Regulation of the EBNA1 Epstein-Barr Virus Protein by Serine Phosphorylation and Arginine Methylation

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The Epstein-Barr virus (EBV) EBNA1 protein is important for the replication and mitotic segregation of EBV genomes in latently infected cells and also activates the transcription of some of the viral latency genes. A Gly-Arg-rich region between amino acids 325 and 376 is required for both the segregation and transcriptional activation functions of EBNA1. Here we show that this region is modified by both arginine methylation and serine phosphorylation. Mutagenesis of the four potentially phosphorylated serines in this region indicated that phosphorylation of multiple serines contributes to the efficient segregation of EBV-based plasmids by EBNA1, at least in part by increasing EBNA1 binding to hEBP2. EBNA1 was also found to bind the arginine methyltransferases PRMT1 and PRMT5. Multiple arginines in the 325-376 region were methylated in vitro by PRMT1 and PRMT5, as was an N-terminal Gly-Arg-rich region between amino acids 41 and 50. EBNA1 was also shown to be methylated in vivo, predominantly in the 325-376 region. Treatment of cells with a methylation inhibitor or down-regulation of PRMT1 altered EBNA1 localization, resulting in the formation of EBNA1 rings around the nucleoli. The results indicate that EBNA1 function is influenced by both serine phosphorylation and arginine methylation.

The EBNA1 protein of Epstein-Barr Virus (EBV) enables the persistence of the episomal viral genome in latently infected cells, cycling B lymphocytes, which can lead to malignant transformation under some circumstances. EBNA1 makes several contributions to EBV latent infection. First, EBNA1 is required for the initiation of DNA replication from the EBV latent origin, oriP (69). This involves the binding of EBNA1 to the dyad symmetry (DS) element of oriP and recruitment of cellular replication initiation proteins (9, 13, 55). Second, EBNA1 is required for the stable segregation of the viral genomes during cell division. Segregation requires EBNA1 binding to the family of repeats (FR) element of oriP and attachment of EBNA1 to the host mitotic chromosomes through an interaction with the cellular hEBP2 protein present on the mitotic chromosomes (25, 26, 28, 66). Third, through binding to the FR element, EBNA1 can activate the transcription of other EBV latency genes, although the mechanism of this activation is not known (19, 52). Finally, EBNA1 can counteract the stabilization of p53 by USP7 that occurs in response to DNA damage, thereby decreasing apoptosis and increasing cell survival (30, 54).

Several functionally important regions of EBNA1 have been defined. The replication, segregation, and transcriptional activation functions of EBNA1 all require the DNA binding and dimerization domain, located near the C terminus between amino acids 459 and 607 (6, 50, 68), in order to bind an 18-bp recognition site present in multiple copies in the oriP DS and FR elements (51) (Fig. 1A). However, this domain is not sufficient for any EBNA1 function (8, 39, 67). The replication function also requires sequences in the N-terminal half of EBNA1, which likely play redundant roles in replication, since no small deletion has been found to abrogate this EBNA1 function (39, 67). The EBNA1 segregation function requires a central Gly-Arg-rich region between amino acids 325 and 376 and is stimulated by residues 8 to 67 (67). These regions appear to contribute to segregation by mediating an interaction with the hEBP2 protein on the cellular mitotic chromosomes (25, 40, 67). Transcriptional activation by EBNA1 requires both the 325-376 Gly-Arg-rich region and amino acids 61 to 83, in addition to the DNA binding and dimerization domain (31, 67). The interaction of EBNA1 with USP7, which can destabilize p53 by blocking the USP7-p53 interaction, occurs through EBNA1 residues 442 to 448 and, unlike the other EBNA1 functions, does not require the DNA binding and dimerization domain (22, 23, 54).

The 325-376 Gly-Arg-rich region is of major importance for both the segregation and transcription activation functions of EBNA1. This contains three tandem, identical 8-amino-acid sequences (GGRGRRGS) as well as a fourth partial match to this sequence (RERARGGS) (34). While a second, smaller Gly-Arg-rich region is present between residues 33 and 53 (Fig. 1A), this sequence is not functionally equivalent to the 325-376 sequence, since an EBNA1 mutant lacking amino acids 34 to 52 has wild-type activity for EBNA1 functions (67). The 325-376 region mediates interactions with at least four cellular proteins, hEBP2, Nap1, importin α, and P32/TAP (14, 23, 32, 56, 62, 65). The interaction with hEBP2, which occurs in mitosis, is important for the segregation function of EBNA1 (28), while the interaction with importin α is implicated in nuclear uptake. The functional significance of EBNA1 binding to Nap1 and P32/TAP is not yet clear. The 325-376 region also mediates the homotypic association of EBNA1 molecules bound to the FR and DS elements of oriP, resulting in the
looping and linking of the oriP DNA (1, 20, 38). This looping interaction stabilizes EBNA1 binding to the DS element and may contribute to DNA replication (17, 39). How the various protein interactions with the EBNA1 325-376 region are regulated remains to be determined.

The activities of many proteins are regulated by posttranslational modifications. EBNA1 is known to be phosphorylated on serine residues (18, 21, 49) and has also been shown to be poly(ADP) ribosylated, which appears to negatively regulate the replication activity of EBNA1 (12). The functional effect of serine phosphorylation in EBNA1 has not been determined, and the locations of the phosphorylated residues have not been mapped. The 325-376 region contains four serines, all of which are predicted phosphorylation sites for calmodulin-dependent kinase II, a kinase implicated in many cellular processes (Fig. 1B). In addition, both the 33-53 and 325-376 EBNA1 regions contain RGG and GRG motifs that are predicted sites of arginine methylation (10 sites in the 325-376 region and 6 in the 33-53 sequence), a modification found to regulate the activity of many proteins (4). Arginine methylation can be catalyzed by at least six different protein arginine methyltransferases (PRMTs), resulting in either the symmetric (for PRMT5 and -7) or asymmetric (for PRMT1, -3, -4, and -6) addition of two methyl groups (4). EBNA1 was previously found to stably interact with PRMT5, suggesting that EBNA1 might be a substrate for symmetric arginine methylation by PRMT5 (23).

In order to better understand how the segregation and transcriptional activation functions and protein interactions mediated by the 325-376 region are regulated, we have investigated whether these sequences are modified by Ser phosphorylation and Arg methylation. Here we show that this region can be methylated by PRMT1 and -5 and that methylation affects EBNA1 localization. Through mutational analyses, we also find that phosphorylation of the serines in the 325-376 region contributes to the segregation function of EBNA1 by affecting binding to hEBP2.

**MATERIALS AND METHODS**

**Identification of phosphorylated serine in EBNA1.** Thirty milligrams of EBNA1 protein, purified from Sf9 insect cells as previously described (23), was treated with 200 ng of trypsin overnight at 37°C and analyzed on an Applied Biosystems/MDS Sciex QTrap LC/MS/MS mass spectrometer in the Proteomic and Mass Spectrometry Centre at the University of Toronto, Faculty of Medicine. The digested protein was directly infused to the mass spectrometer. A neutral loss scan of H$_2$PO$_4$ (98 Da) was performed first to specifically identify the phosphorylated peptides, and then an enhanced product ion scan was performed to sequence the selected phosphorylated peptide.

**Construction of plasmids expressing EBNA1 serine mutants.** EBNA1 serine mutants, in which the four serines in the EBNA1 325-376 region are converted to alanine or aspartic acid individually or in combination, were generated in the mammalian expression plasmid pc3oriP (56). Mutants are named according to the position of the mutated serine(s), where SS5S refers to Ser334, Ser342, Ser350, and Ser365, respectively, pc3oriP expressing ASSS or SSAAS was created using pc3oriPE (56) as a template for QuikChange (Stratagene) mutagenesis. pc3oriP expressing ASSA, SSAAS, ASSS, SDDS, or DDDD was constructed using the following cloning strategy. The EBNA1 coding sequence was PCR amplified from pc3oriPE using primers containing NdeI and BamHI sites and inserted into NdeI and BamHI sites of the pUC19 plasmid. The resulting plasmid was digested with BamHI, which released an EBNA1 fragment containing residues 325 to 376. The BamHI-digested plasmid was ligated to two sets of complementary oligonucleotide cassettes containing BamHI ends. All clones in pc3oriPE were sequenced to confirm the mutations.

For expression in Saccharomyces cerevisiae, the EBNA1 serine mutants were cloned in p416MET25. This involved PCR amplification of the EBNA1 serine mutants in pc3oriP, phosphorylation of the ends of the PCR fragment by T4 polynucleotide kinase, and ligation into the Smal site of pc416MET25.

**S. cerevisiae plasmid loss assays.** Yeast strain KY320 (MATa leu2-2,112 trp1-116 his3-200 GAL-110) was transformed with the segregation test plasmid YRp7FR, the hEBP2-expressing plasmid p425PGK.hEBP2, and p416MET25 expressing EBNA1, an EBNA1 serine mutant, or no EBNA1. The plasmids and plasmid loss assays were as described by Kapoor et al. (29). Briefly, positive transformants were grown in selective medium and then diluted into medium nonselective for the segregation test plasmid and grown for 11 generations. Ten-fold serial dilutions of the resulting cultures were then spotted on selective and nonselective plates to assess the stability of the segregation plasmid. The percentage of cells that retained the segregation plasmid was quantified by counting colonies grown on selective and nonselective plates from equal amounts of culture.

**Mammalian transient-replication and long-term maintenance assays.** HeLa cells were plated at 1 × 10⁶ cells per 6-cm plate 24 h before transfection with the ESCORT II reagent (Siga) as per the manufacturer’s directions. Cells were transfected with pc3oriP (negative control), pc3oriPE expressing EBNA1 (56), or a pc3oriP plasmid expressing an EBNA1 serine mutant. Transfected cells were grown for 3 days without selection (transient replication) or 2 weeks in G418 (300 μg/ml) to select for cells containing the plasmid (long-term maintenance). Cells were then grown in selective medium and then diluted into medium nonselective for the segregation test plasmid and grown for 11 generations. Ten-fold serial dilutions of the resulting cultures were then spotted on selective and nonselective plates to assess the stability of the segregation plasmid. The percentage of cells that retained the segregation plasmid was quantified by counting colonies grown on selective and nonselective plates from equal amounts of culture.

**Transcription activation assays.** HeLa (Tet-On) cells were plated at 2.5 × 10⁶ cells per 6-cm plate 24 h prior to transfection using ESCORT II reagent. Cells were cotransfected with 2 μg of the pFRKTCAT reporter construct and 5 μg pc3oriP expressing EBNA1 or EBNA1 mutants or lacking the EBNA1 cassette. At 48 h posttransfection, cells were lysed and 50 μg of lysate was assayed for chlorophenol red acetyltransferase (CAT) activity as previously described (6, 67). The amount of acetylated product generated by each lysate was quantified for several reaction times and used to determine the acetylation rate.

**Two-hybrid assays.** Coding sequences for EBNA1 serine mutants were PCR amplified from the p416MET25 expression constructs and cloned between NdeI and BamHI sites in pAS2. The mutants were then subcloned into pLexA-kan (a kind gift from Igor Stagljar) containing NdeI and BamHI sites for expression as a LexA fusion protein. pc416MET25 expressing EBNA1 and EBNA1 mutants or lacking the EBNA1 cassette. At 48 h posttransfection, cells were lysed and 50 μg of lysate was assayed for chlorophenol red acetyltransferase (CAT) activity as previously described (6, 67). The amount of acetylated product generated by each lysate was quantified for several reaction times and used to determine the acetylation rate.
EBNA1 proteins for affinity columns and methylation assays. The highly purified EBNA1 proteins were used as substrates in the methylation reactions. The version of EBNA1 used lacks most of the Gly/Ala repeat region, as do the EBNA1 internal deletion mutants. EBNA1 (23), EBNA1GA (with full Gly/Ala repeat [5]), and ΔA1-376 (EBNA1 lacking amino acids 41-376 [1]) were purified from insect cells as previously described. ΔA1-376 (EBNA1 lacking amino acids 61 to 83 [67]) was purified from insect cells on a metal chelating column by virtue of an N-terminal histidine tag. The EBNA1 truncation mutants containing the indicated amino acids, 351 to 641, 363 to 641, 377 to 641, and 452 to 641, were purified from Escherichia coli as previously described (3, 17), as was ΔA525-376 (23). The EBNA1 mutant 320-355/459-607, in which amino acids 320 to 355 are fused at the N terminus to amino acids 459 to 607, was purified from E. coli on a metal chelating column by virtue of a N-terminal six-histidine tag.

EBNA1 affinity columns. Affinity columns containing purified EBNA1 and whole-cell extracts from HeLa cells were generated as described by Holowaty et al. (23). A 400-μl aliquot of the HeLa lysate (at 14 mg/ml) was applied under physiological salt conditions to a 40-μl column containing EBNA1 or deletion mutants coupled at 1 mg/ml. After washing, bound proteins were eluted in high salt followed by 1% sodium dodecyl sulfate (SDS) and identified by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry as previously described (23). SDS eluates were also analyzed by Western blotting using rabbit anti-PRMT1 (Upstate Biochemicals) or mouse anti-PRMT5 (Transduction Laboratories) antibodies.

Purification of PRMT enzymes and GAR substrate. PRMT1, PRMT3, PRMT5, and PRMT6 and GAR substrate were expressed as glutathione S-transferase (GST)-fusion proteins from pGEX plasmids in E. coli strain BL2I(DE3)pLyS8. Plasmids were kindly provided by Harvey Herschman, Steven Clarke, and Sidney Pestka and have been described previously (15, 16, 37, 59). Expression of GST-fusion protein was induced with 0.5 mM isopropyl-1-thio-

PRMT1 silencing. U2OS cells were seeded at 50% confluence in six-well plates containing coverslips, 24 h prior to Lipofectamine 2000 transfection with 3 μg of pSilencer3.1-U1hygro (Ambion) expressing PRMT1 hairpin RNA (5′AAAGGAG GTGGACATCTATAC). The cells were grown for 4 days in the presence of 100 μg/ml hygromycin and then transfected as above with 3 μg pCsiORiP expressing EBNA1. Two days later, the cells were prepared for immunofluorescence microscopy as described above. PRMT1 was detected with rabbit antibody (07-404; Upstate Biochemicals) followed by FITC-conjugated goat anti-rabbit antibody (FITC; Santa Cruz). EBNA1 was detected with the mouse monoclonal antibody OT1x followed by Texas Red-conjugated goat anti-mouse antibody (Molecular Probes). In all cases, DNA was visualized by DAPI staining and microscopy as described above.

RESULTS

In vivo methylation. U2OS cells were grown in H21 medium to 90% confluency in a 15-cm dish and then were transfected with 10 μg of pcDNA3 plasmid expressing EBNA1 or EBNA1 mutant ΔA32-54, ΔA325-376, or ΔA40-376 (described in references 56 and 67) using Lipofectamine 2000 (Invitrogen). After a 4-h incubation, cells were split into two 15-cm dishes. Three days posttransfection, the medium was replaced with 8 ml of H21 containing 100 μg/ml cycloheximide and 10 μg/ml chloramphenicol. After 30 min at 37°C, the cells were rinsed twice with methionine-free Dulbecco’s modified Eagle’s medium and then incubated for 3 h at 37°C in 4 ml methionine-free Dulbecco’s modified Eagle’s medium containing 100 μg/ml cycloheximide, 10 μg/ml chloramphenicol, and 40 μCi [3H]methionine. Cells were then harvested, rinsed with PBS, and incubated in 700 μl lysis buffer (100 mM Tris-HCl pH 8, 250 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM PMSF, Roche protease inhibitors) for 30 min on ice. After centrifugation for 15 min in a microcentrifuge, a 20-μl volume of protein A-Sepharose and 2 μl of 4 μCi [3H]methionine were added to the supernatant and incubated overnight at 4°C with rocking. The Sepharose beads were then washed three times with lysis buffer, and bound proteins were eluted with 40 μl 2% SDS. All of the eluate was loaded onto a 12% polyacrylamide gel and transferred to polyvinylidene difluoride membrane. EBNA1 proteins were identified by Western blotting using anti-EBNA1 mouse monoclonal antibody OT1x (supplied by J. Middledorp). The membrane was then stripped and exposed to film for 1 month to detect protein bands containing tritium.

MTRA treatment and immunofluorescence microscopy. HeLa cells were plated at 1 × 10^6 cells per 6-cm plate 24 h before transfection with 2 μg of pcDNA3 by using the EFSORT II reagent. Four hours posttransfection, the transfection mixture was washed and cells were thoroughly washed with PBS to terminate the transfection process. Cells were replated and incubated in medium containing 0.3 mM 5’-[3H]methylenodeoxadenosine (MTA) (dissolved in dimethylformamide (DMF)) or an equivalent amount of DMF alone (negative control) for 48 h before processing for immunofluorescence microscopy. For microscopy, log-phase cells were adhered to coverslips, fixed, and permeabilized as described by Wu et al. (66). Cells were stained with mouse anti-EBNA1 monoclonal antibody OT1x and either rabbit anti-HEBP2 (66), rabbit anti-USP7 (23), or goat anti-B23 antibody (Santa Cruz). For samples that were costained for EBNA1 and either HEBP2 or USP7, the secondary antibodies used were goat anti-mouse conjugated to Texas Red and goat anti-rabbit conjugated to fluorescein isothiocyanate (FITC; Santa Cruz). For cells stained for both EBNA1 and B23, the secondary antibodies used were goat anti-mouse conjugated to FITC (Santa Cruz) and donkey anti-goat conjugated to Cy3 (Chemicon). All cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, 25 ng/ml) and visualized at 400× magnification using a Leica DMR microscope and OpenLab software.

In vivo phosphorylation of the EBNA1 325-376 region. EBNA1 is phosphorylated on multiple serine residues when expressed in both human and insect cells (18, 21, 49), and the functionally important 325-376 region contains multiple serines that are highly predicted to be phosphorylated. To investigate the possibility that the functionality of the 325-376 region is modulated by phosphorylation, we first asked whether phosphorylation of these sequences could be detected in EBNA1 purified from insect cells after baculovirus expression. To this end, highly purified EBNA1 was completely digested with trypsin and phosphorylated peptides were examined using a QTrap LC/MS/MS mass spectrometer. A phosphorylated tryptic peptide of the expected size for the 325-376 region was selected, sequenced, and shown to be comprised of GGSSGR. This sequence is found in triplicate in the 325-376 region (Fig. 1B), indicating that one or more copies of this sequence are phosphorylated in the context of the functional EBNA1 protein.

Contribution of serine phosphorylation to the EBNA1 segregation function. To test the possible functional importance of phosphorylation of the serine residues in the EBNA1 325-376 region, each of the serines in the GGSSGR repeated sequence (serines 334, 342, and 350) was mutated to alanine in the context of functional EBNA1, and combinations of serine mutations were also generated. Mutants were named according to the four serines (or alanine mutations) in the 325-376 region, where SSRR refers to serines 334, 342, 350, and 365. EBNA1 proteins containing these mutations were first tested for their ability to segregate plasmids containing the EBV
serine mutants with hEBP2, as determined by activation of a reporter gene. EBNA1 with empty pACT2 (-EBP2) is shown as a negative control (second row). Ten-fold serial dilutions of the cultures were grown on plates selective for the segregation element FR in a reconstituted segregation system in Saccharomyces cerevisiae (29). In this system, plasmids replicate from an ARS element using the yeast replication machinery but can only segregate during cell division by virtue of EBNA1 and the FR element. Segregation occurs through the attachment of EBNA1 to the yeast chromosomes, which requires the human EBP2 protein exogenously expressed in the yeast cells (27), a mechanism that appears to be the same as for EBNA1-mediated plasmid segregation in human cells. The ability of the EBNA1 serine mutants to maintain the segregation test plasmids was determined after 11 cell generations in the absence of selection for these plasmids, and the results were compared to wild-type EBNA1 and no EBNA1 by spotting dilutions of the cultures on plates selective for the segregation plasmid (Fig. 2A) and by comparing the numbers of colonies grown on selective and nonselective plates (Table 1). In all cases expression of the EBNA1 proteins at similar levels was confirmed by Western blotting (data not shown). While mutations of individual serines had no significant effect on the EBNA1 segregation function, mutation of two serines in adjacent repeated sequences consistently caused a twofold reduction in plasmid segregation (AASS and SAAS mutants). This segregation defect was restored by converting the alanines to aspartic acid (mutant SDDS) to mimic the charge of the phospho-serine. Plasmid segregation efficiency was further reduced (threefold relative to wild type) by mutating all four serines in the 325-376 region to alanines (mutant AAAA) and was again restored by conversion of all four alanines to aspartic acid (mutant DDDD), indicating that phosphorylation of these serines plays a role in segregation.

To determine if the serine phosphorylation effect observed for EBNA1-mediated segregation in the yeast system also occurs in human cells, we expressed the EBNA1 serine mutants in human cells from plasmids containing the EBV oriP. We then tested the ability of these plasmids to be stably maintained in dividing cells for a 2-week period, as determined by the level of recovered plasmid relative to that in the presence of wild-type EBNA1 (Fig. 3B and Table 1). Stable maintenance of oriP plasmids is known to require the segregation function of EBNA1 in addition to replication. While all EBNA1 proteins were expressed at the same level (Fig. 3A), the SAAS, AASS, and AAAA EBNA1 mutants were consistently less able to maintain oriP plasmids than wild-type EBNA1. As in the yeast system, this defect was rescued by conversion of the alanines to aspartic acid, indicating that phosphorylation of these serines affects the plasmid maintenance ability of EBNA1. To ensure that these effects were due to the ability of EBNA1 to mediate the segregation of the oriP plasmids as opposed to their replication, we expressed the EBNA1 serine mutants in addition to replication. While all EBNA1 proteins were expressed at the same level (Fig. 3A), the SAAS, AASS, and AAAA EBNA1 mutants were consistently less able to maintain oriP plasmids than wild-type EBNA1. As in the yeast system, this defect was rescued by conversion of the alanines to aspartic acid, indicating that phosphorylation of these serines affects the plasmid maintenance ability of EBNA1. To ensure that these effects were due to the ability of EBNA1 to mediate the segregation of the oriP plasmids as opposed to their replication, we expressed the EBNA1 serine mutants in addition to replication.

### Table 1. Functional effects of EBNA1 serine mutations

<table>
<thead>
<tr>
<th>EBNA1 protein</th>
<th>Plasmid segregation (yeast)</th>
<th>Plasmid maintenance (human cells)</th>
<th>Plasmid replication (human cells)</th>
<th>Transcriptional activation (human cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2 (±0.9)</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>EBNA1</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>ASS</td>
<td>92 (±3)</td>
<td>106 (±17)</td>
<td>113</td>
<td>86 (±7)</td>
</tr>
<tr>
<td>SASS</td>
<td>101 (±9)</td>
<td>88 (±2)</td>
<td>88</td>
<td>71 (±14)</td>
</tr>
<tr>
<td>SSAS</td>
<td>116</td>
<td>116 (±20)</td>
<td>91</td>
<td>71 (±8)</td>
</tr>
<tr>
<td>ASSA</td>
<td>84 (±7)</td>
<td>94 (±16)</td>
<td>113</td>
<td>68 (±16)</td>
</tr>
<tr>
<td>AASS</td>
<td>47 (±14)</td>
<td>53 (±8)</td>
<td>83</td>
<td>52 (±14)</td>
</tr>
<tr>
<td>SAAS</td>
<td>44 (±9)</td>
<td>61 (±12)</td>
<td>92</td>
<td>120 (±15)</td>
</tr>
<tr>
<td>SDDS</td>
<td>85 (±1)</td>
<td>115 (±30)</td>
<td>93</td>
<td>47 (±10)</td>
</tr>
<tr>
<td>AAAA</td>
<td>36 (±11)</td>
<td>65 (±8)</td>
<td>106</td>
<td>64 (±9)</td>
</tr>
<tr>
<td>DDDD</td>
<td>76 (±6)</td>
<td>116 (±25)</td>
<td>96</td>
<td>33 (±18)</td>
</tr>
</tbody>
</table>

- Plasmid loss assays in yeast, as conducted in Fig. 2A, were quantified by counting colonies on selective plates. Mean values are shown relative to EBNA1 as a control. Standard deviations are shown in brackets.
- Plasmids recovered from plasmid maintenance assays in human cells, conducted as in Fig. 3B, were quantified relative to those with wild-type EBNA1. Values shown are averages of three experiments, with standard deviations shown in brackets.
- DpnI-resistant plasmids recovered from a transient-replication assay in human cells were quantified relative to those with wild-type EBNA1. Values shown are averages of three experiments, with standard deviations shown in brackets.
- Acetylation rates were determined for CAT reporter assays as shown in Fig. 4 and expressed relative to EBNA1 after subtraction of background acetylation seen in the absence of EBNA1. Mean values are shown as averages of three experiments, with standard deviations shown in brackets.
lication, we also assessed the transient replication of the oriP plasmids in the presence of each of the EBNA1 mutants. None of the serine mutations was found to significantly affect the replication of the oriP plasmids (Table 1). Therefore, the phosphorylation of multiple serines in the EBNA1 325-376 region positively regulates the ability of EBNA1 to segregate plasmids in both human cells and the reconstituted yeast system.

Serine phosphorylation affects the EBNA1-hEBP2 interaction. One possibility of how phosphorylation of serines in the 325-376 region affects the segregation function of EBNA1 is by its altering EBNA1 binding to hEBP2. hEBP2 interacts with EBNA1 through sequences in the 325-376 region (56). This interaction is absolutely required for EBNA1-mediated segregation in yeast and is also important for oriP segregation in human cells, in both cases serving to tether EBNA1 to the cellular mitotic chromosomes (27, 28). To determine if the decreased segregation function associated with the EBNA1 SAAS and AAAA mutants is due to less efficient binding to hEBP2, we compared the hEBP2 binding ability of these EBNA1 mutants to wild-type EBNA1 in a LexA-based yeast two-hybrid assay, where binding stringency can be assessed by comparing activation of the His3 gene in the presence of increasing amounts of AT (Fig. 2B). We found that, while the SAAS mutant appeared to bind hEBP2 at similar levels as wild-type EBNA1, the SDDS mutant had increased hEBP2 binding under stringent conditions (His 5 SAT panel). We also found that the AAAA mutant was clearly impaired for binding hEBP2 (His and His 2AT panels) and that the binding was restored by converting the four alanines to aspartic acids, resulting in higher binding activity than wild-type EBNA1 (His 2AT and His 5 AT panels). Therefore, the ability of EBNA1 to bind hEBP2 is positively affected by phosphorylation of multiple serines in the 325-376 region, which likely affects the segregation function.

Contribution of serine phosphorylation to the EBNA1 transcription activation function. In addition to its role in segregation, the 325-376 region of EBNA1 is important for transcriptional activation from the FR element. Therefore, we tested the EBNA1 serine mutants for possible effects on activating the expression of a CAT reporter gene under the control of the oriP plasmid expressing EBNA1, no EBNA1, or the EBNA1 serine mutants as indicated. Two days later, equal amounts of cell lysates were then assayed for CAT activity by following the acetylation of chloramphenicol in 5-, 15-, and 30-min reactions. Some of the single and double alanine substitutions were found to decrease transcriptional activity to various degrees, as did the quadruple alanine substitution. However, conversion of the four alanines to aspartic acid did not restore transcription function, but rather further decreased it. The results are more consistent with effects on transcription due to sequence alteration rather than phosphorylation effects.

EBNA1 interactions with protein arginine methyltransferases. We have previously examined cellular protein interactions with EBNA1 using an in vivo TAP-tagging approach as well as an EBNA1 affinity column approach (23). The TAP-tagging experiments showed that EBNA1 stably interacted with the protein arginine methyltransferase PRMT5, in addition to other proteins. Strangely, PRMT5 was not identified as an EBNA1 binding protein using the affinity column approach, where cell lysates were applied to EBNA1 microcolumns under physiological salt conditions and retained proteins were then eluted in high salt. We have recently revisited the EBNA1 affinity column method by adding an SDS elution step following the high-salt elution, in order to elute any proteins that may
bind EBNA1 largely through hydrophobic interactions and hence not be efficiently eluted with salt. In doing so, we found that PRMT5 was retained on the EBNA1 column after the high-salt elution and subsequently eluted with SDS (Fig. 5A). PRMT5 was also found to bind EBNA1 mutants lacking amino acids 325 to 376 or 61 to 83, indicating that these sequences are not required for the PRMT interaction. In the same experiments, a second protein arginine methyltransferase, PRMT1, was found to bind EBNA1 and the Δ325-376 and Δ61-83 mutants, eluting from the columns with SDS (the most efficient retention was seen with the Δ61-83 affinity column). Western blot assays performed on the column eluates in Fig. 5A confirmed that both PRMT1 and PRMT5 were specifically retained on the EBNA1 columns (but not on the control column) (Fig. 5B).

In vitro methylation of EBNA1. The interaction of EBNA1 with PRMT1 and PRMT5 may indicate that EBNA1 is a substrate for methylation by these enzymes. To investigate this possibility, we expressed and purified PRMT1 and PRMT5 as GST fusions in E. coli and tested their ability to methylate EBNA1 in vitro in comparison to equivalent amounts of a positive control substrate (GST-GAR). Both PRMT1 and PRMT5 were found to methylate EBNA1, containing either the small or full-length Gly-Ala repeat (EBNA1 and EBNA1GA, respectively) with similar efficiency as they methylated GST-GAR (Fig. 6A and C), although PRMT5 was less active on both GST-GAR and EBNA1 substrate than PRMT1. We then repeated the methylation assays using a variety of EBNA1 deletion and truncation mutants in order to map the EBNA1 regions that were methylated by these enzymes (Fig. 6A and C). For both PRMT1 and -5, methylation of EBNA1 was decreased but not abolished by deleting amino acids 325 to 376, indicating that this region is methylated but is not the only methylated region. The decreased methylation of Δ325-376 with PRMT1 is most obvious in the time course in Fig. 6B, conducted using equal amounts of EBNA1 substrates and PRMT1 enzyme. Methylation by both PRMT1 and PRMT5 was abrogated by the 41-376 deletion, which deletes multiple GRG motifs between amino acids 41 and 50, in addition to the methylation sites in the 325-376 region (note that amino acids 101 to 324 of the Gly-Ala repeat are missing in all EBNA1 proteins except EBNA1GA). This suggests that sequences in both the 41-50 and the 325-376 regions of EBNA1 are targets for methylation by both PRMT1 and PRMT5.

Further information about which sequences in the 325-376 region are methylated was gained from methylation assays performed with a series of EBNA1 truncation mutants (Fig. 6A and C). EBNA1 N-terminal truncation mutants 351-641 and 363-641 were methylated by both PRMT1 and PRMT5, while neither enzyme methylated the 377-641 mutant. This indicated that one or more methylation sites exist between residues 364 and 377. We also found that an EBNA1 mutant containing residues 320 to 355 fused to the DNA binding domain (459 to 607) was methylated by both PRMT1 and PRMT5, indicating that one or more methylation sites exist in the 320-355 region. Therefore, multiple sites in the 325-355 region can be methylated by PRMT1 and PRMT5.

To further investigate the specificity of EBNA1 methylation, we asked whether other PRMTs that have not been found to bind EBNA1 also methylated EBNA1. To this end, we expressed and purified PRMT3 and PRMT6 as GST fusions in E. coli and compared their abilities to methylate EBNA1 and GST-GAR (positive control) substrates (Fig. 6D and E). While both PRMT3 and -6 resulted in some labeling of EBNA1 and EBNA1GA, this was much less than that seen with an equivalent amount of GST-GAR substrate, indicating that both of these enzymes have very low specificity for EBNA1. The methylation of EBNA1 that was observed occurred within the 41-376 region, since removal of these sequences abrogated labeling. Methylation at levels equivalent to GST-GAR was only seen with the 320-355 fragment when fused to the DNA binding domain, indicating that this sequence can be methylated by PRMT3 and -6 when removed from its normal
FIG. 6. In vitro methylation of EBNA1 and EBNA1 mutants. Equal amounts of the purified EBNA1 proteins indicated or the GST-GAR positive control were incubated with S-methyl-14Cadenosyl methionine and either PRMT1 (A and B), PRMT5 (C), PRMT3 (D), or PRMT6 (E) as described in Materials and Methods. Reaction mixtures were then analyzed by SDS-PAGE and autoradiography. In each case the major labeled band corresponds to the position of the full-length protein. Note that EBNA1 362-641 has a small fusion at the C terminus, making it run larger than 351-641 (17). In panel B, methylation reactions were conducted for various times ranging from 5 to 40 min and the amount of labeled EBNA1 was quantified. A direct comparison of methylation of GST-GAR and EBNA1 substrates by PRMT1, -3, and -6 is shown in panel F and used amounts of enzyme that gave equal methylation of GST-GAR.
context. In Fig. 6F, we directly compared the relative specificities of PRMT3 and PRMT6 to PRMT1, using amounts of each enzyme that gave equivalent methylation of GST-GAR. This clearly showed that the level of EBNA1 methylation by PRMT3 and -6 was much less than for PRMT1. Together, the results suggest that PRMT1 and -5, but not PRMT3 and -6, are likely to methylate EBNA1 in vivo.

**In vivo methylation of EBNA1.** To determine whether EBNA1 is methylated in human cells, we expressed EBNA1 and several deletion mutants in U2OS cells. After blocking protein production with cycloheximide, the cells were grown in the presence of [3H]methyl-methionine and then with [3H]methyl-methionine. EBNA1 proteins were then immunoprecipitated with anti-EBNA1 rabbit serum and separated by SDS-PAGE. A. Western blot of immunoprecipitated EBNA1 probed with mouse anti-EBNA1 monoclonal antibody. B. Autoradiograph of the blot in panel A, showing incorporation of [3H]methyl-methionine.

To determine whether EBNA1 is methylated in human cells, we expressed EBNA1 and several deletion mutants in U2OS cells. After blocking protein production with cycloheximide, the cells were grown in the presence of [3H]methyl-methionine, and incorporation of the tritiated methyl groups into EBNA1 was determined by autoradiography of SDS-polyacrylamide gels containing immunoprecipitated EBNA1. As shown in Fig. 7A, EBNA1 and all of the deletion mutants were recovered at similar levels by immunoprecipitation. Exposure to film showed that EBNA1 and Δ34-52, lacking the N-terminal Gly-Arg-rich region, had been methylated, whereas methylation was not detected in the Δ325-376 and Δ41-376 mutants. This indicates that the 325-376 region is a major site of methylation in vivo. The amount of label incorporated in Δ34-52 was consistently about 60% of that for EBNA1, in keeping with the possibility that some methylation might also occur in the N-terminal Gly-Arg-rich region in vivo.

**Effect of methylation on EBNA1 localization.** To determine what effect methylation might have on EBNA1 functions, we looked at the localization and transcriptional activation of EBNA1 in human cells after treating the EBNA1-expressing cells with the methylation inhibitor MTA. MTA concentrations were used that have been previously known to affect protein methylation (43) but which do not inhibit cell proliferation (data not shown). We did not observe an effect on the transcriptional activity of EBNA1, compared to mock-treated cells, nor did we observe an obvious effect on EBNA1 attachment to mitotic chromosomes by immunofluorescence (data not shown). We did, however, consistently see effects of MTA treatment on the localization of EBNA1 in interphase cells. After treatment of EBNA1-expressing cells with MTA (at concentrations ranging from 0.1 to 0.3 mM), EBNA1 was observed to localize to the outer region of the nucleolus in approximately 25% of the cells (values ranged from 20 to 30% in nine separate experiments), resulting in the formation of nucleolar rings that were not seen in mock-treated cells (Fig. 8). This effect appeared to be specific for EBNA1, because the localization of the nucleolar proteins B23 (Fig. 8A) and hEBP2 (Fig. 8B) was not affected by the MTA treatment, nor was the localization of the nuclear protein USP7 (Fig. 8C). The results suggest that the methylation state of EBNA1 affects its localization.

We also examined the effect of PRMT silencing on EBNA1 localization. To this end, U2OS cells were transfected with a plasmid expressing hairpin RNA for PRMT1 and, 2 days later, transfected with an EBNA1 expression plasmid. Cells were then stained for EBNA1 and PRMT1 and visualized by immunofluorescence microscopy (Fig. 8D). EBNA1 was observed to form rings around the nucleoli in approximately 90% of the cells with little or no PRMT1 expression. In contrast, only 10% of cells in the same experiment that had higher PRMT1 staining levels (and expressed EBNA1) showed the nucleolar ringing of EBNA1. This effect was not seen upon transfection of the same cells with a PRMT5 silencing construct; however, efficient silencing of PRMT5 was not apparent by microscopy (data not shown). Therefore, PRMT1 has a major effect on the nuclear distribution of EBNA1.

**DISCUSSION**

We have examined the possible posttranslational modifications of the functionally important 325-376 Gly-Arg-rich region of EBNA1 and found it to be modified by both serine phosphorylation and arginine methylation. Mass spectrometric analysis indicates that the serine within the GGSGGR sequence, repeated three times in the 325-376 region, is phosphorylated, and mutational analyses were consistent with the phosphorylation of multiple serines in this region positively affecting EBNA1's ability to segregate oriP plasmids. At least part of this effect appears to be due to the ability to bind hEBP2, since the quadruple serine mutations affected both segregation and hEBP2 binding. This suggests that phosphorylation of the EBNA1 325-376 region would occur prior to hEBP2 binding, facilitating the EBNA1-hEBP2 interaction in mitosis that tethers EBNA1 and oriP plasmids to the chromosomes.

The phosphorylation of multiple serine residues has also been found to affect the ability of the bovine papillomavirus E2 protein to segregate bovine papillomavirus genomes. In that case, phosphorylation of a series of four serines in the hinge region of E2 was found to be required for efficient maintenance of viral plasmids and for E2 attachment to mitotic chromosomes (35, 36). As for EBNA1, phosphorylation of these four serines played redundant roles in segregation, as phos-
phorylation of any one of the four serines in E2 was sufficient to restore wild-type activity. For E2, the requirement for serine phosphorylation for segregation does not appear to be due to a direct effect on interactions with a mitotic chromosomal protein, but rather due to effects on binding the viral E1 protein, which can inhibit E2 attachment to mitotic chromosomes (63). Unlike for EBNA1, mutation of one of the serines (S301) to alanine in the E2 hinge also increases the half-life of E2, thereby affecting its functions (47). Serines 298 and 301, which are the most prevalent phosphorylated residues in the E2 hinge, fall within consensus sites for cyclin-dependent, mitogen-activated, and casein kinases, and S301 has been shown to be modified by casein kinase II (41, 46). For EBNA1, all four of the serines in the 325-376 region are predicted targets of the highly conserved, multifunctional calmodulin-dependent kinase II (61).

Arginine methylation has been emerging as a modification that regulates many cellular processes (4), and the functions of
several viral proteins have also been shown to be regulated by this modification (2, 7, 42, 53). PRMT1 and PRMT5, in particular, have been shown to have a variety of cellular and viral targets, thereby affecting diverse processes, including gene expression, signal transduction, and DNA repair. We found that EBNA1 binds and is methylated by both PRMT1 and PRMT5, indicating the EBNA1 can be modified by both symmetric and asymmetric arginine methylation catalyzed by PRMT5 and PRMT1, respectively. Mutational analysis is consistent with multiple methylation sites for both enzymes in the 325-376 region and additional methylation between residues 41 and 50. The lack of methylation of the Δ41-376 mutant indicates that neither enzyme methylates arginine 34, which is located within an RGG motif, at least in the context of the mutant protein. We do not yet know whether PRMT1 and PRMT5 are methylating the same arginine residues in these two regions, but this is a possibility that has preceded. PRMT1 and PRMT5 have been shown to methylate the same arginine residues in both histone H4 and the transcription elongation factor SPT5, and it has been suggested that modifications by these two enzymes might affect the functions of these proteins in opposite ways (4, 33, 45, 64). It is not yet clear whether modifications of EBNA1 by PRMT1 and -5 have similar, perhaps redundant, effects on EBNA1, have antagonistic effects, or contribute to independent EBNA1 functions.

EBNA1 was shown to be methylated in cells, and the presence of the 325-376 region was important for methylation in vivo. Inhibition of methylation by MTA treatment did not produce an obvious effect on EBNA1 transcriptional activation or on its ability to attach to cellular chromosomes (a prerequisite for segregation). However, assessing the functional role of methylation is complicated by the fact that EBNA1 is a very stable protein and that both symmetric and asymmetric dimethylation of arginines is thought to be irreversible. Therefore, methylated forms of EBNA1 may be present in the cell long after methylation is blocked. Despite these caveats, we did see an effect of MTA treatment on EBNA1 localization, which resulted in the formation of a ring of EBNA1 around the outer portion of the nucleolus. A similar nucleolar ringing effect was observed when EBNA1 was expressed in cells where PRMT1 levels had been down-regulated. An effect of methylation inhibitors on the localization of two other methylated proteins, Sam68 and HnRNP A2, has previously been reported, in both cases causes these normally nuclear proteins to accumulate in the cytoplasm (10, 44). The nucleolar rings formed by EBNA1 upon MTA treatment or PRMT1 silencing are reminiscent of those formed by B23 upon inhibition of RNA polymerase I (11, 57); however, MTA treatment did not affect B23 localization (Fig. 8A). A proportion of EBNA1 is normally found throughout the nucleolus, and nucleolar localization requires either of the two Arg-rich regions (41 to 50 or 325 to 376) and is rarely seen with the Δ41-376 EBNA1 mutant (67). The findings that the same two Arg-rich regions are methylated by PRMT1 and -5 and that inhibition of methylation affects EBNA1 localization within the nucleolus suggest that methylation of EBNA1 affects its nucleolar localization. Arginine methylation in EBNA1 may affect interactions with nucleolar proteins or RNA (either positively or negatively). Arginine methylation has long been associated with RNA binding and EBNA1 is known to bind RNA (58), although the functional significance of this is not known.

In summary, we have shown that the 325-376 region is subject to serine phosphorylation and symmetric and asymmetric arginine methylation. These modifications could be independently regulated or related sequential events, as observed for histone tail modifications. For example, the methylation of Arg3 in histone H3 by PRMT1 stimulates lysine acetylation by CBP-p300, which in turn stimulates methylation of additional arginine residues (24, 48). In addition, there is a documented connection between serine phosphorylation and lysine acetylation in H3 tails. It will be interesting to determine the relationship between the observed EBNA1 modifications and how they are regulated through the cell cycle to modulate EBNA1 localization and function.

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