pUL69 of Human Cytomegalovirus Recruits the Cellular Protein Arginine Methyltransferase 6 via a Domain That Is Crucial for mRNA Export and Efficient Viral Replication

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ABSTRACT

The regulatory protein pUL69 of human cytomegalovirus acts as a viral mRNA export factor, facilitating the cytoplasmic accumulation of unspliced RNA via interaction with the cellular mRNA export factor UAP56. Here we provide evidence for a post-translational modification of pUL69 via arginine methylation within the functionally important N terminus. First, we demonstrated a specific immunoprecipitation of full-length pUL69 as well as pUL69aa1-146 by a mono/dimethylarginine-specific antibody. Second, we observed a specific electrophoretic mobility shift upon overexpression of the catalytically active protein arginine methyltransferase 6 (PRMT6). Third, a direct interaction of pUL69 and PRMT6 was confirmed by yeast two-hybrid and coimmunoprecipitation analyses. We mapped the PRMT6 interaction motif to the pUL69 N terminus and identified critical amino acids within the arginine-rich R1 box of pUL69 that were crucial for PRMT6 and/or UAP56 recruitment. In order to test the impact of putative methylation substrates on the functions of pUL69, we constructed various pUL69 derivatives harboring arginine-to-alanine substitutions and tested them for RNA export activity. Thus, we were able to discriminate between arginines within the R1 box of pUL69 that were crucial for UAP56/PRMT6-interaction and/or mRNA export activity. Remarkably, nuclear magnetic resonance (NMR) analyses revealed the same α -helical structures for pUL69 sequences encoding either the wild type R1/R2 boxes or a UAP56/PRMT6 binding-deficient derivative, thereby excluding the possibility that R/A amino acid substitutions within R1 affected the secondary structure of pUL69. We therefore conclude that the pUL69 N terminus is methylated by PRMT6 and that this critically affects the functions of pUL69 for efficient mRNA export and replication of human cytomegalovirus.

IMPORTANCE

The UL69 protein of human cytomegalovirus is a multifunctional regulatory protein that acts as a viral RNA export factor with a critical role for efficient replication. Here, we demonstrate that pUL69 is posttranslationally modified via arginine methylation and that the protein methyltransferase PRMT6 mediates this modification. Furthermore, arginine residues with a crucial function for RNA export and for binding of the cellular RNA export factor UAP56 as well as PRMT6 were mapped within the arginine-rich R1 motif of pUL69. Importantly, we demonstrated that mutation of those arginines did not alter the secondary structure of R1, suggesting that they may serve as critical methylation substrates. In summary, our study reveals a novel posttranslational modification of pUL69 which has a significant impact on the function of this important viral regulatory protein. Since PRMTs appear to be amenable to selective inhibition by small molecules, this may constitute a novel target for antiviral therapy.

Every mammalian or avian herpesvirus encodes a member of the ICP27 protein family which functions as a posttranscriptional activator by facilitating the nuclear export of intronless mRNAs into the cytoplasm (1–3). The prototypic mRNA export factor of the subfamily *Betaherpesvirinae* is pUL69 of human cytomegalovirus (HCMV). This multifunctional nuclear protein of 744 amino acids is expressed in several isoforms with molecular masses of 105 to 116 kDa during the early and late phase of the viral replication cycle. HCMV pUL69 enters the host cell as part of the tegument and functions as a pleiotropic transactivator of gene expression (4, 5). Moreover, pUL69 acts as a posttranscriptional transactivator that mediates the nuclear export of unspliced mRNAs (6). We reported previously that pUL69 binds to RNA *in vitro* and *in vivo* via N-terminal, arginine-rich motifs comprising the amino acids 17 to 50 (R1/R2 motifs) and 123 to 139 (RS motif) (7). While mRNA binding is dispensable for pUL69-mediated export of an artificial reporter construct, recruitment of the cellular RNA helicases UAP56 and URH49 is absolutely essential (6, 7).

The N-terminal UAP56/URH49 interaction motif was narrowed down to amino acids 18 to 30 of pUL69 and thereby partially overlaps the bipartite nuclear localization signal (NLS) and the

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RNA-binding motif. More recently, we extended these findings and showed that UAP56/URH49 recruitment is well conserved within the cytomegaloviral pUL69 homologs pCh69 of chimpanzee cytomegalovirus (CCMV) and pRh69 of rhesus cytomegalovirus (RhCMV). This activity correlated with stimulatory effects of the respective proteins on mRNA export analogously to pUL69 (8, 9). The importance of UAP56/URH49 recruitment for HCMV multiplication was ultimately confirmed by the observation that recombinant cytomegaloviruses that had deletions or point mutations of the UAP56 interaction motif of UL69 (UL69 Δ R1 Δ RS and UL69mutUAP) exhibited a severe replication defect compared to wild-type virus (8).

Previous studies provided evidence for a regulation of the multifunctional herpesviral mRNA export factors by posttranslational modifications. For instance, ICP27 phosphorylation is required for its interaction with the cellular RNA export factors REF and TAP/p15 as well as for efficient HSV-1 replication (10, 11). Analogously, we showed that proper subcellular localization and efficient mRNA export of HCMV pUL69 were dependent on the catalytic activity of the viral serine/threonine protein kinase pUL97 or the cellular kinase orthologs CDK1, -2, -7, and -9 (12, 13). In addition, ICP27 was demonstrated to be arginine methylated. This modification proved to be dispensable for nuclear import but was crucial for interaction with SRPK1 and REF as well as for efficient mRNA export and HSV-1 replication (14–17). To date, there is no information regarding whether HCMV pUL69 is also posttranslationally regulated by methylation.

The human genome encodes approximately 50 protein lysine methyltransferases (PKMTs) and 11 protein arginine methyltransferases (PRMTs) (18, 19). Eight of the 11 human PRMTs (PRMT1 to -8) are well characterized. They share a set of four conserved motifs (I, post-I, II, and III), utilize S-adenosyl-L-methionine as the methyl donor, and transfer a single methyl group to one of the two terminal ω -guanidino nitrogens of arginine to synthesize monomethylarginine (MMA) (18). This MMA then serves as a substrate for type I PRMTs (PRMT1, -2, -3, -4, -6, and -8), which attach the second methyl group to the same nitrogen atom of arginine to generate asymmetric dimethylarginine (ADMA). Alternatively, type II PRMTs (PRMT5 and -7) transfer one methyl group to each of the terminal nitrogen atoms, thereby forming symmetric dimethylarginine (SDMA) (18, 20). Although PRMT7 has weak type II activity, it has been demonstrated to be primarily responsible for generating the MMA modification, thereby representing the sole member of the type III subgroup of methyltransferases (21). As suggested by the varying amino acid sequences and different subcellular localizations, PRMTs have distinct substrate specificities (22). In contrast to PKMTs, which mainly modify histones, PRMTs predominantly target nonhistone substrates, for instance, transcription factors, various RNA-processing factors like hnRNPs, spliceosomal Sm proteins, the DEAD box helicase VASA, and RNA-binding proteins involved in neuronal development, such as FMRP, HuR, and HuD (23). Importantly, arginine methylation was demonstrated to modulate the nucleocytoplasmic transport and the activity of various RNA-processing factors (24–28).

We therefore set out to characterize the impact of posttranslational arginine methylation on the activity of the HCMV-encoded RNA export factor pUL69. We demonstrate a direct interaction of pUL69 with the cellular arginine methyltransferase PRMT6 and identify crucial arginine residues within the functionally impor-

tant R1 box of pUL69 that were important for interaction with UAP56 and PRMT6 but did not affect the alpha-helical structure of the binding motif. Moreover, by performing a well-established mRNA export assay, we unambiguously confirmed that the UAP56/PRMT6-binding deficient mutants lost their mRNA export activity. Furthermore, we identified arginines 28 and 29 as being required for pUL69-mediated mRNA export but not for UAP56/PRMT6 interaction, suggesting that these residues may serve as critical substrates for methylation. In summary, our study reveals a novel posttranslational modification of pUL69 which has a significant impact on the function of this important viral regulatory protein. Since PRMTs appear to be amenable to selective inhibition by small molecules, this may constitute a novel target for antiviral therapy (29, 30).

MATERIALS AND METHODS

Oligonucleotides, plasmids, and BAC constructs. Oligonucleotide primers used for this study were purchased from biomers.net GmbH (Ulm, Germany). The sequences of all primers are listed in Table S1 in the supplemental material. Green fluorescent protein (GFP)-tagged PRMT1 to PRMT8 were kindly provided by F. O. Fackelmayer (22), FLAG-UAP56 was a kind gift from K. Nagata (31), and the chloramphenicol acetyltransferase (CAT) reporter plasmid pDM128/CMV/RRE (described elsewhere [32, 33]) was obtained from J. Hauber (Hamburg, Germany). Yeast expression plasmids encoding pUL69 fused either to the GAL4 activation domain (AD-UL69) or to the GAL4 DNA-binding domain (BD-UL69) were described before (34). PRMT6 was amplified using GFP-PRMT6 as a template, followed by digestion of the PCR product using MfeI and XhoI and ligation into the vectors pGAD424 and pGBT9, digested with EcoRI and SalI, thereby generating AD- and BD-PRMT6, respectively. PCR-amplified PRMT6 was digested with BamHI and XhoI and ligated into pHM971 (35) digested with the same enzymes to generate FLAG-PRMT6. The eukaryotic expression plasmids coding for Myc-UAP56, FLAG-pUL69 (pHM2098), and its derivatives FLAG-pUL69aa177-744 and -aa1-140, as well as the internal-deletion-containing constructs FLAG-pUL69 Δ R1, - Δ R2, - Δ RS, - Δ R1/RS, and - Δ R2/RS (pHM2322 to pHM2326) were described before (6, 7, 13). Site-directed mutagenesis within FLAG-pUL69 or GFP-PRMT6 was performed using the QuikChange site-directed mutagenesis kit as instructed by the manufacturer (Agilent Technologies). The resulting plasmids were termed FLAG-pUL69R20A, -R22,23A, -R25,26A, -R28,29A, -R126A, -R130A, -R137,138A (pHM3166 to -3172) or GFP-PRMT6VLD/KLA. FLAG-pUL69aa1-146, -aa51-268, -aa76-268, -aa269-528, -aa156-744, and -aa526-744 were amplified via PCR using pHM2098 as a template, and subsequently, BamHI- and EcoRI-digested or BamHI- and EcoRV-digested PCR-products were ligated into pHM972 (36), coding for an N-terminal in-frame fusion of a FLAG tag as well as the simian virus 40 (SV40) T antigen nuclear localization signal (NLS). C-terminal deletion constructs of the R/A substitution mutants that lack the amino acids 147 to 744 were generated by PCR using plasmids pHM2322-2326 or pHM3166-3172 as a template, subsequent digestion with BamHI and XhoI, and ligation into pHM971. The integrity of all newly generated plasmids was confirmed by automated DNA sequence analysis. The bacterial artificial chromosome (BAC) HB15, which contains the genomic sequence of the HCMV laboratory strain AD169 and its derivative HB15 Δ pUL69, from which the genomic sequence of UL69 was removed, have been described before (9, 37). To engineer recombinant HCMVs harboring amino acid substitutions within the UL69 gene, linear recombination cassettes were utilized to revert the HB15 Δ pUL69 BACmid by a two-step recombination strategy (38). For this, the kanamycin selection marker gene (*aphA1*) was amplified by PCR using pEPkan-S (kindly provided by K. Osterrieder, Berlin, Germany) as a template and inserted into the NheI single cutter site of the UL69 coding sequences of pHM3166-3169. Then, a linear recombination fragment was generated from these

constructs by PCR and subsequently transformed into chemically competent *Escherichia coli* strain GS1783 (gift from G. A. Smith, Chicago, IL, USA) before homologous recombination was induced. In a second recombination step, the kanamycin cassette was removed as verified by growth selection. Finally, PCR analyses, restriction fragment length polymorphism analysis, and nucleotide sequencing confirmed that the newly generated constructs contained the desired sequences.

Maintenance, transfection, and infection of cells. Primary human foreskin fibroblasts (HFFs) as well as HeLa and HEK293T cells were cultured as described previously (4, 35). HeLa and 293T cells were transfected via the calcium phosphate coprecipitation procedure as described earlier (4, 35, 39). The cytomegalovirus strain employed in this study as a control for growth kinetics analyses was obtained by reconstitution of infectious viruses using the BAC HB15 (37). Stocks of wild-type and recombinant viruses were prepared and titrated by IE1p72 fluorescence exactly as described previously (40). Multistep growth curve analyses as well as the quantification of viral DNA in supernatants from infected HFF cells were performed as described in a previous study (41).

Indirect immunofluorescence analysis, coimmunoprecipitation analysis, yeast two-hybrid assays, and mRNA export assays. Indirect immunofluorescence and coimmunoprecipitation (CoIP) analyses as well as the CAT mRNA export assay were performed using transiently transfected HEK293T or HeLa cells exactly as described in detail in previous publications (8, 9, 13, 39). Yeast two-hybrid analysis was performed by transformation of the yeast strain Y153 with plasmids encoding GAL4-AD- and GAL4-BD-fused PRMT6 or pUL69 followed by growth selection and filter lift assays as described before (42, 43). Immunofluorescence images were collected via confocal laser scanning microscopy by using a TCS SP5 microscope equipped with a 63 $\times$ HCX PL APO CS oil immersion objective (Leica). Data were analyzed with LAS AF software (Leica) and further processed by utilizing the Adobe Photoshop package (Universal Imaging Corp., Brandywine, PA; Adobe Systems Incorporated).

Antibodies. The monoclonal mouse antibodies (MAB) anti-FLAG (M2) and anti- β -actin were purchased from Sigma-Aldrich (Deisenhofen, Germany). Monoclonal anti-Myc (1-9E10.2 antibody) was obtained from ATCC (Wesel, Germany), and the monoclonal antibody mixture anti-GFP (clones 7.1 and 13.1) was purchased from Roche (Mannheim, Germany). The anti-mono- and dimethylarginine-specific antibody 7E6 was from Abcam (Cambridge, United Kingdom). MAB anti-UL69 (clone 69-66) and MAB 63-27 (directed against IE1) were kindly provided by W. Britt (Birmingham, AL, USA) (44). Anti-mouse and anti-rabbit horseradish peroxidase-conjugated secondary antibodies were obtained from Dianova (Hamburg, Germany), and the Alexa-555-conjugated secondary antibody was purchased from Abcam (Cambridge, United Kingdom).

NMR. One-dimensional (1D) and 2D ^1H nuclear magnetic resonance (NMR) experiments (total correlation spectroscopy [TOCSY] and nuclear Overhauser effect spectroscopy [NOESY]) were performed at 600.13 MHz on a Bruker Avance 600 MHz instrument equipped with an Ultra-Shield Plus magnet and a triple-resonance cryoprobe with a gradient unit. Individual peptide samples were synthesized by GenScript (Hong Kong), dissolved in 600 μl 50% aqueous trifluoroethanol- d_2 (TFE- D_2) at pH 3. The peptide concentrations were approximately 1 mM. The 2D NMR experiments were performed at 300 K without spinning and with mixing times of 110 ms for the TOCSY experiments and 250 ms for the NOESY experiments, respectively. Efficient suppression of the water signal was achieved by application of excitation sculpting in the 1D and 2D NMR experiments (45). ^1H signal assignments of the NMR spectra were achieved by identification of the individual spin systems in the 2D ^1H TOCSY spectra, combined with observations of sequence-specific short-distance cross peaks ($\text{H}\alpha\text{-HN}_{i,i+1}$) in the 2D ^1H NOESY spectra (46, 47). Readily recognizable spin systems were used as starting points for correlation of the individual spin systems observed in the TOCSY and NOESY spectra to individual residues in the peptide sequences. Acquisition of data, processing, and spectral analysis were performed with

Bruker Topspin 1.3 software. Assigned ^1H chemical shifts of the peptides are presented in Tables S2 and S3 in the supplemental material. The conformational shifts were calculated using the random-coil values reported by Wüthrich (47).

RESULTS

Posttranslational modification of pUL69 via arginine methylation. In order to investigate whether the HCMV UL69 protein is posttranslationally modified by arginine methylation, we performed an *in silico* analyses of putative methylation sites by using the MeMo 2.0 server. Thereby, 11 arginines (R6, R25, R26, R29, R83, R108, R126, R239, R287, R551, and R662) as well as lysine 407 were predicted as putative methylation substrates within pUL69 (48). Seven of these 11 potential arginine substrates are localized within the functionally important N terminus of pUL69 (Fig. 1A). Strikingly, three of these sites can be detected within the arginine-rich R1 box, which has been demonstrated before to be required for UAP56 interaction and mRNA export activity (6, 8, 9). Additionally, one site is located within the RS box which is relevant for mRNA binding of pUL69 (7).

Immunoprecipitation (IP) analyses with a mono- and dimethylarginine-specific antibody using HEK293T cells that were transfected with either full-length FLAG-pUL69 or N- and C-terminal truncation mutants revealed a precipitation of full-length FLAG-UL69, indicating a modification of pUL69 via protein methylation (Fig. 1B, right, lane 1). In addition, FLAG-pUL69aa177-744 was also coprecipitated, but to a lower extent (Fig. 1B, right; compare lanes 1 and 3). In line with the notion that the N terminus serves as the predominant methylation site of pUL69, FLAG-pUL69aa1-140 as well as the deletion mutants FLAG-pUL69aa51-268 and -aa76-268 were strongly precipitated by the mono-/dimethylarginine-specific antibody (Fig. 1B, lanes 4, 5, and 6). However, when mutants lacking the arginine-rich box R1, R2, or RS were assayed, only the ΔR2 mutant was strongly precipitated (Fig. 1B, lanes 7 to 9). Thus, in agreement with the *in silico* prediction, this experiment indicates that pUL69 is arginine methylated and its N-terminal 140 amino acids contain the predominant methylation substrates. Moreover, the IP revealed that the arginine-rich boxes R1 and RS either are substrates for methylation or are required for interaction with the respective arginine-modifying enzyme(s).

Intranuclear colocalization of pUL69 with PRMT2 and PRMT6. Because arginine methylation is mediated by so-called protein arginine methyltransferases (PRMTs), it was interesting to analyze pUL69 for its putative colocalization and interaction with any of the eight most prominent representatives of this protein family. For this, indirect immunofluorescence analyses using HeLa cells transiently transfected with expression constructs for GFP-tagged PRMT1 through -8 were conducted. As illustrated in Fig. 2, a large proportion of PRMT1, -3, -4, and -7 was evenly distributed throughout the cytoplasm with an exclusion of the nucleus (Fig. 2A, panels a to c, g to i, k to m, and t to v). In contrast, PRMT2 (Fig. 2A, panels d to f) and PRMT6 (Fig. 2A, panels q to s) were exclusively located within the nucleus. A unique localization was observed for PRMT5, which accumulated in cytoplasmic dots (Fig. 2A, panels n to p), as well as for PRMT8, which was associated with the plasma membrane (Fig. 2A, panels w to y). Thus, we observed that the subcellular localization of transiently expressed GFP-PRMT1 to -8 in HeLa cells largely resembled their localization after stable expression in HEK293T cells (22).

FIG 1 Arginine methylation of HCMV pUL69. (A) Schematic representation of HCMV pUL69, including the arginine-rich cluster required for RNA binding (R1, R2, and RS) and the nuclear localization signal (NLS). The ICP27 homology region is located in the center and the nuclear export signal (NES) within the C terminus of the protein. Boxes above the protein indicate interaction motifs with the cellular mRNA export factor UAP56 or the transcription elongation factor hSPT6. Putative methylation sites predicted by the MeMo 2.0 server (www.bioinfo.tsinghua.edu.cn/) are indicated by arrows. (B) Methylation of HCMV pUL69 *in vivo* as determined by immunoprecipitation analyses using HEK293T cells that were transfected with the indicated FLAG-pUL69 derivatives. Immunoprecipitation was performed by using a mono- and dimethylarginine-specific antibody, and coprecipitated pUL69 proteins were detected via staining with a FLAG-specific antibody.

In a second set of experiments, we investigated whether cotransfection of pUL69 leads to a relocalization of pUL69 or the respective PRMT or whether both proteins colocalize with each other in the same subcellular compartment (Fig. 2B). As determined by staining with a FLAG-specific primary antibody, FLAG-pUL69 revealed an intranuclear localization in all cotransfection experiments (Fig. 2B, α FLAG panels). The only PRMTs displaying an intranuclear colocalization with pUL69 were PRMT2 and PRMT6 (Fig. 2B, panels e to h and v to y). For all other PRMTs investigated, no co- or relocalization with/by pUL69 could be detected.

PRMT6 leads to a specific electrophoretic mobility shift of the pUL69 N terminus. Since our experiments demonstrated a methylation of the pUL69 N terminus (amino acids [aa] 1 to 146) and indirect immunofluorescence analyses revealed an intranuclear colocalization of pUL69 with PRMT2 and PRMT6, we were interested in investigating whether pUL69 might serve as a substrate for individual PRMTs. Thus, HEK293T cells were cotransfected with a plasmid encoding FLAG-pUL69aa1-146 and vectors coding for any of the GFP-tagged PRMTs, GFP-PRMT1 to -8 (Fig. 3A, lanes 2 to 9), or empty vector (Fig. 3A, lane 1). Two days later, cells were harvested, and proteins were separated by a 20-cm high-percentage SDS-gel electrophoresis. Our Western blot analysis re-

vealed that expression of the pUL69 N terminus (aa 1 to 146) gave rise to two prominent protein isoforms (Fig. 3A, bottom, lanes 1 to 9). This further indicates that the UL69 N terminus serves as a substrate for posttranslational modification (5). Strikingly, cotransfection of GFP-PRMT6 led to a loss of the lower-molecular-weight isoform of pUL69aa1-146 which was accompanied by a mobility shift to a higher-molecular-weight isoform (Fig. 3A, lane 7). This observation was considered specific, since this shift did not occur upon coexpression of any other PRMT (Fig. 3A; compare lane 7 with all other lanes). All PRMTs were expressed at comparable levels and with their expected sizes, as confirmed by staining of the Western blot with an anti-GFP antibody (Fig. 3A, top, lanes 2 to 9).

Having demonstrated that overexpression of PRMT6 led to a specific increase of a high-molecular-weight isoform of pUL69aa1-146, we wished to analyze whether the catalytic activity of PRMT6 was required for this effect. For this, we generated a catalytically inactive derivative of PRMT6 by mutating valine 81 to lysine and aspartate 83 to alanine, as described by Boulanger and colleagues (49). Next, HEK293T cells were cotransfected with a vector coding for FLAG-pUL69aa1-146 and plasmids for either catalytically active GFP-PRMT6 or inactive GFP-PRMT6-VLD/KLA (Fig. 3B, lanes 1 and 2). Approximately 36 h posttransfection,

FIG 2 Localization of PRMTs in the absence (A) or presence (B) of HCMV pUL69. Immunofluorescence analysis used HeLa cells that were transfected with plasmids encoding GFP-PRMT1 (A, panels a to c; B, panels a to d), GFP-PRMT2 (A, panels d to f; B, panels e to h), GFP-PRMT3 (A, panels g to i; B, panels i to m), GFP-PRMT4 (A, panels k to m; B, panels n to q), GFP-PRMT5 (A, panels n to p; B, panels r to u), GFP-PRMT6 (A, panels q to s; B, panels v to y), GFP-PRMT7 (A, panels t to v; B, panels z to C), or GFP-PRMT8 (A, panels w to y; B, panels D to G). In a second approach, HeLa cells were cotransfected with the various GFP-PRMTs and FLAG-pUL69 (B, panels a to G). Cells were fixed about 36 h posttransfection and analyzed for the subcellular distribution of GFP-PRMTs (A and B) and of pUL69 by using a mouse anti-FLAG and an anti-mouse-Cy3 antibody (B). Nuclei were counterstained with 4',6'-diamidino-2-phenylindole (DAPI).

cells were harvested, and protein lysates were analyzed by Western blotting. Due to better separation of protein isoforms by SDS-PAGE in this experiment, we were able to visualize an additional faint third protein isoform that is expressed after transfection of FLAG-pUL69aa1-146 (Fig. 3B, lane 3). Importantly, wild-type PRMT6 again caused a loss of the lower-molecular-weight isoform that was accompanied by a clear shift toward a higher-molecular-weight isoform (Fig. 3B, lane 2). In contrast, cotransfection of catalytically inactive GFP-PRMT6-VLD/KLA did not lead to an electrophoretic mobility shift but, instead, resulted in an expression pattern comparable to that of the sample where an empty vector was cotransfected (Fig. 3B, lanes 1 and 3). Thus, expression of the high-molecular-weight isoform of pUL69aa1-146 was strictly dependent on the catalytic activity of the methyltransferase PRMT6. In order to ultimately confirm the specificity of this observation we tested whether the central part (aa 269 to 528) or the C-terminal part (aa 526 to 744) of pUL69 was affected upon cotransfection of PRMT6. In contrast to the N-terminal pUL69 protein domain, neither the central nor the C-terminal part showed a shift upon overexpression of wild-type PRMT6

(Fig. 3C). Taken together, our results clearly demonstrate that expression of the pUL69 N terminus results in 3 protein isoforms and that one of these isoforms shifts upon cotransfection of catalytically active PRMT6, suggesting that the pUL69 N terminus represents a methylation substrate for PRMT6.

Interaction between pUL69 and PRMT6 *in vivo*. In order to serve as a methylation substrate, both proteins, the substrate pUL69 and the enzyme PRMT6, should be able to interact with each other, as already suggested by their colocalization within the nucleus (Fig. 2). To reveal a direct protein-protein interaction, we took advantage of the yeast two-hybrid system. Yeast cells were transformed with vectors encoding pUL69 fused to the GAL4 activation domain (AD) and wild-type or mutated PRMT6 fused to the GAL4 DNA-binding domain (BD) followed by growth selection and filter lift assays. As illustrated by blue staining of the respective colonies, we observed a positive interaction between pUL69 and wild-type PRMT6 as well as catalytically inactive PRMT6-VLD/KLA (Fig. 4A, lanes 4 and 5). This result was considered specific, as the cotransformation of pUL69 with an empty BD vector or PRMT6 and PRMT6-VLD/KLA with an empty AD

FIG 3 Effect of PRMT overexpression on the electrophoretic mobility of various pUL69 deletion mutants. Western blot analysis used lysates from HEK293T cells which had been cotransfected with an expression construct for either the N terminus of pUL69 (A and B, FLAG-pUL69aa1-146) or the central part (C, FLAG-pUL69aa269-528, lanes 1 and 2) or the C-terminal part (C, FLAG-pUL69aa526-744, lanes 3 and 4) of pUL69 in combination with the indicated GFP-tagged PRMT. Two days later, cells were harvested, and proteins were separated by SDS-PAGE and visualized via Western blotting by using an anti-Flag antibody. Catalytically inactive PRMT6-VLD/KLA (B, lane 1; C, lanes 1 and 3) and an empty vector (A, lane 1; B, lane 3) served as negative controls. An electrophoretic mobility shift is highlighted by an asterisk.

vector did not activate the reporter gene in the absence of an interactor (Fig. 4A, lanes 1 to 3). Although this experiment revealed a direct interaction of full-length pUL69 with PRMT6 in lower eukaryotes, this recruitment had to be confirmed by coimmunoprecipitation analyses in higher eukaryotic cells. In order to narrow down the PRMT6 interaction region within pUL69, HEK293T cells were cotransfected with vectors encoding truncated versions of FLAG-pUL69 together with a plasmid for GFP-PRMT6 (Fig. 4B, lanes 1 to 4 and 6 to 9). Two days later, cells were harvested, and the correct protein expression was confirmed by Western blotting analyses (Fig. 4B, left). As determined by staining of the CoIP Western blot with an anti-Flag antibody, GFP-PRMT6 was able to coprecipitate FLAG-pUL69aa1-146 but none of the truncated mutants that lack the N terminus (Fig. 4B, top, lanes 6 to 9). This coprecipitation result was considered specific for the pUL69-N terminus because all mutants were equally expressed (Fig. 4B, input, lanes 1 to 4), GFP-PRMT6 was properly precipitated in each sample (Fig. 4B, lower right, lanes 6 to 9) and FLAG-pUL69aa1-146 was not coprecipitated in a nonspecific manner when GFP-PRMT6 was absent (Fig. 4B, lanes 5 and 10). Conclusively, we could demonstrate a direct interaction of

PRMT6 and pUL69 in lower and higher eukaryotes. Furthermore, our mapping study confirmed that the N-terminal 146 amino acids of pUL69 were sufficient for PRMT6 recruitment.

Fine mapping of the PRMT6-interaction motif within the pUL69 N terminus. So far, our experimental results suggest that the pUL69 N terminus recruits PRMT6, which then methylates distinct arginines within the N terminus, as visualized by a clear electrophoretic mobility shift upon overexpression of catalytically active PRMT6. As the initial IP with the mono/dimethylarginine-specific antibody indicated an importance of the arginine-rich boxes for arginine methylation, we were interested in further mapping the PRMT6 interaction region within the pUL69 N terminus. For this we generated mutants of the pUL69 N terminus that lack any of the arginine-rich boxes R1, R2, and RS or combinations of them. Next, HEK293T cells were transfected with plasmids encoding GFP-PRMT6 together with these internal-deletion-containing pUL69 mutants, followed by coimmunoprecipitation experiments. Strikingly, none of the mutants lacking either of the arginine-rich boxes was coprecipitated by GFP-PRMT6, although all proteins were expressed (Fig. 5A; compare lanes 10 to 14 with lanes 3 to 7). However, coprecipitation of

FIG 4 Protein interaction between pUL69 and PRMT6. (A) Yeast two-hybrid analysis. Yeast cells were transformed with expression plasmids encoding catalytically active PRMT6 (lanes 2 and 4) or inactive PRMT6-VLD/KLA (lanes 3 and 5) fused to the GAL4 DNA-binding domain (BD) and pUL69 fused to the GAL4 activation domain (AD) (lanes 4 and 5). Combinations with an empty vector served as specificity controls (lanes 1 to 3). Growth-selected yeast colonies were analyzed by filter lift assays for the expression of β -galactosidase, as indicated by blue staining of the respective colony. (B) Coimmunoprecipitation analyses. HEK293T cells were cotransfected with plasmids encoding GFP-PRMT6 and truncated derivatives of pUL69 (lanes 1 to 4 and 6 to 9). Two days later, cells were harvested, and the amount of protein in the input was analyzed by Western blotting (input [lanes 1 to 5]). Immunoprecipitation was performed by using an anti-GFP antibody, and coprecipitated proteins were visualized by using an anti-Flag antibody (IP α GFP; lanes 6 to 10). Cells cotransfected with an empty expression plasmid (lanes 5 and 10) served as a specificity control. Immunoglobulin light and heavy chains on the IP blot served as an internal control for the presence of the precipitating antibody (Ig_{lc} and Ig_{hc}; lanes 6 to 10).

wild-type pUL69aa1-146 by GFP-PRMT6 was confirmed (Fig. 5A, lane 9). The CoIP blot was additionally stained with an anti-GFP antibody, thereby confirming that equal amounts of PRMT6 were precipitated in each sample (Fig. 5A, lanes 9 to 14). Thus, these results provide evidence for a requirement of each arginine-rich box within the pUL69 N terminus for PRMT6 interaction.

Since our previous experiments demonstrated that arginines 22/23 and 25/26 of the R1 box are essential for both RNA export and interaction with UAP56, we further concentrated on the R1 box to fine map individual arginines with a crucial role for PRMT6 recruitment (6). For this, HEK293T cells were transfected with plasmids encoding GFP-PRMT6 together with mutants of pUL69aa1-146 that carry R/A amino acid substitutions of R1, followed by CoIP analyses. GFP-PRMT6 coprecipitated wild-type pUL69aa1-146, hence serving as the internal positive control (Fig. 5B, lane 9). Similar to wild-type pUL69aa1-146, PRMT6 coprecipitated pUL69 mutants with R/A substitutions at positions 20 or 28 and 29 within the R1 box (Fig. 5B, lanes 10 and 13), suggesting that these residues are not involved in PRMT6 recruitment. In contrast, mutants with R/A substitutions at positions 22 and 23 or 25 and 26 or the combination of all four abrogated the interaction with PRMT6 (Fig. 5B, lanes 11, 12, and 14).

UAP56 does not interact with PRMT6. The finding that arginines 22/23 and 25/26 are both essential for binding to PRMT6 and UAP56 gave rise to the idea that UAP56 might bridge the association of PRMT6 with UL69. In order to answer this question, additional CoIP experiments were performed using HEK293T cells that had been transfected with vectors coding for Flag-UAP56 and GFP-PRMT6 or Myc-UAP56 and Flag-PRMT6

(Fig. 6A and B). This revealed that PRMT6 was able to interact with the N terminus of pUL69 but did not coprecipitate UAP56 (Fig. 6A, lanes 4 and 6). In order to exclude the possibility that the large GFP tag interfered with an interaction of GFP-PRMT6 with UAP56, the CoIP was repeated, this time utilizing FLAG-PRMT6 in combination with Myc-UAP56. Again, PRMT6 was not coprecipitated by UAP56 (Fig. 6B, top, lane 4), confirming that UAP56 does not bind to PRMT6.

Individual arginines of the R1 box are crucial for efficient mRNA export of pUL69. In order to investigate the relevance of individual arginines of the pUL69 N terminus for pUL69-mediated RNA export, we utilized a well-established mRNA export assay (39). Briefly, HEK293T cells were cotransfected with the reporter pDM128/CMV/RRE (Fig. 7A) and wild-type FLAG-pUL69 or various FLAG-pUL69-R/A amino acid exchange mutants (Fig. 7B). Subsequent determination of CAT protein levels in cell lysates served as a direct correlate of pUL69's capacity to export unspliced CAT reporter RNA from the nucleus into the cytoplasm (Fig. 7B). In accordance with previous results, expression of FLAG-pUL69 yielded a significant increase of CAT protein levels compared to the mock-transfected sample, thus confirming that pUL69 exports unspliced CAT reporter RNA (Fig. 7B, compare lanes 2 and 1) (6, 8). Next, pUL69 mutants harboring arginine-to-alanine substitutions of putative methylation sites were tested. While stimulation of RNA export by pUL69R20A did not differ from that by wild-type pUL69, expression of RR22/23AA, RR25/26AA, and RR28/29AA mutants resulted in a clear decrease of CAT protein levels to background (Fig. 7B). In contrast, arginine mutations within the RS box of pUL69 did not affect the

FIG 5 Fine mapping of the PRMT6 interaction motif within the pUL69 N terminus. Coimmunoprecipitation analyses using HEK293T cells that were cotransfected with plasmids encoding GFP-PRMT6 and containing internal deletions (A) or point mutation derivatives of FLAG-pUL69aa1-146 (B), as indicated. The amount of protein in the input was monitored prior to immunoprecipitation. Coprecipitated proteins were visualized by using an anti-Flag antibody (A and B, upper right; IPαGFP), which revealed that GFP-PRMT6 coprecipitated wild-type FLAG-pUL69aa1-146 as well as several of its R/A derivatives. For internal controls, the CoIP blot was restained with an anti-GFP antibody, which confirmed equal precipitation of PRMT6 (A and B, lower right; IPαGFP).

mRNA export activity (Fig. 7B and C, lanes 7 to 9). Protein expression was comparable for all pUL69 mutants (Fig. 7C).

Importantly, since all arginines with relevance for RNA export were located either within or next to the UAP56 interaction motif, we had to clarify whether the pUL69 RR22/23AA, RR25/26AA, and RR28/29AA mutants lost their mRNA export activity due to a defect in binding to UAP56, which was investigated by CoIP analysis. While Myc-UAP56 was able to coprecipitate FLAG-pUL69aa1-146 (Fig. 7C, lane 8), it failed to coprecipitate mutants that lack the arginines at positions 22/23 or 25/26 alone or in combination (Fig. 7D, lanes 8 to 11 and 13). The latter observation confirms our previous finding that the combinatorial change of arginines 22, 23, 25, and 26 to alanine changes pUL69 into a UAP56-binding-deficient mutant (6, 8, 9). Interestingly, however, the arginines at positions 28 and 29 were not required for UAP56 interaction, since a mutant harboring the respective substitutions was coprecipitated by Myc-UAP56 (Fig. 7D, lane 12). Taken together, our results demonstrate that the R1 box arginines 22/23

and 25/26, which are important for PRMT6 interaction (Fig. 5B), are also required for UAP56 interaction (Fig. 6D). Strikingly, mutation of the arginines 22/23 and 25/26 within the pUL69-R1 box abolished its interactions with UAP56 as well as PRMT6, we wondered whether the secondary structure of pUL69 might be altered by the substitution of the respective arginines to alanine (Fig. 8A). Empirical structure prediction using the Phyre2 server indicated that the wild-type peptide exhibits a short C-terminal α -helix comprised by residues R39 to R45 (Fig. 8B) (50). A similar short C-terminal helix was also predicted for the mutant peptide (Fig. 8B). The N terminus of the wild-type peptide was predicted to assume a random structure. The predicted structure of the mutant indicated that an N-terminal α -helical structure is induced when R22,

Secondary structure of wild-type pUL69aa1-146 and its UAP56/PRMT6 binding-deficient derivative. Since mutation of the arginines 22/23 and 25/26 within the pUL69-R1 box abolished its interactions with UAP56 as well as PRMT6, we wondered whether the secondary structure of pUL69 might be altered by the substitution of the respective arginines to alanine (Fig. 8A). Empirical structure prediction using the Phyre2 server indicated that the wild-type peptide exhibits a short C-terminal α -helix comprised by residues R39 to R45 (Fig. 8B) (50). A similar short C-terminal helix was also predicted for the mutant peptide (Fig. 8B). The N terminus of the wild-type peptide was predicted to assume a random structure. The predicted structure of the mutant indicated that an N-terminal α -helical structure is induced when R22,

FIG 6 Capacity of UAP56 to interact with PRMT6. (A and B) CoIP experiments were performed by transfection of HEK293T cells with plasmids encoding the indicated proteins. Two days after transfection, cells were lysed, and immunoprecipitation was performed using either anti-GFP (A) or anti-Myc antibody (B). As a negative control, an empty vector (mock) was cotransfected (A, lanes 2 and 5; B, lanes 1 and 3). The interaction of the pUL69-N terminus with PRMT6 (A, lane 4) served as a positive control for both blots. Immunoglobulin heavy chain (IgHc) and light chain (IgLc) served as internal controls for the presence of precipitation antibody.

R23, R25, and R26 are replaced with A (Fig. 8B). However, even though structure prediction methodology has improved significantly in recent years, it still has limitations. Current structure prediction methods may become particularly unreliable when there are no homologs with solved structures in PDB or when templates under consideration are distant homologs (51, 52). In order to determine the solution structures of the peptides experimentally, we performed NMR spectroscopy of two sequences encoding either wild-type pUL69aa17-46 or its UAP56/PRMT6 binding-deficient derivative pUL69aa17-46-R22/23/25/26A (Fig. 8A). The graphs in Fig. 8C illustrate the H α chemical shift indexes (CSI) for both pUL69 peptides, wherein the experimentally determined H α chemical shifts are compared with H α chemical shifts of analogous residues of random coil peptides. CSI plots of the α -proton (H α) chemical shifts relative to those of residues in a random coil have proved to be an appropriate and powerful tool for determining the presence of secondary structure in peptides and proteins (47, 53). A minimum of four adjacent residues with an upfield shift relative to random coil (negative CSI) are indicative of an α -helix, whereas β -sheets require a minimum of three residues with downfield shifts (positive CSI) (53). Both peptides exhibited a short C-terminal helix similar to that of the predicted structures (Fig. 8B). However, in contrast to what was indicated from the empirical structure predictions, the main α -helical region of the wild-type pUL69aa17-46 in aqueous TFE solution is located at the N terminus of the peptide. The CSI plot indicates that α -helix 1 includes residues 17 to 34 (Fig. 8C and D). The downfield shift of H α of Glu-35 is normally observed

for residues preceding a Pro unit and is not related to secondary structure (46). The localization and extent of helix 1 were supported by the presence of a considerable number of medium-range cross peaks in the NOESY spectrum that are indicative of helical conformation in this region of the molecule ($^1\text{H}\alpha$ Ser-18/ ^1HN Glu-21, $^1\text{H}\alpha$ Glu-19/ ^1HN Arg-22, $^1\text{H}\alpha$ Glu-21/ ^1HN Arg-24, $^1\text{H}\alpha$ Arg-22/ ^1HN Arg-25, $^1\text{H}\alpha$ Arg-23/ ^1HN Arg-26, $^1\text{H}\alpha$ Ala-27/ ^1HN Phe-30, $^1\text{H}\alpha$ Glu-21/ $^1\text{H}\beta$ Ala-24, $^1\text{H}\alpha$ Ala-27/ $^1\text{H}\beta$ Phe-30, and $^1\text{H}\alpha$ Phe-30/ $^1\text{H}\beta$ Asp-33). The presence of a helical structure was further supported by the observation of NH-NH $_{i,i+1}$ cross peaks, including residues 18 to 35 in the 2D ^1H NOESY spectrum. The region containing Pro-36 and Pro-38 acts as helix breaker; thus, the residues Pro-36, Val-37, and Pro-38 comprise an interhelical loop region. The NH-NH $_{i,i+1}$ cross peaks observed for residues F41 to S46 in the NOESY spectrum indicate that this region of the peptide is also able to assume an α -helical conformation, indicating that these residues are included in a second short α -helical region which is destabilized, as usual, at the end of the peptide. It is likely that the region containing Pro-47 and Pro-50 would terminate the second short α -helix in the full-length protein; however, residues 47 to 50 are not included in the peptides analyzed. Notably, and in contrast to the empirical predictions, the mutant peptide HCMV pUL69(17-46R22,23,25,26A) exhibited a similar localization and extent of α -helical structure to that of the wild-type peptide (Fig. 8C and D). As the combinatorial mutation of all 4 arginines did not affect the secondary structure of pUL69, we can consider it ascertained that their individual

exchange would also not affect the α -helical structure of the R1 box.

Arginines 28 and 29 of pUL69 are required for efficient HCMV replication. Our previous studies already demonstrated that mutation of the arginines 22/23 and 25/26 within the pUL69-R1 box, which abrogates UAP56 and, as shown in this study, PRMT6 interaction, severely affects the replication of human cytomegalovirus (6, 8). Since we detected that mutation of

arginines 28 and 29 compromised the RNA export activity of pUL69, we were interested in investigating the effect of these mutations in the context of viral replication. Using the two-step red-mediated recombination for markerless DNA manipulation, the wild-type AD169-based HCMV bacterial artificial chromosome (BAC) pHB15 was first used to delete the genomic region of UL69, resulting in HB15 Δ UL69-BAC (9, 38) (Fig. 9A). Subsequently, pUL69-RR28/29AA was reinserted at

FIG 7 Nuclear mRNA export and UAP56 binding of wild-type pUL69 and R/A amino acid exchange mutants. (A) Schematic representation of the mRNA export reporter pDM128/CMV/RRE, which was used in cotransfection assays with pUL69 variants. This vector contains the CAT gene within an intron (SD and SA, splice donor and acceptor sites) as well as an SV40 late poly(A) signal (SV40PA). Coexpression of this reporter with pUL69 results in the cytoplasmic accumulation of unspliced reporter mRNA, thus leading to increased CAT protein synthesis. (B) Relative amounts of CAT protein measured 36 h posttransfection of HEK293T cells with a mixture of the reporter pDM128/CMV/RRE and the indicated expression plasmids for FLAG-pUL69 or R/A mutants. Transfections were performed in triplicate, and relative values (means and standard deviations) of CAT activity relative to wild-type pUL69 are given. (C) UL69 protein expression was monitored prior to CAT assays via Western blotting using a FLAG-specific antibody. (D) Coimmunoprecipitation analyses using HEK293T cells that were cotransfected with plasmids encoding Myc-UAP56 and point mutation-containing derivatives of FLAG-pUL69aa1-146. The amount of protein in the input was monitored prior to CoIP (input lanes). After immunoprecipitation of UAP56 via its Myc tag, coprecipitated proteins were visualized by using a FLAG-specific antibody (D, upper right; IPαMyc).

FIG 8 Substitution of arginines 22, 23, 25, and 28 by alanine does not change the secondary structure of the R1 and R2 boxes of pUL69. (A) Amino acid sequences of wild-type pUL69aa17-46 and its UAP56/PRMT6 binding-deficient derivative pUL69aa17-46-R22,23,25,26A. (B) Predicted structures of both peptides as determined by the Phyre2 server (see the text for details). (C) Chemical shift differences (in parts per million) of the α -protons between the experimental values and those for residues in a random coil for the N-terminal peptides of wild type pUL69aa17-46 (left) and the mutant pUL69aa17-46-R22,23,25,26A (right) obtained at 300 K in 50% aqueous TFE at pH 3. The localizations of R/A substitutions are indicated by arrows (see also Tables S2 and S3 in the supplemental material). (D) Schematic illustration of the secondary structure of both peptides determined by NMR (see panel C and the text for details).

the position of the UL69 open reading frame (ORF) (Fig. 9A). In order to validate the genomic integrity of the newly generated BAC distinct PCRs, restriction enzyme cleavage with *Afl*II or *Asc*I and nucleotide sequence analysis of the recombined region were performed and confirmed the correctness of the BAC (data not shown). After reconstitution of infectious viruses, multistep growth curve analyses of wild-type and mutant AD169 viruses were performed (Fig. 9B). For this, HFFs were infected in triplicate (multiplicity of infection [MOI], 0.001) with viral inocula normalized for equal immediate early (IE) units. After harvesting of the supernatants at 4, 6, 8, 10, and 12 days postinfection (dpi), samples were subjected to TaqMan PCR (qPCR) to quantify the amount of viral IE1 gene corresponding to HCMV genome equivalents. As reported previously, the growth properties of AD169- Δ UL69 were considerably lower than those of wild-type virus, confirming the important role of pUL69 for efficient viral replication (Fig. 9B) (9). Interestingly, mutation of R28/29 to alanine resulted in a significant decrease in the release of progeny virions, indicating that these two arginine residues are critical for the correct function of pUL69 during viral replication.

DISCUSSION

Over the past decade, it has become apparent that RNA processing factors are regulated by posttranslational modifications, including arginine methylation (23). We reported previously that the viral mRNA export factor pUL69 of HCMV is expressed in at least three distinct isoforms that are modified via phosphorylation mediated by both viral and cellular protein kinases (4, 5, 12, 13). Here, we extend these findings and provide evidence for an additional post-translational modification of pUL69 by arginine methylation. Importantly, coexpression of FLAG-pUL69aa1-146 with protein methyltransferases PRMT1 to -8 revealed that only PRMT6 induced an electrophoretic mobility shift of the respective UL69aa1-146 protein. While the fastest-migrating isoform of pUL69 disappeared upon PRMT6 coexpression, the isoform exhibiting the lowest electrophoretic mobility in SDS-PAGE was significantly enhanced (Fig. 6B, lane 3). This strongly suggests that PRMT6 catalyzes a specific methylation of arginine residues within the N terminus of pUL69.

Modification of a herpesviral RNA export factor by a specific protein methyltransferase is not without precedence. For instance, the multifunctional regulatory protein ICP27 of herpes

FIG 9 Generation and characterization of a recombinant cytomegalovirus carrying pUL69 arginine mutations 28 and 29, which affect the mRNA export activity of pUL69. (A) Schematic diagram illustrating recombinant HCMV bacmids generated via homologous recombination in *E. coli*. (B). Multistep growth curve analyses of HFF cells that were infected with equal IE units (MOI, 0.001) of wild-type HCMV AD169, the UL69 deletion virus AD169-ΔUL69, or the R/A mutant virus AD169-UL69RR28/29AA. Viral supernatants were harvested at the indicated time points (d, days postinfection), followed by the determination of viral genomes released into the supernatants by quantitative real-time PCR. Each infection was performed in triplicate, and the standard deviations are shown.

simplex virus type I has previously been shown to be methylated in its RGG box motif, and this was critical for viral replication (14, 57). A subsequent study suggested that PRMT1 acts as a cellular mediator of ICP27 RGG box methylation (17). Interestingly, we observed that PRMT1 was predominantly cytoplasmic, whereas only PRMT2 and PRMT6 exhibited a distinct colocalization with pUL69 within the cell nucleus. Furthermore, we demonstrate a specific protein interaction of pUL69aa1-146 with PRMT6 which could be detected both by yeast two-hybrid and coimmunoprecipitation analyses. This strengthens our assumption that PRMT6 plays an important role in posttranslational modification of pUL69 via methylation. However, since our experiments suggest that the C terminus of pUL69, which contains four additional putative arginine methylation substrates, may also serve as a target for methylation, the contribution of additional PRMTs cannot be excluded. In particular, we observed strong arginine methylation of a pUL69 mutant lacking the R2 box. Since the R2 box contains the bipartite nuclear localization signal of pUL69, and consequently, mutants lacking this sequence are excluded from the nucleus, cytoplasmic PRMTs may also be able to modify pUL69 depending on the specific subcellular localization of pUL69 (7, 56).

The assumption that the mono/dimethylarginine-modified N terminus of pUL69 results from a PRMT6-mediated enzymatic reaction is further strengthened by our finding that coexpression of catalytically active but not of catalytically inactive PRMT6 induced an electrophoretic mobility shift of pUL69aa1-146 (Fig. 3B, lanes 1 and 2). This suggests that the pUL69 N terminus is modified by asymmetric dimethylarginines, which are transferred by the catalytic activity of the type I PRMT6 but not by PRMT2.

Notably, expression of hypomethylated pUL69 seems to be weaker in the presence of catalytically inactive PRMT6-VLD/KLA (Fig. 3B, lane 1). This may be due to the fact that PRMT6-VLD/KLA can act as a dominant negative inhibitor that prevents methylation by endogenous PRMT6 and may thus affect the stability of pUL69. As demonstrated previously, PRMT6 stability itself is regulated by methylation, as its expression drastically decreases when automethylation of the arginine at the position 35 is prevented (59).

In order to decipher the impact of PRMT6 methylation on the functions of pUL69, we focused on a mutational analysis of the viral substrate pUL69, since a global knockdown of PRMT6 is thought to affect a multitude of cellular functions. For instance, PRMT6 has been shown to be involved in transcriptional regulation, DNA base excision repair, and cell cycle regulation, and a knockdown of PRMT6 results in an accumulation of cells at the G₂ checkpoint (55, 58, 60). Furthermore, global inhibitors of protein methylation, such as adenosine dialdehyde, broadly affect the function of many important cellular proteins and may also fail to inhibit the modification of stably methylated proteins (61). By mutational analyses, we could exclude the possibility that putative arginine methylation substrates were required for intranuclear localization of pUL69. We performed indirect immunofluorescence analyses and showed that arginines within the R1 or RS box were not required for intranuclear localization of pUL69 (data not shown). This finding is in agreement with previous reports demonstrating that pUL69 targets the nucleus independently from its R1 or RS boxes (7). Moreover, nuclear import of ICP27, the al-

phaherperviral homolog of pUL69, is also not modulated by methylation (15).

In particular, we concentrated on the R1 box motif of pUL69, since previous studies had demonstrated that a quadruple mutation of arginines 22/23 and 25/26 of this sequence abrogated the interaction with the cellular RNA export factor UAP56 and drastically reduced the RNA export activity of pUL69 (6). This correlated with a severe replication defect of recombinant viruses harboring the respective UL69 mutation (8, 9). Interestingly, we observed that the quadruple mutation of arginines 22/23 and 25/26 as well as double mutations of either arginines 22/23 or 25/26 both affected the binding of UAP56 and PRMT6. One explanation for this might be an alteration of the secondary structure of pUL69 by the respective mutations. Importantly, however, NMR analyses of peptides corresponding to the wild-type and the quadruple mutant sequence revealed that the secondary structure is well preserved within the mutant.

RNA export assays revealed that not only arginines 22/23 and 25/26 of the R1 box but also arginines 27/28 were essential for the RNA export activity of pUL69. Consequently, we investigated whether these arginines might also be relevant for efficient viral replication, which could be confirmed by growth curve analysis with a recombinant virus harboring the respective double mutation within pUL69. Interestingly, arginines 27/28 turned out to be not required for a direct protein interaction with either UAP56 or PRMT6. Thus, we propose that these two arginines may serve as methylation substrates of pUL69 that play a critical role in the stimulation of RNA export and, consequently, efficient viral replication. How methylation of pUL69 mechanistically modulates the RNA export activity is presently not clear. It has been demonstrated that the UAP56 interactor REF is methylated by PRMT1 and that this decreases its affinity for RNA, hence driving the handover of the RNA to the TAP/p15 export receptor (54). Since the arginine-rich box R1 of pUL69 is required not only for interaction with UAP56 or PRMT6 but also for RNA binding, a similar scenario may be true for UL69 (7). Alternatively, methylated arginines of pUL69 might provide a binding platform for additional cellular interaction partners, which is suggested by the finding that MMA, ADMA, and SDMA serve as binding motifs for proteins containing Tudor domains, such as the splicing factor survival of motor neuron (SMN) protein (62). However, additional studies will be required to further prove these hypotheses.

In summary, our study reveals a novel posttranslational modification of pUL69 which has a significant impact on the function of this important viral regulatory protein. Since not only pUL69 but also ICP27 exhibits this modification, regulation via posttranslational protein methylation may constitute a conserved feature of the ICP27 protein family.

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