Serine 220 phosphorylation of the Merkel cell polyomavirus large T antigen crucially supports growth of Merkel cell carcinoma cells

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Merkel cell polyomavirus (MCPyV) is regarded as a major causal factor for Merkel cell carcinoma (MCC). Indeed, tumor cell growth of MCPyV-positive MCC cells is dependent on the expression of a truncated viral Large T antigen (LT) with an intact retinoblastoma protein (RB)-binding site. Here we determined the phosphorylation pattern of a truncated MCPyV-LT characteristically for MCC by mass spectrometry revealing MCPyV-LT as multi-phospho-protein phosphorylated at several serine and threonine residues. Remarkably, disruption of most of these phosphorylation sites did not affect its ability to rescue knockdown of endogenous T antigens in MCC cells indicating that phosphorylation of the respective amino acids is not essential for the growth promoting function of MCPyV-LT. However, alteration of serine 220 to alanine completely abolished the ability of MCPyV-LT to support proliferation of MCC cells. Conversely, mimicking the phosphorylated state by mutation of serine 220 to glutamic acid resulted in a fully functional LT. Moreover, MCPyV-LTS220A demonstrated reduced binding to RB in co-immunoprecipitation experiments as well as weaker induction of RB target genes in MCC cells. In conclusion, we provide evidence that phosphorylation of serine 220 is required for efficient RB inactivation in MCC and may therefore be a potential target for future therapeutic approaches.

Merkel cell carcinoma (MCC) is a very aggressive skin cancer translating into high mortality rates. Immunosuppression is one of the risk factors for developing MCC.1 This may be explained by the fact that a viral infection contributes to MCC. In this regard, DNA of the Merkel cell polyomavirus (MCPyV) is detected in the vast majority of MCC cases,2 and monoclonal integration of MCPyV into the tumor genome indicates that viral integration occurs prior to clonal expansion of the tumor cells.3

Polyomaviruses are small DNA viruses with a circular double-stranded genome encoding five – nine proteins.4 From the early region different oncproteins termed T antigens (TA) are derived by translation of differentially spliced mRNAs. In MCPyV-positive MCC cells the viral small and Large T antigen (sT and LT) are expressed.5,6 LT and sT share the same N-terminal region comprising 78 amino acids but differ in their C-terminus. Importantly, MCC-associated MCPyV-LTs are characterized by large C-terminal deletions.5,7 These truncated LTs result from point mutations leading to premature stop codons, or are due to integration break points. Generally, however, the retinoblastoma protein (RB) binding motif is preserved, and a mutation affecting RB binding abolishes the ability of MCPyV-LT to promote growth of MCC cells.8

The molecular function of a protein is not only dependent on its amino acid sequence, but also determined by post-translational modifications. A prominent type of post-translational modification is phosphorylation.9 It has been estimated that 30% of all cellular proteins contain at least one phosphorylated residue.10 Hence, it is not surprising that polyomavirus T antigens are also subject to phosphorylation by cellular protein kinases.11 For example, middle T antigen, i.e. the major transforming protein of the murine polyomavirus, is phosphorylated at several tyrosine residues, and these phosphorylation events affect different signal transduction pathways as well as transformation.11 In contrast, SV40-LT is phosphorylated at many serine and threonine residues, and some of these sites have been implicated in either facilitating or repressing LT-mediated SV40 replication.12

Key words: merkel cell carcinoma, polyomavirus, Large T antigen, phosphorylation

Abbreviations: LT: Large T antigen; MCC: Merkel cell carcinoma; RB: retinoblastoma; TA: T antigens

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Grant sponsor: German Research Foundation; Grant number: HO5280/2-1

DOI: 10.1002/ijc.29862

History: Received 30 June 2015; Accepted 10 Sep 2015; Online 18 Sep 2015

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What's new?
To find possible points of attack, this study investigated the phosphorylation pattern of a viral protein that causes skin cancer. The large T antigen produced by Merkel cell polyomavirus contains a retinoblastoma protein binding site and drives tumor growth. These authors located several serine and threonine residues in the protein which become phosphorylated. Upon testing several variants, each containing a disruption in one of these phosphorylation sites, they found one, serine 220, which had lost the ability to bind RB and failed to promote cell proliferation. Disrupting this site, then, could halt the spread of Merkel cell carcinoma.

MCPyV-LT is the first polyomavirus LT known to be essential in human tumor cells. Elucidating the functional consequence of post-translational modifications of MCPyV-LT in MCC cells bears the potential of identifying molecular targets for future therapies. Therefore, we applied mass spectrometry to identify phosphorylated amino acids in MCPyV-LT. Subsequently, we performed rescue experiments in which we re-introduced several LT phospho-site mutants into MCC depleted of endogenous LT by lentiviral shRNA expression to determine the relevance of phosphorylation for protein function. Our results suggest that phosphorylation of serine 220 in MCPyV-LT is required for RB inactivation and thus is essential to support growth of MCC cells.

Material and Methods
Cloning and mutagenesis
To allow affinity purification of MCPyV-LT, a His-tag was fused to the C-terminus of a truncated MCPyV-LT. By PCR using as reverse primer 5’-CTA TCA GTG GTG ATG ATG GCC TGA TCC AGA CTT TCT TGA GAA the coding sequences for a 6xHis-tag and the coding sequences for a 6xHis-tag and a GSGS spacer were added to the 3’-end of a cDNA coding for the 278 amino acid LT protein as it is expressed in the MCC cell line WaGa.13 The PCR product was cloned into the pCDH vector.

For inducible knockdown of MCPyV-LT, we used the lentiviral single vector TA.shRNA.tet which allows constitutive GFP expression and doxycycline-inducible expression of an shRNA targeting all transcripts derived from MCPyV early region.8 A cDNA coding for GFP C-terminally fused to truncated MCPyV-LT was generated by first cloning a GFP sequence derived by PCR from the pGreenFire vector (System Biosciences) using the primers 5’-ACA CAC AGA GCG ACG AGA GCC GC-3’ and 5’-ACA CAC GAA TTC TCA GGC GAA GAC GAT GGG GGT C-3’ into the Xmal/EcoRI sites of the lentiviral vector pLVX-TRE3G-ires (Clontech). Next, truncated MCPyV-LT was cloned 5’ and in frame with GFP into the BamHI/Xmal sites thereby removing the IRES. As forward primer we used 5’-ACA CAC AGA TCT ACC GCC ATG ATG TTA GTC CTA AAT AG-3’, and to generate the coding sequence for LT334-GFP and LT254-GFP (the 334 and 254 N-terminal amino acids of MCPyV-LT) the primers 5’-ACA CAC CCC GGG TTA CGT TTT TAT TAC TAT ATA-3’ and 5’-ACA CAT CCC GGG AGA TAA CGA GCC TCT CTC GGC-3’ were applied, respectively, using a cDNA coding for MCC206 MCPyV-LT (GenBank: FJ173812.1) as template. Several point mutations in the LT coding sequence were introduced using the Quik change lightning site directed mutagenesis kit (Stratagene).

MCC cell lines stably expressing ectopic shRNA-insensitive MCPyV-LT were generated by stable transduction with a LT-cDNA cloned into the retroviral vector pH containing a hygromycin resistance gene. Using the quick change mutagenesis kit (Stratagene) the ectopically expressed MCPyV mRNAs were made insensitive to the TA-shRNA by introduction of 6 silent mutations in the TA-shRNA target sequence.8 Furthermore, for the exchange of amino acids identified as potential phosphorylation sites either to alanine (A) or to glutamic acid (E), mutagenesis was used to alter the coding sequence of MCPyV-LT. The intended constructs were verified by sequencing of the complete coding sequence. Retrovirus-containing supernatants were generated by transient transfection of HEK293T, and infection of target cells was carried out as previously described.8

Purification of a His-tagged MCPyV-LT
HEK 293T cells were transfected with pCDH LT-His and RIPA-Buffer lysates were prepared on day 2. After mixing with an equal volume of equilibration/wash buffer (50 mM sodium phosphate, 300 mM sodium chloride, 10 mM imidazole; pH 7.4), the lysate was transferred to a cobalt spin column (Thermo Scientific) and incubated for 30 min on a rotator. Following two wash steps according to the manufacturer’s instructions, the protein was eluted with 50 mM sodium phosphate, 300 mM sodium chloride, 150 mM imidazole; pH 7.4. The eluted protein was concentrated under vacuum and then subjected to SDS-PAGE followed by staining of the gel with Coomassie Blue (Thermo Scientific).

Mass spectrometry
For in-gel digestion, the excised gel bands were destained with 30% ACN, shrun with 100% ACN and dried in a Vacuum Concentrator (Concentrator 5301, Eppendorf, Hamburg, Germany). Digests with trypsin and elastase were performed overnight at 37°C in 0.05 M NH4HCO3 (pH 8). Digests with thermolysin were performed for 2 h at 60°C in 0.05 M NH4HCO3 (pH 8). About 0.1 μg of protease was used for one gel band. Peptides were extracted from the gel slices with 5% formic acid. NanoLC-MS/MS analyses were performed on an LTQ-Orbitrap Velos Pro (Thermo Scientific) equipped
with an EASY-Spray Ion Source and coupled to an EASY-nLC 1000 (Thermo Scientific). Peptides were loaded on a trapping column (2 cm x 75 μm ID. PepMap C18 3 μm particles, 100 Å pore size) and separated on an EASY-Spray column (25 cm x 75 μm ID, PepMap C18 2 μm particles, 100 Å pore size) with a 45 min linear gradient from 3% to 30% acetonitrile and 0.1% formic acid. MS scans were acquired in the Orbitrap analyzer with a resolution of 30,000 at m/z 400, MS/MS scans were acquired in the Orbitrap analyzer with a resolution of 7,500 at m/z 400 using HCD fragmentation with 30% normalized collision energy. A TOP5 data-dependent MS/MS method was used; dynamic exclusion was applied with a repeat count of 1 and exclusion duration of 30 sec; singly charged precursors were excluded from selection. Minimum signal threshold for precursor selection was set to 50,000. Predictive AGC was used with AGC target a value of 1e6 for MS scans and 5e4 for MS/MS scans. The same options were used for ETD fragmentation except for the following settings: A TOP3 method was applied, singly and doubly charged precursors were excluded, ETD activation time was set to 60 ms for triply and 45 ms for quadruply charged precursors, AGC target was set to 300,000 for fluoranthene. Lock mass option was applied for internal calibration in all runs using background ions from protonated decamethylcyclopentasiloxane (m/z 371.10124).

Mascot Distiller 2.4 was used for raw data processing and for generating peak lists, essentially with standard settings for the Orbitrap Velos (high/high settings). Mascot Server 2.4 was used for database searching with the following parameters: peptide mass tolerance: 8 ppm, MS/MS mass tolerance: 0.02 Da, enzyme: "trypsin" with three missed cleavage sites allowed for trypsin or "none" for elastase and thermolysin; fixed modification: carbamidomethyl (C), variable modifications: Gln->pyroGlu (N-term. Q), oxidation (M) and phosphorylation (STY). Database searching was performed against a custom database containing the His-tagged sequence of MCPyV-LT. All identified phosphopeptides were verified manually.

Cell culture
The MCPyV-positive MCC cell lines MKL-114 and PeTa15 as well as MCC1316 and HEK293T cells were grown in RPMI 1640 supplemented with 10% FCS, 100 U/mL penicillin and 0.1 mg/mL streptomycin. To establish pure populations of cells with integrated retroviral pH expression vectors 100 μg/mL Hygromycin B was added to the culture medium for 2 weeks. Pure cell populations carrying the TA.shRNA.tet coding for GFP were established by fluorescence activated cell sorting.

Mixed cell culture assay
GFP expression of TA.shRNA.tet transduced cells was used to compare the growth behavior of transduced and untransduced cells: TA.shRNA.tet cells were mixed with approximately 20% of untransduced cells, and changes in the frequency of GFP-positive TA.shRNA cells were determined by flow cytometry over time in the absence or presence of 1 μM doxycycline.

Cell cycle analysis
Cells were fixed with ethanol, and DNA was stained with propidium iodide as described. Analysis of the cellular DNA content was performed on a FACSCanto flow cytometer (BD Biosciences).

Immunoblotting
Immunoblotting was performed as previously described with total cell lysates harvested on Day 5 following addition of doxycycline to the TA.shRNA.tet cells. The antibodies used in this study were directed against MCPyV-LT (CM2B4; Santa Cruz Biotechnologies), the V5 tag (SV5-Pk1; Abcam), His tag (D311O; Cell Signaling) or β-tubulin (TUB 2.1; Sigma-Aldrich).

Co-immunoprecipitation
Using TurboFect transfection reagent (Thermo Scientific) 293T cells were co-transfected with expression constructs coding for 6xHIS tagged RB1 and different V5 tagged MCPyV-LT278 versions with either serine, alanine or glutamic acid at position 220. 24 h after transfection, total cell lysates were harvested with lysis buffer (50 mM Tris-HCl, 0.15 M NaCl, 1% Triton-X100, pH 7.4) supplemented with protease and phosphatase inhibitors. Lysates were incubated with constant shaking overnight at 4°C with anti-His tag antibody (D311O; Cell Signaling). Then, protein G sepharose beads (Roche) were incubated with the lysates for 3 h at 4°C. Beads were collected by centrifugation and, following 4 wash steps with lysis buffer, boiled with SDS loading buffer. The supernatant was subsequently loaded on to a SDS/PAGE gel, and proteins were analyzed by immunoblotting.

Real time PCR
Total RNA was isolated with Qiagen RNAeasy columns and reverse transcribed using the Superscript II RT First Strand Kit (Invitrogen). Real time PCR was conducted in the ABI 7500 Fast Real-Time PCR cycler (Applied Biosystems) using a SybrGreen I Low Rox Mastermix (Eurogentec GmbH) and the following primers:

- MYB: TCCACACTGCCAAGTCTCT; AGCAAGCTGTTCATTTGGA
- CDC6: CCTGTTTCTTCGTAAGA; GTGTGCACAGTTGTCATCG;
PLK1: AAGATCTGGAGTGAAATAGGG; AGGAGTCCCACACAGGGTC;
CCNB1: GCCTGAGCCTATTTTGGT, GCACATCCAGATGGTCCCT;
RPLP0: CCATCAGCACCAGCCCTTC; GGCAGGCTGGAAGTCCAACT;

Relative expression levels were determined applying the comparative ΔΔCT method, and given as percent relative to the vector control.

Results and Discussion

Truncated MCPyV-LT is a multi-phosphoprotein

Phosphorylation of proteins at distinct sites is an important mechanism to regulate their activity.9 To identify potential phosphorylation sites in truncated MCPyV-LT mass spectrometry analysis was performed. To this end, a 6xHis tag was added to the C-terminus of the truncated LT protein expressed in the MCC cell line WaGa,13 and ectopically expressed in HEK 293T cells. Following affinity purification and digestion, the peptides were analyzed by nanoLC-MS/MS. Based on these analyses we conclude that at least 17 serine (S) or threonine (T) residues in the truncated MCPyV-LT that includes the first 278 N-terminal amino acids of wild type LT can be modified by phosphorylation (Fig. 1b) while we could not detect any phosphorylated tyrosine. 9 single phosphorylation-sites could be unambiguously identified: S100, S134, S147, T192, S239, S254, T257, S265 and T271. For other regions the distinct phosphorylation-sites could not unequivocally be concluded since the analyzed peptides contained more potential phospho-acceptor sites than detectable phosphates. For instance, at least one amino acid within a peptide containing S202, S203 and T205 and at least two residues of the threonine/serine cluster between amino acids 172 and 179, the 3 consecutive serines S225-S227, the serine-rich regions comprising S217-S220 and S245-S247 are phosphorylated in truncated MCPyV-LT expressed in 293T cells (Fig. 1b). It has to be noted that some of these potential phospho-acceptor sites are not present in all MCPyV-LTs due to naturally occurring polymorphisms, i.e. S177Y, S226P, S245C and S254F, suggesting that phosphorylation at these specific sites is probably not necessary for LT function. Notably, identification of phospho-T271 confirms findings of a recent paper reporting phosphorylation of T271, T295 and T299 in full length MCPyV-LT ecotopically expressed in HEK 293 cells.17 Functional analyses demonstrated that phosphorylation of T295 and T299, which lack in our examined truncated LT, may regulate the initiation of MCPyV replication in a repressive or supportive fashion, respectively, while phosphorylation of T271 had no impact on this function of MCPyV-LT.17 Surprisingly, Diaz and colleagues did not report any of the other 17 phosphorylation sites within the N-terminal 278 amino acids of MCPyV-LT identified in our current study. This discrepancy could represent differences between the full length and the truncated protein. More likely, however, the diverse findings may be due to the limited coverage of only 45% and 25% of the LT sequence that these authors obtained following analysis of peptides derived from trypsin digestion and purification of full length MCPyV-LT using two different protocols.17 In contrast, our peptide analyses were derived from separate digestions with three different enzymes, which yielded 100% coverage of the amino acid sequence of truncated MCPyV-LT.18

Figure 1. Potential phosphorylation sites detected in MCPyV-LT278. (a) Schematic illustration of the truncated MCPyV Large T proteins analyzed in this study. LT278-His was used for mass spectrometry analysis (Fig. 1b). LT278 proteins carrying different point mutations were expressed in the rescue assays (Figs. 2 and 6) and in the cell cycle experiments (Fig. 3). The two different LT-GFP proteins either possessing or lacking the nuclear localization sequence (NLS) were analyzed in the localization studies (Fig. 5). (b) His tagged MCPyV-LT278 was expressed in 293T cells. Following affinity purification and digestion, the peptides were analyzed by nanoLC-MS/MS. Depicted is the amino acid sequence with the 278 amino acids of the analyzed MCPyV-LT278 in bold, and the sequence proceeding in larger MCPyV-LTs in italic. The RB-binding motif (LxCxE) is highlighted in blue, the nuclear localization sequence (NLS) in orange, and the regions of the protein which can be deleted without affecting the function of MCPyV-LT in grey. Additionally, serine or threonine residues which unambiguously were found phosphorylated are marked in green and serine/threonine cluster where at least one or two of several amino acids are phosphorylated are marked in yellow and red, respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
LT S220A mutation impairs growth supporting activity of MCPyV-LT in MCC cells

Next, we evaluated the impact of phosphorylation of several of the identified phosphorylation sites on function of truncated MCPyV-LT in MCC cells. Since our previous work had demonstrated that RB binding is essential for MCPyV-LT function, and large parts of the protein upstream or downstream of this region can be deleted without affecting its function, we focused our analysis on those sites located closely to the RB binding motif (Figs. 1 and 2). To this end, we determined the ability of mutant LT proteins to rescue the growth arrest induced by knockdown of endogenous MCPyV-LT in MCC cells. These analyses were performed in the MCC cell line MKL-1 engineered for doxycycline-inducible expression of an shRNA targeting all T antigen mRNAs (TA.shRNA.tet) by transducing them with an empty vector or with expression constructs coding for different shRNA-insensitive MCPyV-LTs. Since the TA.shRNA.tet vector also encodes for a green fluorescent protein, this system allows to measure TA-shRNA-induced changes on proliferation in mixed cultures with unmarked control cells. As expected from our previous work, addition of doxycycline to the culture medium induced an efficient knockdown of endogenous MCPyV-LT translating into reduced cell growth (Fig. 2; panel vector). In contrast, growth of MKL-1 cells stably transduced with TA-shRNA-insensitive, truncated MCPyV-LT consisting of the 278 N-terminal amino acids (LTWT) was not affected by doxycycline-induced shRNA-expression (Fig. 2; panel LTWT). This unambiguously demonstrates that inhibition of proliferation induced by the TA-shRNA is due to knockdown of MCPyV-LT expression.
Moreover, this rescue assay can be used for functional characterization of the putative MCPyV-LT phosphorylation sites in the natural tumor host cells of the viral protein. Hence, we modified the selected serines or threonines either to alanine (A) precluding addition of a phosphate group, or to glutamic acid (E) mimicking phosphorylation. Strikingly, the exchange of most of the potential phosphorylation sites (T192, S202, S203, T205, S217, S225, S226, S227 and S239) to alanine or to glutamic acid did not alter the rescue activity of MCPyV-LT in MKL-1 cells (data for T192, S217 and S239 mutants are shown in Figure 2; the full data set is depicted in the Supporting Information Fig. S1) demonstrating that phosphorylation of all these individual amino acids by itself is not critically regulating MCPyV-LT’s function on MCC cell proliferation. In contrast, mutation of S220 to alanine and to a lesser extent the S219A mutation reduced the ability of MCPyV-LT to support proliferation of MKL-1 cells (Fig. 2; panel LT$^{S219A}$ and LT$^{S220A}$). Thus, phosphate residues at these two acceptor sites very close to the RB-binding motif (Fig. 1) seem to be required for full activity of MCPyV-LT. Accordingly, for both serines the exchange to glutamic acid mimicking phosphorylation fully restored the rescue activity of MCPyV-LT (Fig. 2; panel LT$^{S219E}$ and LT$^{S220E}$). Since the impact of the S220A mutation on MCPyV-LT function was more pronounced than for S219A, we focused on S220 in our further investigations.

**The S220A mutation affects the ability of MCPyV-LT to promote cell cycle progression**

To test whether the observed impairment of LT function by the S220A mutation is restricted to established MCPyV-positive tumor cells, we analyzed the cell cycle distribution of the MCPyV-negative cell line MCC13 following transduction with truncated LT. While ectopic expression of LT$^{wt}$ and LT$^{S220E}$ was associated with a modest but consistent increase of cells in S and G2/M phases, LT$^{S220A}$ did not change the cell cycle distribution measured 24 h after lentiviral infection (Fig. 3) further supporting that phosphorylation of S220 is essential for LT-driven proliferation.

**S220 does not fulfill the minimal requirements of a CKII target site but may be targeted by a proline-directed kinase**

Generally, in polyomavirus LT proteins as well as in other viral RB1-binding proteins like papillomavirus E7 or adenovirus E1A one or more serine residues are located a few amino acids C-terminal of the LxCxE motif (Fig. 4). For some of these serines (e.g. S in position +5 in SV-40 LT or S in position +6 relative to LxCxE in HPV-16 E7) their phosphorylation by casein kinase II (CKII) has been reported.20,21 Indeed, these residues - as well as many of the corresponding serines in other LT proteins and in E1A - perfectly match the CKII target consensus sequence S/T-E/D-x-E/D, i.e. a serine or threonine followed by acidic amino acids in positions 1 and 3 (Fig. 4; 9). Although exceptions from this consensus sequence have been described, all described CKII target sites (in 2003 already 308 different sites had been reported) contain as minimum requirement an acidic amino acid in either position +1 or +3 relative to the phospho-acceptor site.22 Consequently, from the two potential phosphorylation sites adjacent to the LxCxE in MCPyV-LT, only S219 may be targeted by CKII, due to the presence of an E in +3. For S220, however, its phosphorylation by CKII seems unlikely, since this site has two prolines at positions +1 and +3. Indeed, pharmacological inhibition of CKII did not select for LT$^{S220E}$ expressing MCC cells in mixed cultures with MCC cells expressing only wild type LT (data not shown). Both, the lack of a CKII consensus site and the presence of proline (P) residues +1 and/or +3 relative to the serine is shared with the LT proteins from Ki and murine polyomavirus as well as with two very closely related LTs from gorilla and chimpanzee (GggPyV1 and MpyV1)
which, however, lacks an LxCxE motif.37 [Color figure can be viewed online.]

One more human polyomavirus (HPyV12) has been described especially when S220 is phosphorylated, matches the CKII consensus. (GggPyV1) and chimpanzee (PTVPyV2a). S219 in MCPyV-LT, especially two LT proteins from the highly related polyomaviruses from gorilla (GggPyV1) and chimpanzee (PTVPyV2a). S219 in MCPyV-LT, especially when S220 is phosphorylated, matches the CKII consensus. One more human polyomavirus (HPyV12) has been described which, however, lacks an LxCxE motif.37 [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

PTVPyV2a, respectively). Notably, only in the latter and in MCPyV-LT this serine is located four amino acids C-terminal of the LxCxE motif (Fig. 4). S/T-P is a major regulatory phosphorylation motif that is targeted by a large family of so-called proline-directed protein kinases including cyclin-dependent protein kinases (CDKs), mitogen-activated protein kinases (MAPks) and glycosynthetic kinases-3 (GSK-3).39 Only upon phosphorylation, S/T-P motifs can be further targeted by the peptidyl-prolyl isomerase Pin1. In this regard, proline residues bear among the proteinogenic amino acids the unique feature of being able to switch between two distinct conformational states and Pin1-mediated prolyl isomerization can induce significant changes in protein structure regulating functions like protein stability, protein–protein interactions or subcellular localization.24

No evidence for a role of phosphorylation in nuclear import of MCPyV-LT

For SV40-LT it has been demonstrated that phosphorylation of S112 (+5 relative to LxCxE) is critical for subcellular localization of the protein by enhancing its nuclear import.25 Indeed, substitution of S111 and S112 (both are targeted by CKII with S112 as the preferred phosphorylation site), or mutation of the adjacent CKII recognition sequence DDE to NQQ resulted in nuclear import rates <4% compared to wild type.26 To study the impact of the phospho-sites identified in MCPyV-LT on nuclear import, we expressed LT-GFP fusion proteins in NIH3T3 cells. As expected, MCPyV-LT truncated after amino acid 334 (LT334-GFP) containing the nuclear localization sequence (NLS; amino acids 277–280; Fig. 1) was strictly located in the nucleus, while LT truncated at amino acid 254 (LT254-GFP) resided in the cytoplasm (Fig. 1a). When an S220A or an S220E mutation was introduced in LT254-GFP this did not alter nuclear localization of the protein suggesting that phosphorylation of S220 is not relevant for nuclear import (Fig. 1a for S220A; data not shown for S220E). This result was expected since studies reported that phospho-amino acids facilitating nuclear import are generally located 10 to 30 amino acids upstream of a corresponding nuclear localization sequence NLS.28,29 This holds true for S112 in SV40-LT being 12 amino acids upstream of the NLS. In contrast, S220 in MCPyV-LT is located 58 amino acids away from the NLS (Fig. 1). Consequently, to investigate whether any of the potential phospho-acceptor sites identified by mass spectrometry located between 6 and 31 amino acids upstream of the NLS in MCPyV-LT (Fig. 1b) is involved in regulation of the nuclear import, we exchanged all the respective serines and threonines (S246, S247, S254, T257, S265, T271) to alanine in LT334-GFP. This protein, however, still demonstrated strict nuclear localization indicating that phosphorylation of none of these sites is required for nuclear localization (Fig. 5a). However, phosphorylation might have an impact on the nuclear import rate which is not detectable under steady state conditions. Therefore, we used time lapse microscopy to analyze the distribution of LT-GFP in the course of mitosis. Upon breakdown of the nuclear envelope in prophase LT-GFP is evenly distributed all over the cell, and starts to accumulate in the reforming nucleus in telophase (Fig. 5b). Neither the time from breakdown of the nuclear envelope to complete nuclear localization (X-Z) nor the mere accumulation period (Y-Z) (Fig. 5b) is substantially affected by introducing the respective mutations (S246, 247, 254, 264A; T257, 271A) in LT334-GFP (Fig. 5c). Thus, in contrast to many other viral and non-viral nucleoproteins,29 the nuclear import rate of MCPyV-LT seems not to be dependent on phosphorylation of residues upstream of the NLS.

The S220A mutation impairs binding to the retinoblastoma protein

Since phosphorylation did not affect localization of MCPyV-LT, we wondered if it might be involved in regulating its interaction with a cellular target protein. In this regard, the
observation that the RB binding motif (LxChE) is generally preserved in the truncated MCPyV-LT proteins present in MCC suggests that its interaction with RB1 might be an essential function of LT.7 Indeed, an E216K mutation within this motif not only represses binding of MCPyV-LT to RB1,5 but – similar to the S220A mutation—interfered with LT’s ability to drive proliferation of MCC cells.8 Since S220 is also in close proximity to the LxChE motif (Fig. 1), we speculated that phosphorylation of S220 in MCPyV-LT might be important for its interaction with RB1. Consequently, we performed immunoprecipitation experiments with ectopically expressed tagged RB1 in combination with three different LT variants in 293T cells. Importantly, immunoprecipitation of RB1 from lysates of cells co-expressing RB1 and either wild type or S220E mutated truncated LT distinctly co-immunoprecipitated LT. In contrast, when S220A mutated LT was co-expressed, co-precipitation of MCPyV-LT was largely reduced (Fig. 6a) suggesting that phosphorylation of S220, i.e., position +4 relative to the LxChE motif, promotes binding to RB1. Several studies addressing the relevance of phosphorylation events for other viral RB1 interaction partners reported partially conflicting results. For example, Barbosa et al. proposed that, although phosphorylation of the S31 and S32 in HPV-16 E7 (+5 and +6 relative to LxChE; Fig. 4) by CKII is required for transformation, it is not essential for RB binding.20 More recently, however, Chemes et al., demonstrated that phosphorylation of S31 and S32 in a synthetic E7 fragment increased the affinity towards the central domain of RB1 by fourfold.30 Moreover, in keratinocytes efficient binding of HPV-18 and HPV-11 E7 protein to RB1 and

Figure 5. Mutation of six putative phosphorylation sites N-terminal to the NLS does not affect nuclear import of MCPyV-LT. (a) The indicated MCPyV-LT-GFP fusion proteins were expressed in NIH3T3 cells, and subcellular localization was analyzed using a fluorescence microscope. LT254-GFP and LT334-GFP consist of a green fluorescence protein C-terminally fused to an LT protein consisting of the 254 and 334 N-terminal amino acids of MCPyV-LT, respectively. Furthermore, LT334-GFP contained either wild type MCPyV-LT or carried the indicated mutations. (b and c) Subcellular localization of LT334-GFP was monitored by fluorescence microscopy under standard culture conditions over time by taking pictures every 2 min. (b) Selected pictures taken during mitosis of one cell demonstrate LT334-GFP evenly distributed in the whole cell before cell division and followed by re-accumulation in the nuclei of the daughter cells. (c) The duration of cytoplasmic localization (X-Z; see Fig. 3b) as well as the length of the nuclear accumulation period (Y-Z; see Fig. 3b) was measured for either wild type or LT334-GFP carrying the indicated mutations. Mean values (±SD) are depicted. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
The RB-family member p130 seems to depend on the CKII site adjacent to the LxGxE motif. For HPV-18 E7, however, a contradictory report suggested that phosphorylation by CKII and RB-interaction are independent events.

The S220A mutation impairs induction of E2F target genes
To further confirm that targeting of RB1 by MCPyV-LT is dependent on S220 phosphorylation, we analyzed the impact of the 220 mutants on RB1 function. The tumor suppressor protein RB1 is an established key regulator of cell cycle progression exerting its function for example by regulation of E2F transcription factor dependent gene expression. In this respect, RB1 inactivates the E2F family members 1, 2 and 3 thereby repressing transcription of their target genes. Different viral proteins like SV40-LT or HPV-E7 are capable of interfering with the RB mediated repression of E2F target gene expression. Since we observed reduced binding of MCPyV-LT<sub>S220A</sub> to RB1 in the co-immunoprecipitation

Figure 6. The S220A mutation in MCPyV-LT impairs binding to the retinoblastoma protein and induction of E2F target gene expression. (a) Co-immunoprecipitation. One of the three different MCPyV-LT<sup>WT</sup> versions with the indicated amino acid at position 220 was co-transfected with empty vector or His-tagged RB1 into 293T cells. After 24 h, total cell lysates were harvested and RB1 was immunoprecipitated with a His-tag-antibody. Co-immunoprecipitation of MCPyV-LT was analyzed by immunoblot. (b and c) The MCPyV-positive MCC cell lines PeTa and BroLi were stably transduced with TA.shRNA.tet driving GFP expression and allowing doxycycline-inducible expression of TA-shRNA. Additionally, the cells were stably transduced with an empty vector or with the indicated shRNA-insensitive versions of MCPyV-LT<sup>WT</sup>. (b) After treatment with doxycycline for five days, MCPyV-LT was analyzed by immunoblot. The relative ratio of a mixed population of green fluorescent TA.shRNA.tet cells and parental PeTa or BroLi cells 13 days after addition of doxycycline is depicted above the immunoblots. Given are mean values (±SD) of the ratios on Day 13 relative to Day 0 derived by at least three independent experiments. (c) Relative mRNA expression levels of the RB target genes CDC6, MYB, PLK1 and CCNB1 were determined by real-time PCR and application of the ΔΔC<sub>T</sub> method in TA.shRNA.tet PeTa and BroLi cells transduced with the indicated expression vectors on Day 5 following addition of doxycycline. RPLP0 served as endogenous control, and the untreated vector control as calibrator. Depicted are mean values of two independent experiments.
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