proteins were identified as PP2A A and PP2A C subunits, respectively. PP4C was also recovered from a 35-kDa band, consistent with a previous report describing PP4C binding to MCV sT (51). Other MCV sT-binding proteins with multiple peptide identifications included heat shock protein 70 (HSP70), STK38, tubulins α1 and β2A, mevalonate kinase, STUB1/CHIP E3 ligase, and ADP/ATP translocase 3.

Catalytic core PP2A activity in HEK293 cells expressing either MCV sT or SV40 sT was determined *in vitro* using a phosphopeptide substrate assay (Fig. 1B). This peptide assay only measures total catalytic PP2A activity and does not rely on substrate specificity provided by the B subunit. The expression of MCV sT and SV40 sT produced similar reductions in PP2A activity in this assay. Catalytic PP2A activity was restored by alanine substitutions that disrupt the binding between PP2A and MCV sT (L142A) (39) or SV40 sT (L133A). SV40 sT is a smaller molecule than MCV sT, and the SV40 L133 residue corresponds to the MCV L142 residue.

We next assessed the potential for MCV or SV40 sT to bind with PP2A A and C subunits. This was determined by immunopurifying ectopically expressed FLAG-tagged PP2A subunit protein complexes in HEK293 cells expressing each sT protein. MCV sT was found to bind to PP2A Aα and Aβ subunits while SV40 bound only to PP2A Aα (Fig. 2A). In contrast, both MCV sT and SV40 sT were immunoprecipitated in equivalent amounts by PP2A Cα and Cβ subunits that differ only by 8 amino acid residues in their N terminus (97% homology) (18) (Fig. 2B). Although the binding between either MCV sT or SV40 sT and PP2A Cα or PP2A Cβ was stable, weaker binding between both sTs and PP1C or PP4C were also detected (Fig. 2C and D), consistent with previous findings of PP4C interaction with MCV sT (51). Despite repeated attempts, we were unable to evaluate PP6C interaction

TABLE 2 Mutational analysis of MCV sT[a]

sT mutation	Expression	PP2A Aα binding	4EBP1 phosphorylation	Enhanced DNA replication
Wild type	+	+	+	+
Exon1 (DNA J domain)				
R7A	+	–	+	+
D44N	+	+	+	+
P49A	+	+	+	+
Exon1A (Core domain)				
S80A	+	+	+	+
P84A	+	+	+	+
Y88A	+	+	+	+
K92A	+	+	+	+
Q96A	+	+	+	+
N100A	+	+	+	+
C104S	+	+	+	+
C109S	+/–	–	–	–
L114A	+	+	+	+
K118A	+	–	+	+
I122A	+/–	–	–	+
L126A	+/–	–	–	+
H130A	–	–	NT	NT
K134A	+	–	+	+
Q138A	+	+	+	+
L142A	+	–	+	+
E146A	+/–	–	–	+
Y150A	+/–	–	–	+
I154A	+	+	+	+
T162A	+	+	+	+
F166A	+	+	+	+
Q170A	+	+	+	+
E174A	+	+	+	+
Y178A	+	+	+	+
H182A	+	+	+	+
LSD_{91-95A}	+	+	–	–

[a] Mutants that do not bind PP2A but retain 4EBP1 phosphorylation and DNA replication function are shaded. NT, not tested.

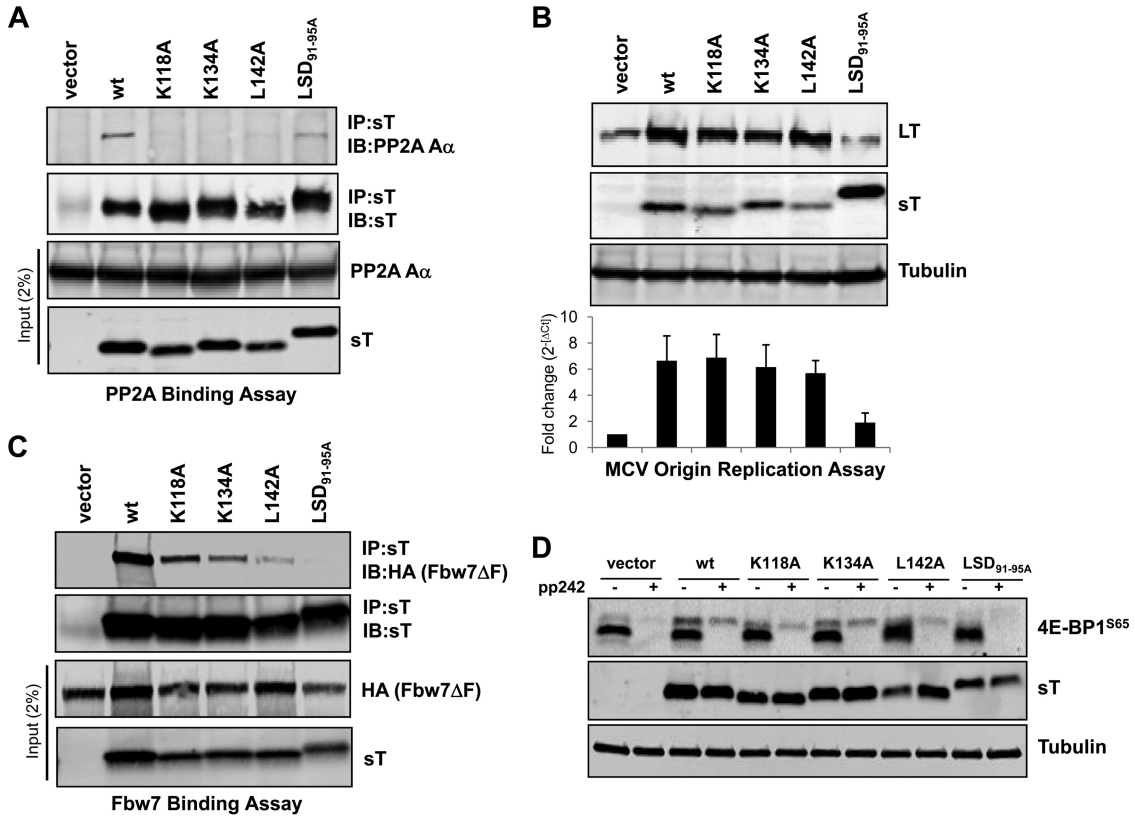


FIG 3 Mutational analysis of sT. (A) Alanine substitution mutation at MCV sT residues K118, K134, and L142 results in loss of binding to endogenous PP2A Aα subunit. The MCV sT.LSD$_{91\text{-}95A}$ mutant protein, however, retained PP2A Aα binding. (B) MCV sT enhances LT expression and origin replication in a PP2A-independent manner. MCV sT.LSD$_{91\text{-}95A}$ mutant is unable to enhance LT expression or MCV origin replication, as described previously (42). The data show the means ± the SE from three quantitative real-time PCR experiments. (C) MCV sT binds to Fbw7 independent of PP2A binding. PP2A binding mutants retained Fbw7-binding activity, whereas MCV sT.LSD$_{91\text{-}95A}$ mutant did not. The F-box deletion mutant form of Fbw7 (Fbw7ΔF) was used for the binding assay to eliminate substrate degradation (68). (D) sT hyperphosphorylates 4E-BP1 in a PP2A-independent manner. mTOR-dependent 4E-BP1 S^{65} phosphorylation was inhibited by pp242 (5 μM) for 6 h to enhance MCV sT-induced phosphorylation.

with either MCV sT or SV40 sT due to poor expression of the phosphatase protein (data not shown).

Mapping of MCV sT residues involved in PP2A A interactions. To map amino acid residues in MCV sT required for its interaction with PP2A Aα, we generated 29 alanine substitutions in MCV sT (Table 2). We first determined whether the individual alanine mutations altered the stability of the sT protein. Mutation of sT with alanine substitutions at six sites (including C109 [for which the corresponding residue has been commonly used in SV40 sT studies as a negative control], I122, L126, H130, E146, and Y150) markedly reduced sT protein stability and limited our ability to assess interactions and functional activities for these mutants (Table 2). No protein was detected for the H130A substitution mutant. We next determined whether the remaining 23 alanine substitution mutants altered binding to PP2A Aα as well as induced 4E-BP1 hyperphosphorylation (39) and promoted MCV LT-mediated DNA replication in an MCV origin replication assay (42). Alanine substitution at four destabilizing residues (I122, L126, E146, and Y150) were negative for PP2A binding and 4E-BP1 phosphorylation, presumably due to reduced sT expression but retained activity for MCV DNA replication (Table 2), consistent with our previous report showing that minute amounts of sT coexpression are capable of maximally activating LT-induced DNA replication (42).

Four substitution mutations (R7, K118, K134, and L142) were found to inhibit PP2A Aα subunit binding despite abundant sT protein expression. These interactions for K118, K134, and L142 are illustrated in Fig. 3A (see also references 39 and 42). These mutations did not lose LT stabilization or DNA replication functions (Fig. 3B), and they retained Fbw7 binding and induced hyperphosphorylation at S65 of 4E-BP1 (Fig. 3C and D). Only mutations to the LSD (91-95A) reduced Fbw7 binding and sT-induced 4E-BP1 hyperphosphorylation (Fig. 3C and D); however, this mutant retained PP2A Aα binding. (Fig. 3A). Figure 4 shows the similarity in predicted models for MCV and SV40 sT structures. For SV40, residues (R7, R21, and P132) have been previously reported to be critical for sT interaction with PP2A Aα (28). Three MCV sT residues that we found critical for PP2A binding (R7, L142, and K134) are on the same planar surface as the reported SV40 sT PP2A binding sites. Comparison of the MCV sT alanine substitution data shown here and previously published SV40 sT data are consistent with binding of PP2A Aα to corresponding surfaces on both viral sT proteins (Fig. 4).

A computational docking model suggests that MCV sT competes with the regulatory B subunits for assembly of the holoenzyme (Fig. 5A), indicating that sT domains might displace the regulatory B subunit from binding to PP2A core enzyme. R7 and L142 lie on an exposed ridge that is predicted to facilitate MCV sT

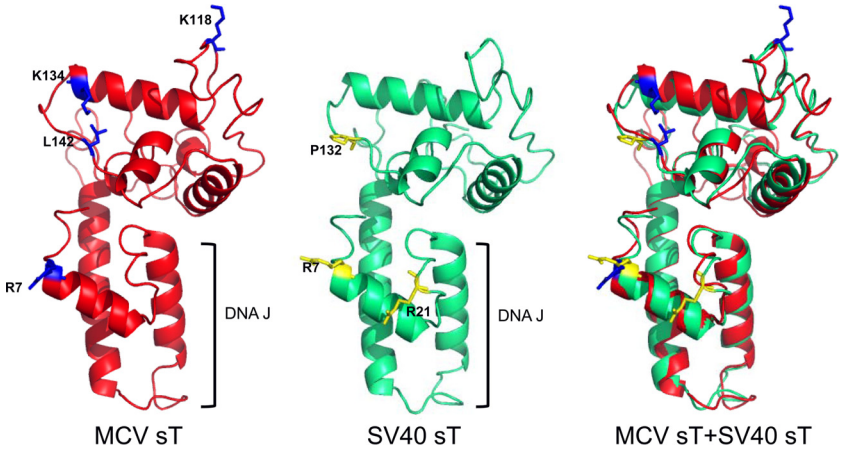


FIG 4 Predicted ribbon structures for MCV (red) and SV40 (green) showing critical PP2A interaction residues. Scanning alanine mutations demonstrated that MCV sT amino acids R7, K118, K134, and L142 (blue) are critical PP2A interaction sites (Table 2 and (39). These residues are compared to similar sites in SV40 sT (yellow) (14). Superimposed structures of MCV sT and SV40 sT are shown on right (the root mean square deviation value is 1.254). Images were generated using PyMOL.

binding to PP2A Aα (Fig. 5B) (42). K118 may be critical for PP2A C docking, although both PP2A Aα and PP2A C failed to coimmunoprecipitate with this MCV sT mutant, suggesting that it may play a role in proper alignment of both PP2A subunits on sT, as well as direct interaction with PP2C. All four PP2A A binding residues (R7, K118, K134, and L142) lie on the opposite planar face from the LSD loop.

MCV sT displaces a specific regulatory subunit, B56α, in the PP2A complex. SV40 sT binding to PP2A Aα competes with regulatory PP2A B subunits binding to PP2A Aα (48). To determine whether MCV sT can displace PP2A B subunits from the PP2A holoenzyme via a similar mechanism, we immunoprecipitated HA epitope-tagged PP2A B subunits with either PP2A A or PP2A C from HEK293 cells expressing either MCV sT or SV40 sT (Fig. 6). MCV sT_{L142A} and SV40 sT_{L133A} were used as negative controls. Five PP2A B subunits (B55α, B56α, B56γ3, B56δ, and B56ε) were immunoprecipitatable with PP2A A and PP2A C proteins in 293 cells. Of these five, MCV sT efficiently inhibited only B56α (also called B′α/PPP2R5A/PR61α) interaction with PP2A A and PP2A C, an effect not seen with the MCV sT_{L142A} protein (Fig. 6). In contrast, SV40 sT had broader PP2A B subunit displacement activity and diminished or completely eliminated B55α, B56α, and B56ε interactions with PP2A A and C proteins. This displacement was abolished with an alanine substitution at L133 in SV40 sT that corresponds to L142 in MCV sT.

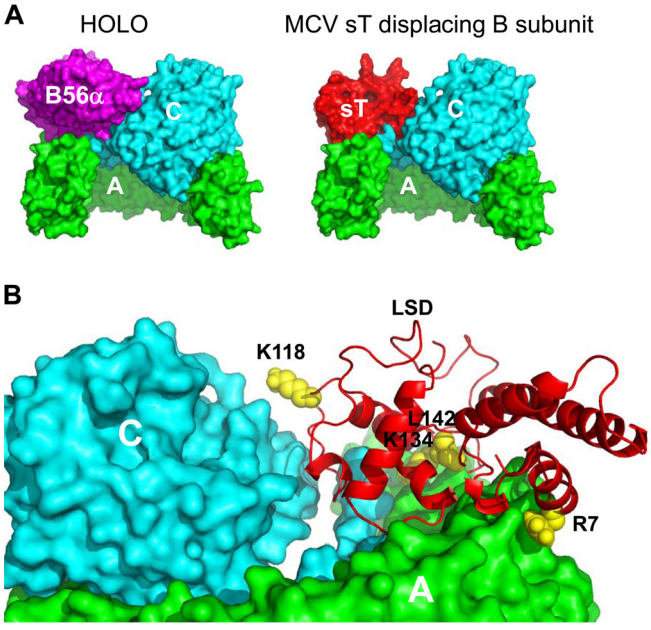


FIG 5 Docking model for MCV sT with PP2A A-C core enzyme complex. (A) A surface representation of the PP2A holoenzyme is shown on the left (28). MCV sT directly interacts with both PP2A scaffolding A and catalytic C subunits, displacing the regulatory B subunit (right). (B) Residues R7, K118, K134, and L142 are situated in close contact with the PP2A complex but lie on the opposite planar face of the sT molecule from the LSD loop responsible for Fbw7 binding.

DISCUSSION

The downregulation of protein phosphatase activity by viral proteins is believed to be a principal mechanism through which viruses change multiple intracellular signaling pathways. We show here that MCV sT binds to PP2A A and C proteins similar to well-characterized SV40 sT-PP2A interactions but that MCV sT displacement of PP2A B subunits is more restricted since it only displaces B56α from the PP2A holoenzyme. In contrast, while SV40 sT only binds to the PP2A Aα subunit, SV40 sT displaces more PP2A B subunits, including B55α, B56α, and B56ε, possibly due to higher affinity of SV40 sT binding to PP2A A or C proteins. One caveat to our study is that it does not take into account differences in PP2A interactions within the natural host, which is the rhesus macaque for SV40. Importantly, however, we show here that MCV sT interaction with human PP2A is genetically separable from Fbw7 targeting. Recent studies (M. Shuda, C. Velasquez, H. J. Kwun, P. S. Moore, and Y. Chang, unpublished observations) reveal the MCV LSD to be a promiscuous E3 ligase targeting domain interacting with multiple E3 ligases.

MCV sT, but not SV40 sT expression is sufficient to transform rodent fibroblasts (39, 42, 52). However, despite the importance of PP2A targeting in defined oncogene transformation studies in human cells (15), MCV sT transformation in rodent cells depends

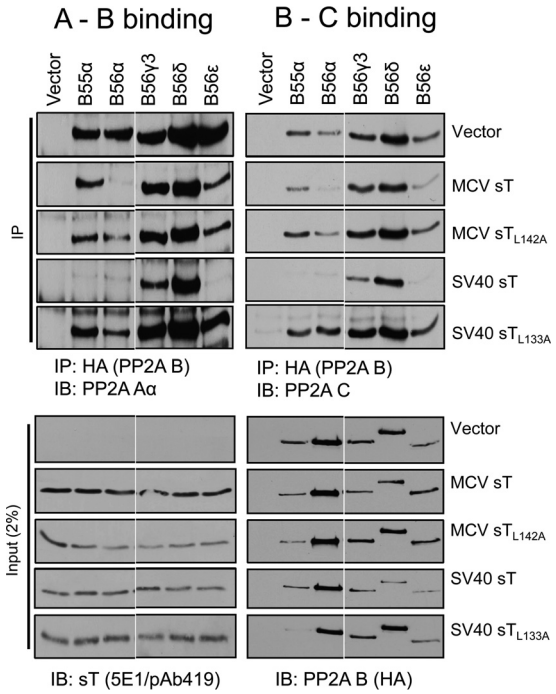


FIG 6 MCV sT inhibits B56α complex incorporation into the PP2A holoenzyme. The holoenzyme complex formation of various HA-tagged PP2A B subunits (B55α, B56α, B56γ3, B56δ, and B56ε) in the presence of small T antigens was assessed by immunoprecipitation in 293 cells. PP2A Aα and PP2A B subunit (left upper panel) or PP2A C and PP2A B subunit (right upper panel) interactions were compared in the presence of sT (MCV sT or SV40 sT) or PP2A binding mutants (MCV sT$_{L142A}$ or SV40 sT$_{L133A}$). MCV sT expression specifically attenuates B56α interaction to PP2A Aα and PP2A C. In contrast, SV40 sT displaces B55α, B56α, and B56ε incorporation into the holoenzyme. The expression level of input proteins (2%) was confirmed by immunoblotting (lower panels).

on activities that map to the LSD domain rather than the PP2A targeting domain. The MCV sT LSD inhibits specific E3 ligases, including Fbw7 (42, 45), highlighting the alternative contribution of protein stability to cancer cell signaling. We did not find that MCV sT expression alone is capable of transforming primary human fibroblasts (39).

MCV sT binds to PP4C, which disrupts NF-κB essential modulator (NEMO) to limit activation of NF-κB (51), as well as PP2A. MCV sT binding to PP4C has also been found to promote cell motility through microtubule destabilization, and this may contribute to the aggressiveness of MCV-MCC tumors (53). Our MS and immunoprecipitation analyses confirm the binding of MCV sT and PP4C. Various catalytic subunits of protein phosphatases share extensive sequence similarity with the PP2A C subunit, including PP4C and PP6C, and PP4C that bind to the PP2A scaffold A subunit (54). This suggests that the disruption of PP4C by both MCV and SV40 sT may be related to PP2A A subunit interactions. Although we have not examined it here, PP1 disruption by sT is significant in tumorigenesis studies since PP1 is required for the activation of the retinoblastoma tumor suppressor, Rb (55–57). Although MCV sT does not contain the consensus PP1 binding motif (RVxF), a potential mechanistic connection between MCV sT and PP1 remains to be addressed.

Tumor induction by viruses is a biological accident that provides the virus no selective advantage (58, 59). Evidence, however, suggests functional overlap between innate immune signaling and tumor suppressor pathways (59). This may help explain why viruses disrupt many tumor suppressors if these tumor suppressor proteins have dual innate immune activity as well. Akt and mTOR activation are induced by several unrelated tumor viruses (60–63), underscoring the importance of this pathway for virus propagation and proliferation (64–66). SV40 sT activates Akt (43), a protein serine/threonine kinase, causing increased mTORC1 activation associated with decreased inhibitory PP2A dephosphorylation (67). MCV sT targets an end-effector of this pathway to promote cap-dependent translation but does so through a novel mTOR-independent mechanism (38, 39). We find that both viruses disrupt PP2A B56α phosphatase activity, suggesting that inhibition of this portion of PP2A activity may be important for polyomavirus survival in cells.

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