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Antibodies against the mono-methylated arginine-glycine repeat (MMA-RG) of the Epstein–Barr virus nuclear antigen 2 (EBNA2) identify potential cellular proteins targeted in viral transformation

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Abstract

The Epstein–Barr virus is a human herpes virus with oncogenic potential. The virus-encoded nuclear antigen 2 (EBNA2) is a key mediator of viral tumorigenesis. EBNA2 features an arginine-glycine (RG) repeat at amino acids (aa)339–354 that is essential for the transformation of lymphocytes and contains symmetrically (SDMA) and asymmetrically (ADMA) di-methylated arginine residues. The SDMA-modified EBNA2 binds the survival motor neuron protein (SMN), thus mimicking SMD3, a cellular SDMA-containing protein that interacts with SMN. Accordingly, a monoclonal antibody (mAb) specific for the SDMA-modified RG repeat of EBNA2 also binds to SMD3. With the novel mAb 19D4 we now show that EBNA2 contains mono-methylated arginine (MMA) residues within the RG repeat. Using 19D4, we immune-precipitated and analysed by mass spectrometry cellular proteins in EBV-transformed B-cells that feature MMA motifs that are similar to the one in EBNA2. Among the cellular proteins identified, we confirmed by immunoprecipitation and/or Western blot analyses Aly/REF, Coilin, DDX5, FXR1, HNRNPK, LSM4, MRE11, NRIP, nucleolin, PRPF8, RBM26, SMD1 (SNRDP1) and THRAP3 proteins that are either known to contain MMA residues or feature RG repeat sequences that probably serve as methylation substrates. The identified proteins are involved in splicing, tumorigenesis, transcriptional activation, DNA stability and RNA processing or export. Furthermore, we found that several proteins involved in energy metabolism are associated with MMA-modified proteins. Interestingly, the viral EBNA1 protein that features methylated RG repeat motifs also reacted with the antibodies. Our results indicate that the region between aa 34–52 of EBNA1 contains ADMA or SDMA residues, while the region between aa 328–377 mainly contains MMA residues.

INTRODUCTION

The Epstein–Barr virus (EBV) is an oncogenic human herpes virus that usually infects young children without causing symptoms [1] and establishes a latent infection of resting memory B-cells [2]. Infection at adolescence or later often results in the development of infectious mononucleosis [3]. More than 95 % of the adult population worldwide shows serological markers of infection with EBV. About 1 % of all tumours worldwide are caused by EBV [4]. EBV is found in virtually all cases of undifferentiated nasopharyngeal carcinoma (NPC), as well as those for nasal NK/T-cell lymphoma (NKTL). The virus is also present in up to 95 % of endemic Burkitt's lymphoma (BL) cases, 60 % of Hodgkin's lymphoma (HL) cases and about 10 % of gastric carcinoma (GC) cases [4]. EBV efficiently transforms B-cells to generate so-called lymphoblastoid cell lines, the *in vitro* complement to post-transplant lymphoproliferative disease (PTLD) arising under immunosuppression [5]. In tumours, different sets of viral proteins are expressed depending on the type of latency [6].

The *in vitro* transformation of B-cells is critically dependent on the presence of the nuclear antigen 2 (EBNA2) [7].

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Abbreviations: ADMA, asymmetrically di-methylated arginine; AdOx, per-Iodate oxidized adenosine; EBNA1, Epstein–Barr Virus (EBV)–encoded nuclear antigen 1; EBNA2, Epstein–Barr Virus (EBV)–encoded nuclear antigen 2; MMA, mono-methylated arginine; NMA, non-methylated arginine; SDMA, symmetrically di-methylated arginine; TPA, 12-O-tetra-dodecanoylphorbol-13-acetate.

One supplementary table and six supplementary figures are available with the online Supplementary Material.

EBNA2 activates gene expression, mainly through interaction with the transcriptional repressor RBPjk/CBF1 and the PU.1 protein. For a recent review on EBNA2 function, see [8]. EBNA2 binds to CBF1 via a conserved TrpTrpPro motif ('WWP') at amino acid (aa) position 323–325 of its polypeptide chain. In addition to phosphorylation [9], EBNA2 contains both symmetrically and asymmetrically di-methylated arginine residues (SDMA and ADMA, respectively) within its arginine-glycine (RG) repeat at aa 339–354 of the polypeptide chain [10–13]. Following the definition by Richard and colleagues [14], EBNA2 features a tri-RG repeat. The aa sequence of EBNA2 is shown in Fig. S1a (available with the online Supplementary Material). SDMA-EBNA2 binds to the 'Tudor' domain of the survival motor neuron protein (SMN), which is mutated or deleted in spinal muscular atrophy (SMA) [15]. On the one hand, antibodies against SDMA-EBNA2 recognize the cellular SMD3, which in turn associates with SMN via its SDMA-modified RG repeat [16]. Apparently, EBNA2 mimics SMD3 binding to SMN. On the other hand, antibodies against ADMA-modified EBNA2 bind the heterogeneous nuclear ribonuclear protein K (HNRNPK), another interacting partner of EBNA2 [12].

The EBV-encoded EBNA1 protein is also essential for efficient transformation and for autonomous maintenance of the EBV episome in the infected cell. For a recent review on EBNA1, see [17]. The primary sequence of EBNA1 is shown in Fig. S1b. EBNA1 features RG as well as RGG repeat elements that are probably involved in RNA binding [18]. It was shown that EBNA1, in addition to being phosphorylated, is methylated at its arginine residues [19]. The RG repeats are located in two regions, denoted linking regions 1 and 2 (LR1, aa 34–52, and LR2, aa 328–377, respectively), which are critical for replication of the viral episome by targeting EBNA1 to the origin recognition complex (ORC) [20].

Post-translational methylation of arginine residues in proteins plays a pivotal role in RNA binding and metabolism, protein–protein interactions, transcriptional regulation, signal transduction and DNA repair (for reviews, see [14, 21–23]). The methylation of arginine residues in various histone proteins also regulates gene expression, with ADMA modification activating transcription and SDMA modification being inhibiting [24]. Further, demethylation of ADMA-modified histones to citrulline residues regulates methylation in an antagonistic manner [25, 26]. Previous studies also identified the MMA-containing proteins involved in chromatin remodelling [27]. Of the presently known nine human protein arginine methyl transferases (PRMTs), PRMT-1−3, CARM1 (PRMT4) and PRMT-6–8 are class I PRMTs, which catalyze the formation of either mono-methylated (MMA; ω-N^G-methyl arginine) or asymmetrically di-methylated arginines (ADMA; ω-N^G,N^G-dimethyl arginine); the class II PRMTs consist of PRMT-5 and PRMT-9 and generate either MMA or symmetrically di-methylated arginine (SDMA; ω-N^G,N'^G-di-methyl arginine) [28, 29]. PRMT-7 is classified as a type III enzyme that appears to preferentially generate MMA residues [30]. PRMT-5 has oncogenic properties [31, 32]. The methylated arginines are mostly found in the context of conserved repeat structures featuring either RGG and/or RG repeat elements that are clustered as Di-RGG, tri-RGG, Di-RG or tri-RG repeats, which may be interrupted by 0–4 amino acids (aa) [14]. Following this definition, EBNA2 has one tri-RG motif between aa 339–354 (Fig. S1a), while EBNA1 contains two tri-RG repeats (aa 34–52 and aa 372–377) and one Di-RG motif at aa 354–357. EBNA1 further contains, at aa 329–357, one repeat element consisting of three RGRGGs with the sequence SGG and SGGR in-between them (Fig. S1b).

The first aim of this analysis was to determine with specific monoclonal antibodies whether EBNA2, in addition to SDMA- or ADMA-modified arginines, also contains MMA-modified residues within its RG repeat. Using monoclonal antibodies against SDMA and ADMA-EBNA2, we previously demonstrated that SMD3, HNRNPK and the C9orf72 protein involved in familial frontotemporal lobar degeneration (FTLD) and amyotrophic lateral sclerosis (ALS) have methylated surface epitopes that are probably similar to those featured by the methylated RG repeat of EBNA2 [10, 12, 13, 33, 34]. The hypothesis of this work was that antibodies detecting the presence of an MMA motif in EBNA2 might help to identify cellular proteins which feature comparably methylated protein surface epitopes. We hypothesize that the methylated surface of EBNA2 mimics such epitopes to interfere with or engage in the processes carried out by these cell proteins. The identification of such proteins might help to elucidate unknown pathways employed by EBV during the transformation of B-cells, and might also help to identify the proteins involved in diseases other than FTLD or ALS.

RESULTS

Generation of monoclonal antibodies against mono-methylated (MMA)-EBNA2

The proposition of this work was that an antibody against the MMA-modified RG repeat of EBNA2 canrecognize cell proteins with a comparable surface epitope. The first aim was to generate a monoclonal antibody by immunizing Lou/C rats with an Ova-coupled EBNA2-RG-peptide containing MMA residues. The clones obtained were screened against the same peptide sequence featuring either unmodified arginines or MMA, SDMA or ADMA residues [12]. A clone designated 19D4 binding to the MMA-modified peptide was characterized by enzyme-linked immunosorbent assay (ELISA) (Fig. 1a). We found that the antibody only reacted with the non-methylated peptide at the highest concentration (2.7 ng well^{-1}), while it still gave a signal above background at a concentration of 0.08 ng well^{-1}. We further used 19D4 in a Western blot analysis using Raji cells derived from a Burkitt's lymphoma, which contains a type I EBV expressing EBNA2A [35] in comparison to non-infected

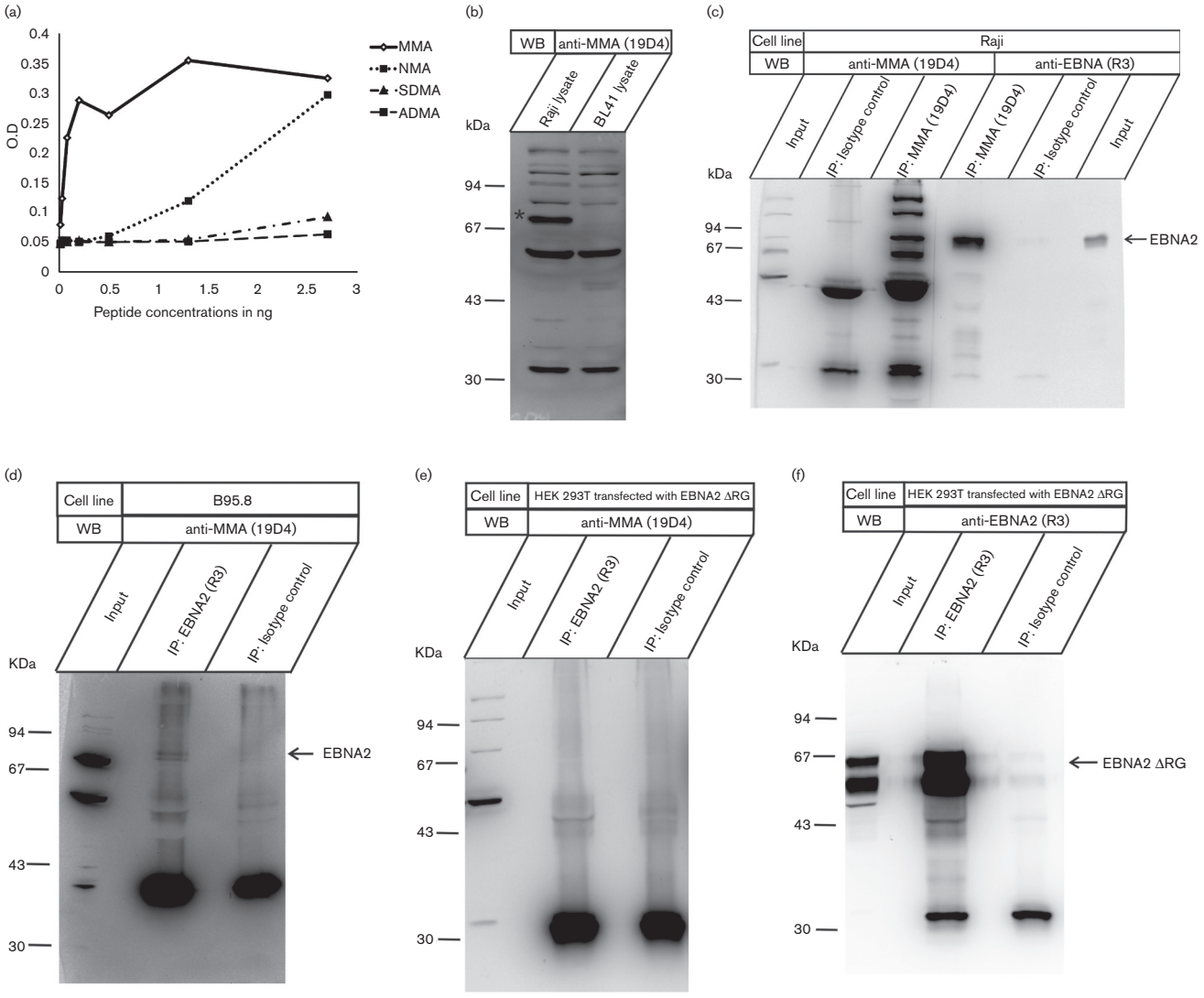

Fig. 1. Characterization of rat monoclonal antibody (mAb) 19D4 directed against MMA-EBNA2. (a) The binding of 19D4 to peptides with the non-methylated (NMA-), mono-methylated (MMA), symmetrically (SDMA) and asymmetrically (ADMA) di-methylated EBNA2-derived RG repeat was tested by ELISA. Antibody binding to the peptides at different concentrations was determined as described in the Methods section. (b) Extract of the BL cell lines Raji (EBV-positive) and BL41 (EBV-negative) were analysed with the mAb 19D4. Bound antibodies were visualized using ECL. (c) Raji cell extract immunoprecipitated with 19D4 and an appropriate isotype control was analysed by Western blot using either 19D4 or the methylation-unspecific antibody R3 directed against EBNA2. The position of EBNA2 is indicated by an arrow. (d) Extract of the EBV-positive lymphoblastoid cell line B95.8 was precipitated using R3 and an appropriate isotype control and then analysed using 19D4. (e, f) HEK293T-cells were transfected with a plasmid expressing EBNA2 with a deletion of the RG repeat (EBNA2ΔRG) and then analysed sequentially with 19D4 (e) followed by R3 (f). The molecular mass marker proteins separated on the same gel were, in descending order: phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carboanhydrase, 30 kDa.

BL41 Burkitt's lymphoma cells [36]. As shown in Fig. 1(b), the antibodies recognized various proteins in both cell lines, indicating that it reacted with cell proteins sharing epitopes similar to the MMA-modified RG repeat of EBNA2; in addition to those observed in BL41, the antibodies strongly reacted with a protein of approximately 75–80 kDa in the Raji cell line. However, as outlined below, mass spectrometry-based analysis of this band revealed predominantly EBV-encoded EBNA1 protein (marked with a star). Raji cell extract was then immune-precipitated using 19D4 and probed in a Western blot with the EBNA2A-specific antibody R3 [37]. As shown in Fig. 1(c), the antibody 19D4 recognized EBNA2. Fig. 1(c) also indicates that the 19D4 precipitate, when stained with 19D4, not only showed the band of EBNA2, but also bands of cell proteins that were either co-precipitated with EBNA1/EBNA2 or contained

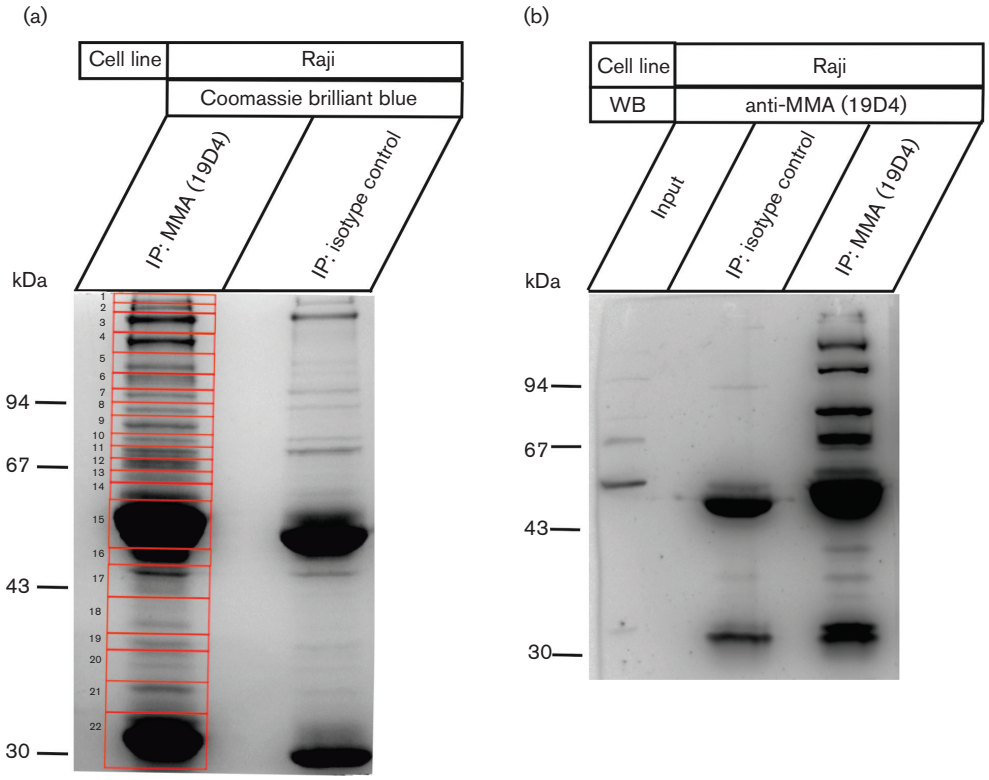

Fig. 2. Immunoprecipitation of MMA-modified proteins from Raji cells. (a) Raji cell extract was subjected to immunoprecipitaton using 19D4 and an appropriate isotype control. The proteins obtained were separated by SDS-PAGE. Twenty-two Coomassie-stained bands were excised from the gel as indicated, and analysed by LC-MS/MS and quantitatively compared with the contralateral band from the isotype control. (b) An aliquot of the precipitate shown in (a) was analysed by Western blot with 19D4 to show the absence of 19D4-reactive proteins in the isotype control precipitate. Molecular mass marker proteins were as shown in Fig. 1.

MMA residues themselves. In the reverse experiment, R3 was used to precipitate EBNA2 from EBV-transformed B95.8 cells and then probed with 19D4 (Fig. 1d). Again, we obtained a signal with 19D4. Further, 19D4 also reacted with the EBNA2B protein precipitated from the type II EBV-infected Jijoye cell line [37] (not shown). These results indicated that EBNA2 from unrelated EBV strains can be detected by 19D4. Further, we transiently expressed an EBNA2 mutant with a deletion of the RG repeat (EBNA2ΔRG) in HEK 293 T-cells as described earlier [10]. The proteins were precipitated with R3 and the blot was sequentially probed with 19D4 followed by R3. As shown in Fig. 1(e, f), 19D4 did not yield a signal, while R3 recognized dRG-EBNA2. These experiments showed that 19D4 reacted specifically with the RG repeat of EBNA2, indicating that it contains MMA residues. Finally, we treated cells with the methylation inhibitor AdOx, which is known to impede the generation of SDMA and ADMA while increasing the amount of MMA [38]. The treatment of Raji cells with AdOx resulted in a decrease in the signal of ADMA-modified EBNA2 (when stained with the ADMA-EBNA2-specific antibody 6F12 [12]), but not the signal of the EBNA2 protein itself (not shown), while the bands reacting with MMA clearly increased further, indicating that 19D4 indeed detects MMA-modified EBNA2 and also MMA-containing cellular proteins. This is shown in Fig. S2.

Precipitation of MMA-containing proteins from the EBV-positive Burkitt's lymphoma Raji cell line

An extract corresponding to approximately 10^8 Raji cells was immune-precipitated using the 19D4 antibody and an appropriate isotype control antibody. The proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue to visualize the precipitated bands. Fig. 2(a) shows that the 19D4 precipitate contained bands of various sizes that were mostly not detectable in the control precipitate. The precipitate was assayed by Western blot analysis to ascertain that the negative control was devoid of precipitated 19D4-reactive proteins (Fig. 2b). Twenty-two bands from the 19D4 precipitate and the corresponding lanes from the control (as indicated in Fig. 2a) were analysed by mass spectrometry as outlined in the Methods section. Table S1 lists the proteins that were significantly enriched in the 19D4 precipitate and also summarizes which of these proteins are known to be arginine-methylated, which contain MMA residues and which feature RG repeats, but with an unknown methylation status. We note that bands 11 and

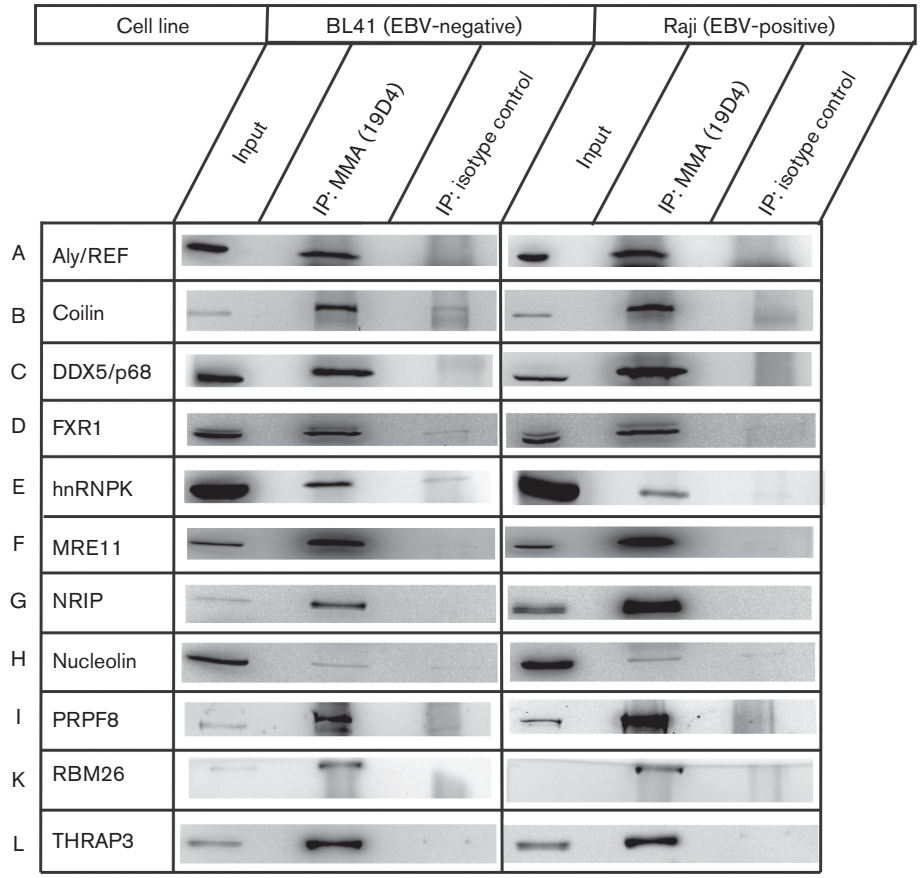

Fig. 3. Confirmation of proteins precipitated with the MMA-specific mAb 19D4. Extracts from the BL cell lines Raji (EBV-positive) and BL41 (EBV-negative) were immunoprecipitated with the mAb 19D4 and an appropriate isotype control antibody. The individual proteins as indicated were then stained with specific antibodies described in the Methods section. The complete Western blots are shown in Fig. S4(a–l).

12 contained peptides derived from the EBNA1 protein, while EBNA2 was not detectable by mass spectrometry in any of the analysed bands. The failure to detect EBNA2 had already been observed in our previous analysis with monoclonal antibodies against SDMA- or ADMA-EBNA2, where the antibodies clearly precipitated EBNA2, as shown by Western blot, while the EBNA2 protein was not detectable by mass spectrometry [10, 12]. We assayed to establish whether induction of the EBV lytic replication cycle of B95.8 or Jijoye cells by treatment with TPA resulted in a change of methylation, but could not detect differences in the signals of the treated versus the control cells (data not shown).

Precipitation of MMA-containing proteins from the EBV-negative Burkitt's lymphoma BL41 cell line

Cell extract corresponding to approximately 10^8 BL41 cells was immune-precipitated using 19D4 and an appropriate isotype control. The Coomassie-stained gel of the precipitation is shown in Supporting Fig. Fig. S3(a). The precipitate was assayed by Western blot analysis to ascertain that the negative control was devoid of precipitated 19D4-reacting proteins as depicted in Fig. S3b. From the Coomassie-stained gel, 19 protein bands were excised and analysed with the contralateral gel slices from the control precipitate. The proteins significantly enriched by 19D4 are shown in Table S1. We note that some proteins, such as MRE11, Rad50, BRD4 and Aly/REF, were detected in both Raji and BL41, while others, such as PRR12 and FXR2, were only found in one precipitate.

Confirmation of cellular proteins precipitated with 19D4 by Western blot analysis

The precipitation by 19D4 by some of the proteins shown in Table S1 was verified using commercially available antibodies. These results are summarized in Fig. 3. The complete blots are shown in Fig. S4(a–l)L. We observed that some proteins (i.e. Coilin or DDX5) migrated with a slightly reduced mobility in the lanes of the immune precipitation. We assume that the large amount of IgG heavy and light chains or, alternatively, a different salt concentration in the precipitate, was the reason for this observation. The proteins analysed were: THO complex subunit 4 (Aly/REF; Fig. S4a),

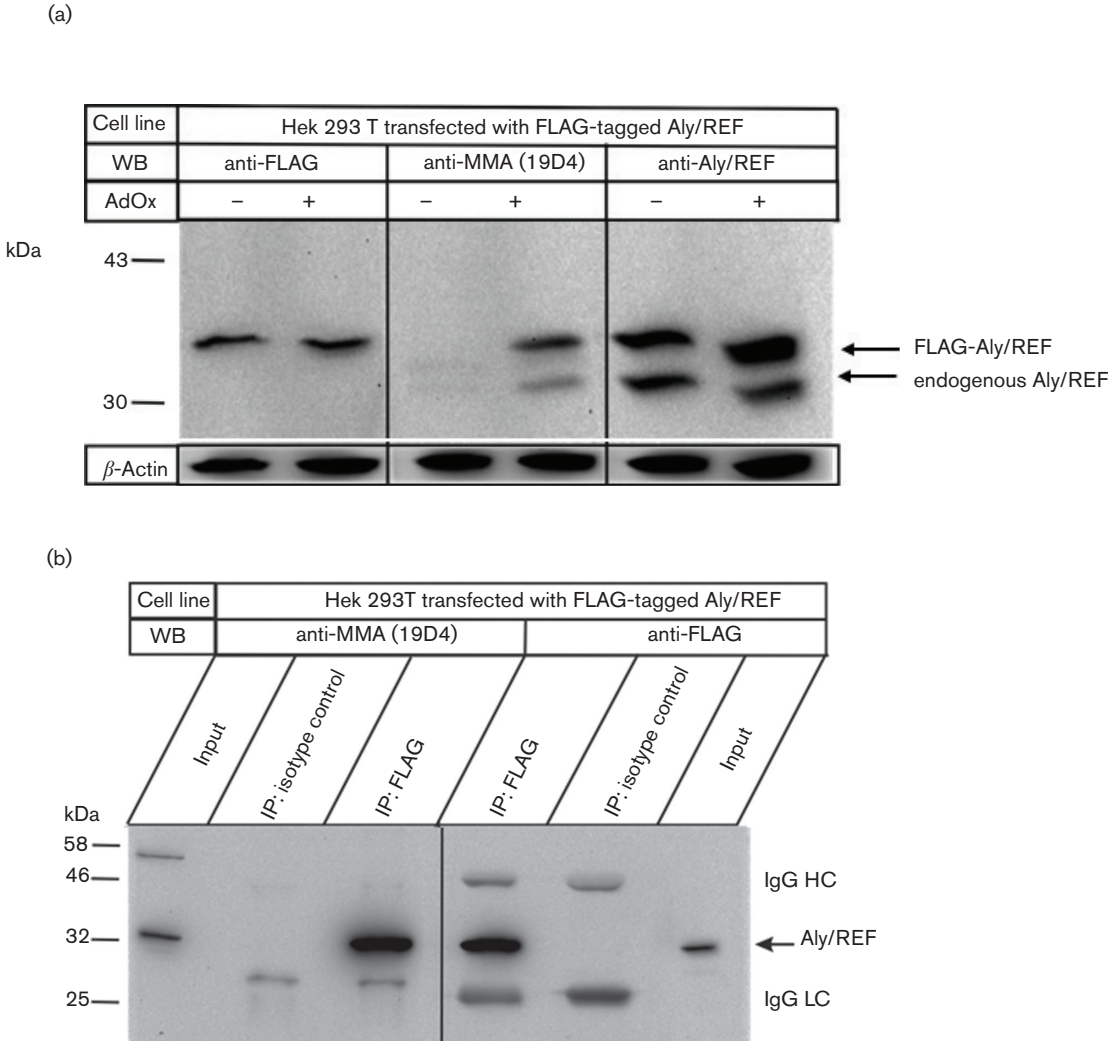

Fig. 4. Analysis of arginine methylation of Aly/REF. (a) FLAG-tagged Aly/REF protein was transiently expressed in HEK293T, which was subsequently untreated or AdOx-treated. The cell extracts were assayed using anti-FLAG, 19D4 or commercial anti-Aly/REF antibody. The positions of the FLAG-Aly/REF and the endogenous Aly/REF are indicated. (b) FLAG-tagged Aly/REF protein transiently expressed in HEK293T was immunoprecipitated using anti-FLAG and an appropriate isotype control antibody. The precipitated FLAG-Aly/REF was visualized using anti-FLAG and 19D4. The positions of the FLAG-Aly/REF and IgG heavy (IgG HC) and light chains (IgG LC) are indicated. the molecular mass marker proteins were from New England Biolabs (11–190 kDa, # P7706S).

Coilin (COIL; Fig. S4b), RNA helicase DDX5/p68 (DDX5; Fig. S4c), a cluster of fragile X mental retardation syndrome-related protein 1 (FXR1; Fig. S4d), heterogeneous ribonucleoprotein K (HNRNPK; Fig. S4e), double-strand break repair protein 11 (MRE11; Fig. S4f), nuclear receptor-interacting protein 1 (NRIP/DCAF6; Fig. S4g), nucleolin (NCL, Fig. S4h), a cluster of pre-mRNA-processing-splicing factor 8 (PRPF8; Fig. S4i), a cluster of RNA-binding protein 26 (RBM26; Fig. S4k) and thyroid hormone receptor-associated protein 3 (THRAP3; Fig. S4l). Aly/REF, Coilin, DDX5, FXR1, HNRNPK, MRE11, nucleolin and THRAP3 contain RG-repeat elements, while PRPF8 features five isolated RGs distributed throughout the polypeptide chain and, for instance, RBM26 contains only one RG. We found that 19D4 precipitated these proteins from b oth EBV-positive Raji extract and EBV-negative BL41 extract. Nucleolin, a known binding partner of EBNA1 [39], and DDX5, Coilin, HNRNPK and LSM4 (see below), were previously shown to contain MMA residues [27]. MRE11 had been immune-precipitated using the ASYM24 antibodies [23] against ADMA proteins, along with their interaction partners Rad50 and Nibrin, and both were also detected in the present study (Table S1). MRE11, however, was not found by Sylvestersen and co-workers, who identified MMA-containing proteins [27]. To test whether MRE11 contains MMA residues, the protein was precipitated from HEK 293 T-cell extract using a commercially available polyclonal rabbit antibody (A303-998A-T; Bethyl Laboratories) and then

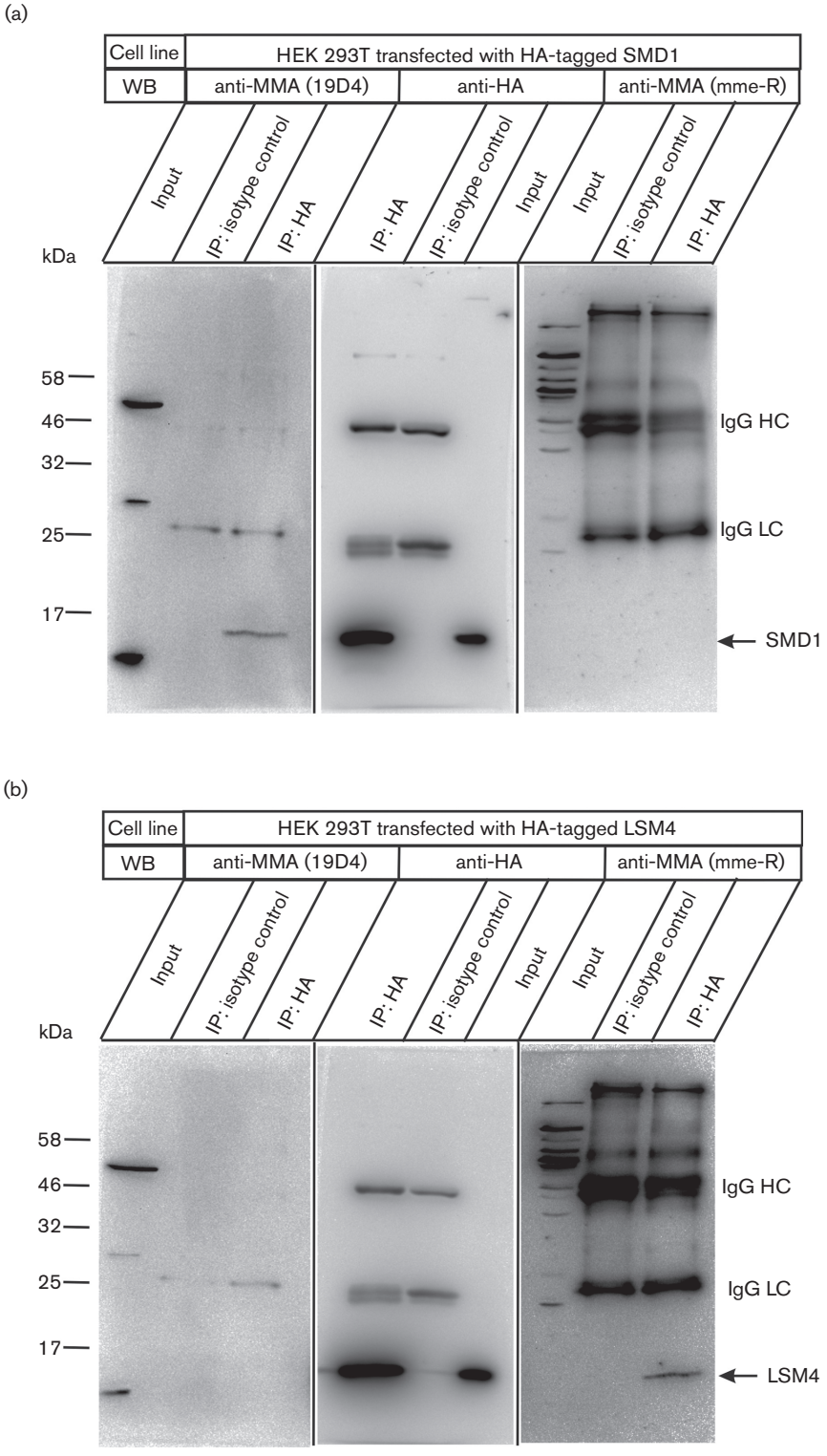

Fig. 5. Analysis of arginine methylation of SMD1 and LSM4. (a) HA-tagged SMD1 transiently expressed in HEK293T was immunoprecipitated using anti-HA and an appropriate isotype control antibody. The precipitate was analysed in a Western blot with 19D4, 6F10 anti-HA and mme-R anti-MMA, as indicated. The positions of HA-SMD1 and the IgG heavy (IgG-h) and light chains (IgG-l) are indicated. (b). HA-tagged LSM4 transiently expressed in HEK293T was immunoprecipitated using anti-HA and an appropriate isotype control antibody. The precipitate was analysed in a Western blot with 19D4, 6F10 anti-HA and mme-R anti-MMA, as indicated. The positions of HA-LSM4 and the IgG heavy (IgG HC) and light chains (IgG LC) are indicated. The molecular mass marker proteins were as indicated in Fig. 4.

detected in a Western blot with our MMA antibody 19D4, confirming the presence of MMA residues. This is shown in Fig. S5. We note that some of the proteins, although only identified in either the Raji or the BL41 analysis, were nevertheless precipitated from both cell lines. Of all the proteins identified by mass spectrometry, only PSAT1 was not be confirmed by Western blot with specific antibodies after precipitation with 19D4 (data not shown). We assume that the level of precipitated PSAT1 was too low for detection by the antibody used.

It was previously demonstrated that the THO complex subunit 4 protein (THOC4),referred to below as Aly/REF, is mono-methylated or asymmetrically di-methylated at arginine residues [27, 38]. To confirm that 19D4 recognized the MMA-containing Aly/REF, FLAG-tagged Aly/REF expressing HEK 293 T-cells were treated with AdOx. As shown in Fig. 4(a), the FLAG antibody clearly recognized the band corresponding to FLAG-tagged Aly/REF with the same intensity in the treated or untreated cell extracts; the employed Aly/REF-specific antibody stained both the endogenous protein migrating at about 30 kDa and the ectopically expressed Aly/REF that migrated with a slightly reduced mobility due to the presence of the tag sequence, while the 19D4 antibody only detected both proteins in the treated cell extracts, indicating that a large part of the Aly/REF is di-methylated at the arginines. We further precipitated the FLAG-tagged protein from untreated cell extracts and analysed the precipitate with 19D4 and in parallel with anti-FLAG (Fig. 4b). We obtained a clear signal with both antibodies, confirming that some part of Aly/REF is mono-methylated. In summary, this also showed that the antibodies indeed specifically react with MMA-containing RG repeat elements.

The SMD1 protein contains MMA residues

A Western blot of a Raji cell extract separated on a 15 % SDS-polyacrylamide gel revealed the presence of a protein (or proteins) at about 11 kDa that was (were) reactive with 19D4 (see, e.g. the protein labelled with * in the left-most lane of Fig. 5(a)). We therefore subjected an extract from approximately 10^8 Raji cells to immune precipitation with 19D4 and analysed the bands obtained in the 11 kDa range. The proteins identified by mass spectrometry included SMD1, SMD2, SMD3, LSM3 and LSM4 (Table S1). The SMD1-3 (SNRDP1-3) proteins are part of the methylosome complex [40–42], SMD1 and SMD3 feature RG repeats, and SMD2 has only one RG. SMD3 is known to bind to the SMN protein via its SDMA-modified RG repeat [16] and is recognized by a monoclonal antibody raised against the SDMA-modified RG repeat of EBNA2 [10]. Putatively SDMA-modified SMD1 was identified after immunoprecipitation using the SYM10 and Sym11 antibodies specific for SDMA [23]. To determine which of these proteins contain the MMA modification, we transiently expressed the HA-tagged SMD1, SMD2 and SMD3 proteins in HEK 293 T-cells, immune-precipitated them with HA-specific antibody, and probed with 19D4. As shown in Fig. 5(a), 19D4 reacted strongly with SMD1, while SMD2 and SMD3 yielded only faint signals (Fig. S6a, possibly from associated SMD1. All three proteins yielded clear signals with the HA antibody, as shown in Fig. S6b. The HA-tagged SMD1 yielded a clear signal with 19D4, but the major signal in this molecular mass range was seen at approximately 11 kDa (marked with *). To analyse this further, we precipitated the Raji cell extract again using 19D4 and analysed Coomassie-stained bands migrating with a molecular mass of about 11–17 kDa (not shown) by mass spectrometry and again found SMD1. Apparently, the addition of the HA tag resulted in a shift of the HA-SMD1 signal to a molecular mass of about 17 kDa. Surprisingly, the precipitated HA-SMD1 did not yield a signal with the mme-R antibody, but did with 19D4.

In addition to SMD1-3, we identified the Sm-like proteins LSM3 and LSM4 by mass spectrometry (see above). It is known that Sm proteins form a complex with SMN and Sm-like proteins, such as LSM3 and LSM4 [43]. LSM3 only contains one RG, while LSM4 has one tri-RG repeat between aa 109 and 118 of its polypeptide chain of 139 aa. We expressed HA-tagged LSM4 from the pSG5-HA vector and detected a band of approximately 15 kDa by Western blot using an HA-specific antibody (Fig. 5b). The transiently expressed LSM4 was HA-precipitated and analysed in a Western blot using either the HA antibody, the antibody 19D4 or the MMA-specific antibody mme-R. As seen in Fig. 5(b), the HA and mme-R antibodies clearly yielded a signal that migrated with the same mobility, indicating that LSM4 indeed contains MMA residues. However, the failure to detect the precipitated LSM4 with 19D4 indicated that it was probably co-precipitated with other 19D4-reactive proteins (presumably SMD1), and again indicating that the 19D4 antibody reacts specifically with MMA-containing surface epitopes resembling EBNA2, but not with every protein (e.g. LSM4) that contains MMA in the context of a tri-RG repeat.

Analysis of MMA residues of EBNA1

The arginine methylation of EBNA1 had previously been analysed by Frappier and co-workers, who found that both of the regions between aa 40–51 and 325–377 can be methylated by PRMT1 and/or PRMT5 [19]. To verify the detection of EBNA1 by the 19D4 antibody, Raji cell extract was subjected to precipitation by the EBNA1-specific antibody 1H4 [44]. The precipitate was then assayed in a Western blot with either 1H4 or 19D4. As can be seen in Fig. 6(a), the antibody 19D4 clearly detected the EBNA1 protein purified by precipitation. In addition, Raji cell extract precipitated with 1H4 was analysed with the commercially available antibody mme-R against MMA-modified proteins [27], and also gave a clear signal (Fig. 6b). We then tested baculovirus-derived EBNA1 expressed in SF158 insect cells [45] with 1H4, 19D4 and commercially available antibodies against MMA [27] or ADMA residues. The antibody adme-R had been used in an analysis of ADMA-modified proteins [46]. As shown in Fig. 6(c), 1H4, 19D4 and mme-R gave a

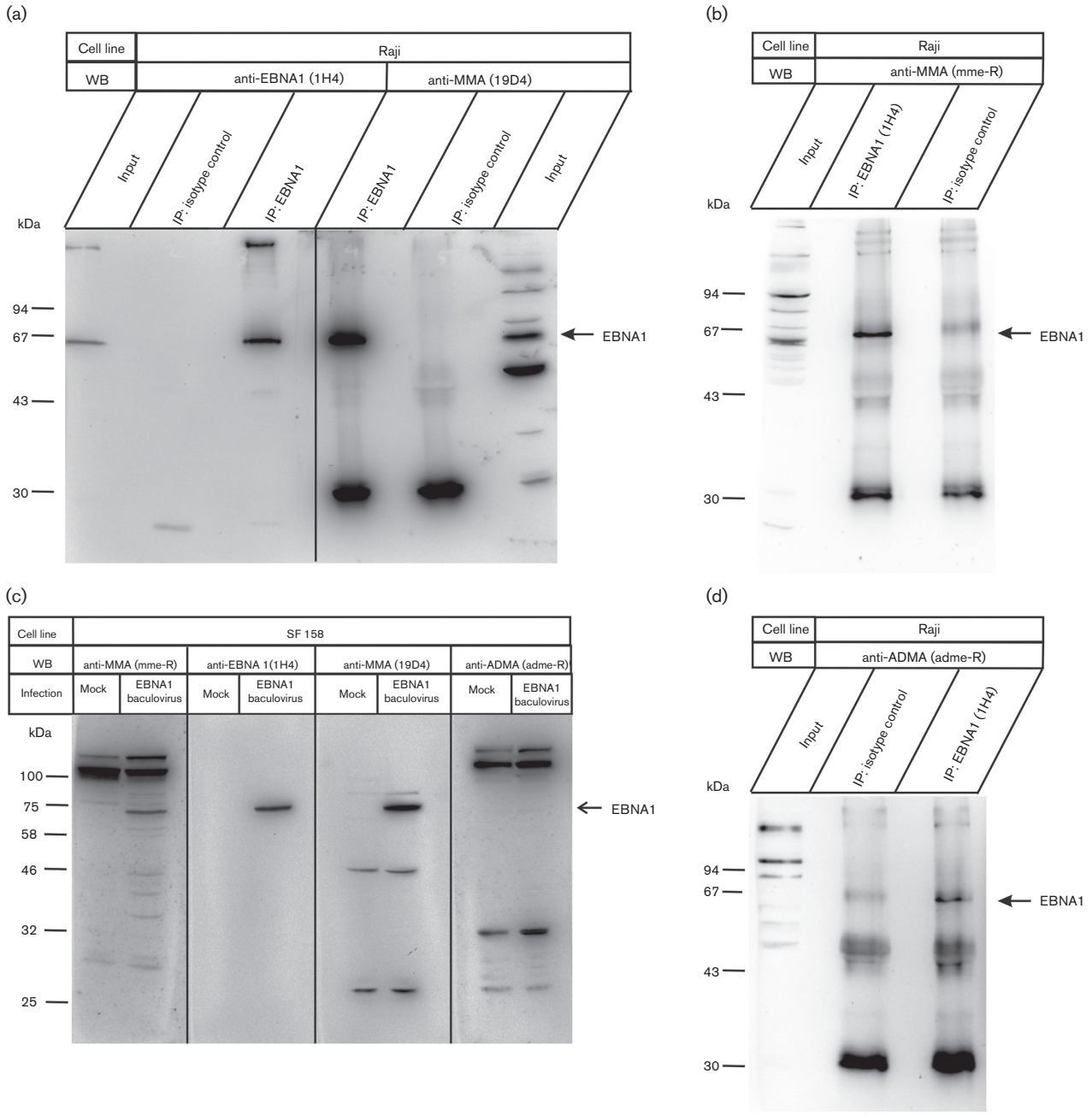

Fig. 6. Reactivity of EBNA1 with the MMA-specific mAb 19D4. (a) Extract from Raji cells was immunoprecipitated using the EBNA1-specific mAb 1H4 and an appropriate isotype control. The precipitate was analysed by Western blot with 1H4 and 19D4. (b) Immunoprecipitated EBNA1 was stained with the commercial antibody mme-R against MMA-containing proteins. (c) Analysis of baculovirus-expressed EBNA1 with the antibody mme-R, EBNA1-specific 1H4, the MMA-specific antibody 19D4, and the commercial ADMA-specific antibody #13522, adme-R. (d) EBNA1 precipitated with 1H4 was stained with the commercial antibody adme-R against ADMA-containing proteins. The position of EBNA1 is indicated by an arrow in (a–d). The molecular mass marker proteins were as indicated in Figs 1 and 4.

clear signal with EBNA1, while no signal for ADMA-modified EBNA1 was obtained. Because the methylation of EBNA1 with PRMT1 and PRMT5 should yield an EBNA1 protein with ADMA or SDMA modification, Raji cell extract was precipitated with 1H4 and then stained with the adme-R antibody. We obtained a faint but clear signal, indicating that at least a sub-fraction of EBNA1 contains ADMA residues. This is shown in Fig. 6(d).

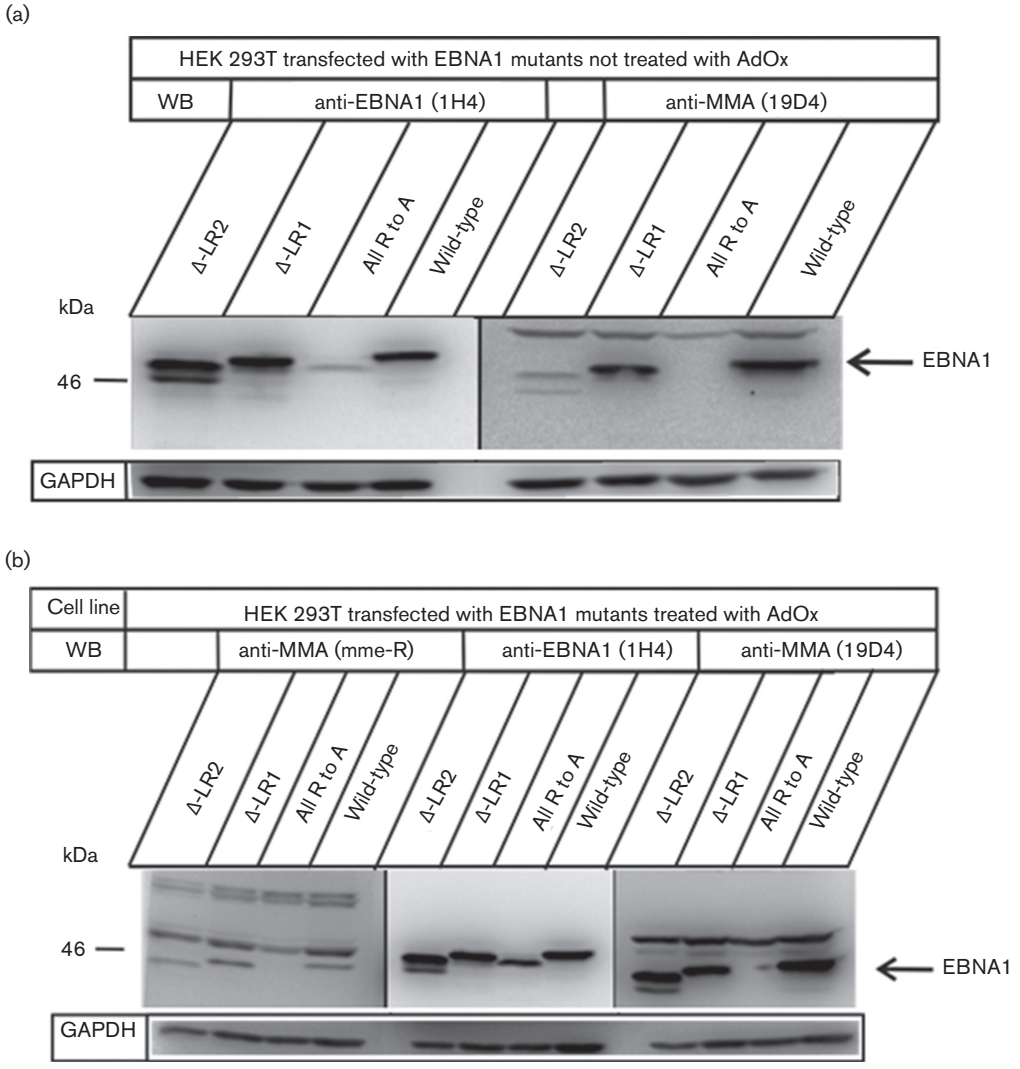

Fig. 7. Analysis of EBNA1 mutants. (a) HEK293T-cells transfected with WT-EBNA1 and the indicated EBNA1 mutants were analysed in a Western blot with 19D4 and 1H4. (b) HEK293T-cells transfected with WT-EBNA1 and the indicated EBNA1 mutants were treated with AdOx and then analysed with mme-R, 1H4 and 19D4. The position of EBNA1 is indicated. The blot was then restained with GAPDH-specific antibody as a loading control. The molecular mass marker proteins were as indicated in Fig. 4.

The arginine methylation of EBNA1 was further analysed using either EBNA1 mutants deleting the RG repeat between aa 34–51, previously termed LR1, the region between aa 328–377 designated LR2 [20], and an EBNA1 mutant where all the arginine residues were exchanged to alanine; these mutants were based on an EBNA1 with a deletion of the glycine alanine-repeat that migrates with a molecular mass of about 46 kDa. These were expressed along with the non-deleted EBNA1-WT in HEK 293 T-cells and analysed with 19D4, mme-R and1H4. The cell extracts yielded a clear signal with 1H4 (Fig. 7a), while 19D4 only reacted with the wild-type (WT) and the delta LR1and LR2 mutants, but not with the R to A mutant. Likewise, the mme-R yielded a signal with WT-, LR1- and LR2-EBNA1, but not with the R to A mutant. In a parallel experiment, we treated the transfected cells with AdoX. Under these conditions, the signal obtained with the ΔLR2 mutant increased appreciably, indicating that linking region 1, which contains a stretch of RGs, contains more ADMA and/or SDMA than LR2 (Fig. 7b). The signal obtained with the EBNA1-1H4 antibody showed that the amount of EBNA1 in the lanes with the ΔLR1 and ΔLR2 mutants did not change due to the AdOx treatment. The blots were additionally stained with the GAPDH antibody to demonstrate equal loading.

DISCUSSION

The intention of the experiments presented in this paper was to identify proteins that share antigenically similar mono-methylated surface epitopes such as EBNA2 by

generating monoclonal antibodies recognizing these structurally similar epitopes

In a previous analysis, we had found that the EBV-encoded EBNA2 protein interacts via its SDMA-modified RG repeat with the survival motor neuron protein (SMN), and with its ADMA-containing RG repeat with the HNRNPK protein [12, 13]. Accordingly, a monoclonal antibody against SDMA-EBNA2 recognized SMD3 [12]. Because we had also shown that the unmethylated form of EBNA2 is hardly present in EBV-transformed cells [10], we wanted to use novel monoclonal antibodies against the mono-methylated RG repeat to determine whether EBNA2 also featured this modification. From our data, we concluded that EBNA2, in addition to carrying SDMA and ADMA modifications, also has MMA residues, albeit at relatively low levels. At present, it is unclear whether these represent a functional subspecies of EBNA2 or are only a transient product during di-methylation of the RG repeat.

Surprisingly, the antibodies also recognized EBNA1, where a previous analysis suggested that it might be either SDMA- or ADMA-modified [19]. The LR1 repeat of EBNA1 features a run of four RGs between aa 41–48 flanked by two RGGs (aa 34–36 and 49–52), which is reminiscent of the RG repeat of EBNA2. We interpret the observations described above such that the LR2 probably contains ADMA- and/or SDMA-modified EBNA1, while LR1 probably mainly (or possibly exclusively) contains MMA residues, because the signal obtained with the EBNA1 mutant that only carries LR1 does not increase after AdOx treatment. The signal of EBNA1 obtained with 19D4 only slightly increases in AdOx-treated Raji cells, while the EBNA2 signal obtained with our ADMA-specific antibody 6F12 is reduced (Fig. S2). This indicates that only a small part of the normal ADMA/SDMA-EBNA1 is arrested at the MMA-stage by AdOx treatment post-translationally, and that a relatively large fraction of EBNA1 contains MMA residues.

The question arises of whether the detection of both EBNA1 and EBNA2 by 19D4 points to a functional overlap between the two proteins. Both proteins are essential for *in vitro* transformation by EBV, while only EBNA1 is detectable in all EBV-induced malignancies. It might be possible that EBNA1 assumes some functions of EBNA2 by its LR1 repeat that are initially carried out by EBNA2 via its RG repeat. For instance, EBNA2 binds to HNRNPK [12], while EBNA1 associates with various other members of the HNRNP family [47]. Here we found HNRNPD. We note that we only obtained a faint signal from EBNA2, but a strong signal from EBNA1, indicating that EBNA1 carries a substantial amount of MMA modification. The antibody 19D4 was generated against the MMA-modified arginines of EBNA2 within the sequence GQSRGQSRGRGRGRGRG RGKGK; however, 19D4 only reacts strongly with the EBNA1 mutant ΔLR2 (which contains the sequence R_{41} GRGRGRGRGG$_{51}$ within LR1) upon AdOx treatment, which indicates that this region mainly contains di-methylated arginines. In contrast, the LR2 region encompassing the sequence R_{370}GRGRGRG$_{377}$ was insensitive to the AdOx treatment, indicating that it probably contained the MMA residues.

We then asked which classes of proteins could be detected by 19D4. The proteins we identified as being involved in DNA double-strand break repair were the three in the MRE11/Rad50/NSB1 (nibrin) complex, and here these were identified in both Raji and BL41 cells [48]. Of these three proteins, MRE11 features five consecutive RGs, and the purified protein was shown here to react directly with the novel antibody, suggesting that it is indeed mono-methylated (Fig. S5).

A number of the proteins detected here play a role in oncogenesis. Activation of the proto-oncogene c-myc is a hallmark Burkitt's lymphoma [49]. EBNA1 forms a complex with BRD4, which is involved in chromosomal segregation of EBV episomes, and also in EBNA1-mediated transcription and activation from the FR repeats in the EBV genome [50]. Further, the BRD4 gene is amplified in cancer [51] and the RG repeat-containing BRD4 protein plays a role in c-myc activation [52]. In line with this, the arginine-methylated, Tudor-domain-containing TDRD3 protein detected here also plays a role in c-myc activation [53], and is a component of the FXR1 complex [54]. The FXR1/FXR2 complex binds the ADMA/MMA methylated caprin-1 protein that was also detected in the present analysis [27]. Caprin-1 has tumour-promoting activity [55] and binds to the c-myc RNA. In both Raji and BL41 cells, 19D4 recognized members of the type II chaperonin TRiC/CCT (T-complex protein-1 ring complex, TCP1/CCT1) [56], which have been linked to carcinogenesis [57]. In BL41, we identified TCP1/CCT1 and CCT6A, while in Raji we found TCP1, CCT2, CCT4, CCT7 and CCT8.

We found a variety of proteins involved in RNA processing, export, transcriptional activation or splicing. Aly/REF (Tho complex 4, THOC4) is known to contain MMA residues [27], while it binds to RNA polymerase II and is involved in RNA splicing and export [58, 59]. It forms a complex with the HSV1 ICP27 protein and is associated with HSV1 promotor sequences [60]. Likewise, Aly/REF was found to be a co-activator of the T-cell receptor alpha [61]. Although our data suggest that Aly/REF is mostly di-methylated, its MMA form might nevertheless carry out an important function (or functions), as mono-methylated Aly/REF in AdOx-treated cells binds RNA more efficiently than the di-methylated protein from untreated cells [38]. PRPF8, which was found in both Raji und BL41, has been reported to carry di-methylated arginines [27] and forms a complex with SNRNP200 [62], which was also detected in this analysis, and it also appears to play a role in leukemogenesis [63]. Also present in this complex and identified in addition to SNRP200, were PRPF3, PRPF4, PRPF6, PRPF8, PRPF31, DDX23, SART1, WDR57 and EFTUD2. Finally, KHRSP, detected here, is functionally associated with SMN [64], which we showed to be functionally associated with EBNA2 [13].

Further, various proteins involved in energy metabolism were found in Raji cells, i.e. fructose bi-phosphate aldolase or malate dehydrogenase. The significance of these interactions needs to be evaluated.

In summary, we identified various MMA-modified proteins binding to the novel antibody 19D4 that are involved in different cellular functions, such as DNA repair, transcriptional activation, splicing and RNA processing. Further studies will be needed to evaluate the significance of these similarities.

METHODS

Cell lines and transfection

The adherent HEK 293 T-cells were grown in Dulbeccos's modified Eagle's medium (DMEM; Sigma, Munich, Germany) and passaged twice per week [65, 66]. The EBV-positive Raji, B95.8 and Jijoye cells, as well as the EBV-negative BL41 cells, were grown in RPMI-1640 medium (Sigma, Munich, Germany) supplemented with 10 % foetal calf serum (FCS; Biochrom, Berlin, Germany) and antibiotics (40 IU/ml penicillin and 50 $\mu g\,ml^{-1}$ streptomycin; Sigma, Munich, Germany; neomycin-sulfate, 1 IU/mL; Roth, Karlsruhe, Germany; nystatin, 90 iU/mL; Fagrom, Barsbüttel, Germany), and sub-cultured routinely once per week [11, 12, 37]. The insect cell line SF158 [67] was kept at 29 °C in TC100 medium supplemented as above and infected with recombinant baculoviruses expressing EBNA1 and EBNA2, as described earlier [45]. Transient transfection was performed in HEK-293T-cells, using either a conventional calcium phosphate technique [68] or the Polyfect transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Treatment of cells with periodate-oxidized adenosine (AdOx) was carried out at 25 µM AdOx for 48–72 h as described [12]. Periodate-oxidized adenosine (adenosine-2′,3′-dialdehyde) was obtained from Sigma (A7154). Reactivation of EBV was carried out by treatment of the latently infected B95.8 and Jijoye cells using tetradecanoylphorbol-acetat TPA (*Phorbol*-12-myristat-13-acetat) at 20 $ng\,ml^{-1}$ for 24 h [9].

Animals and antibodies

Lou/C rats were bred and maintained at the animal facility at the Helmholtz Zentrum München in accordance with German animal welfare legislation and the Government of Upper Bavaria, Germany (Gz 55.2-1-54-2532.6-4-99). The monoclonal antibodies against the peptide NH_2-C-GQSRG QSRGRGRGRGRGKGK featuring monomethylated arginine (MMA) residues were established as described earlier [10]. The peptides were synthesized by Peps4LS GmbH, Heidelberg, Germany. In short, the MMA-modified peptide coupled via the additional N-terminal cysteine residue to ovalbumin (OVA) was used to immunize Lou/C rats. The clones secreting antibodies were screened in an ELISA assay against the unmethylated (NMA) peptide or the peptide containing symmetrically (SDMA) or asymmetrically dimethylated arginine (ADMA) residues coupled to bovine serum albumin (BSA) [10]. A clone designated MMA 19D4 (IgG2c) that was reactive with the MMA-containing BSA peptide was used after subcloning for further analyses. The fine specificity of this clone was determined using the above-mentioned biotin-coupled peptides (Peps4LS GmbH, Heidelberg, Germany) in an ELISA assay [69]. For this assay, ELISA plates were coated with 50 µl avidin (Sigma A9275) at 3 $\mu g\,ml^{-1}$ in PBS overnight. Blocking was performed with 2 % FCS in PBS. The biotin-coupled peptides were applied in a serial dilution in PBS/2%FCS/0.01 % sodium azide (NaN_3) starting at 27 ng $well^{-1}$. After washing, 50 µl of the tissue culture supernatant containing the 19D4 antibody was added in a 1 : 10 dilution in the above buffer. For detection of the bound mAb, a mouse mAb against rat IgG2c (Helmholtz Zentrum, Munich, Germany, unpublished) coupled to horseradish peroxidase was used, employing TMB (1-Step Ultra TMB-ELISA substrate, Thermo, 34029) as a substrate.

The rat monoclonal antibody R3 against EBNA2A encoded by type I EBV that binds irrespective of secondary post-translational modifications was described previously [37]. The rat monoclonal antibody 1H4 detects the EBNA1 protein in both Western blots and immune precipitation [44]. The rat monoclonal antibody 6F7 was produced against the published FLAG sequence (Kremmer, unpublished), while the monoclonal antibody 6F12 against ADMA-modified EBNA2 was described previously [10]. The following antibodies are commercially available and were purchased from the companies noted in parenthesis: rat monoclonal anti-HA (Roche, Mannheim, Germany; #11867423001), monomethyl arginine (mme-R) MultiMab rabbit mAb mix and ADMA-specific antibody (Cell SignallingTechnology, New England Biolabs GmbH, Frankfurt/M., Germany; CST #8015 and #13522, respectively), PRPF8 (GeneTex Irvine, CA, USA; #GTX108046), RBM26 (Bioorbyt, Cambridge, UK; #orb127180), hnRNPK (D6, Santa Cruz Biotechnology, USA; #sc-28380) and GAPDH (Cell Signalling Technology; 14C10), PSAT1 (Gene Tex, Irvine, CA, USA; GTX110576). The following antibodies were purchased from Bethyl Laboratories (Montgomery, TX, USA): Aly/REF (THOC4) (#A302-892A), Coilin (#A303-760A), FXR1 (#A303-892A), nucleolin/NCL (#A300-709A), MRE11(#A303-998A), NRIP (#A302-435A) and THRAP3/TRAP150 (#A300-956A). Antibody C10 against DDX5/p68 [70] wasa generous gift from Hans Stahl, Department of Biochemistry, Saarland University Medical School, Homburg/Saar, Germany.

Vectors

The pcDNA 3.1-based vectors for the eukaryotic expression of HA-tagged SMD1, SMD2 and SMD3 were kindly supplied by Utz Fischer, Department of Biochemistry, University of Würzburg, Würzburg, Germany. The expression vector for pCI-FLAG-Aly/REF (THOC4) protein was a generous gift from Niels Gehring, Institute of Genetics, University of Cologne, Cologne, Germany. The human LSM4 gene was PCR-amplified from the vector pQE30-LSM4 kindly supplied by Christian Kambach, Department

of Biochemistry, University of Bayreuth, Bayreuth, Germany, using the primers For-Bgl II-LSM4 GAAGATCTACCATGCTTCCCTTGTCACTGC and Rev-EcoRI-LSM4 CTGCAGAATTCGACTGTTTGCCCGCCTGTCTGC. The PCR product was inserted into the vector pSG5-HA [12], digested with Bgl II and EcoR I, generating a C-terminally HA-tagged LSM4.

EBNA1 and EBNA2 mutants

The EBNA1 mutants used in this paper were based on an EBNA1-WT (missing the glycine-alanine repeat). The mutants with deletions of either linking region 1 (aa 34–52; ΔLR1) or linking region 2 (aa 328–377; ΔLR2), respectively, were generated using site-directed mutagenesis (Stratagene) according to the manufacturer's instructions. The mutant with all of the arginines changed to alanine has already been described [20]. The vectors pSG5-EBNA2-WT and pSG5-EBNA2ΔRG were used as described previously [10].

Cell lysis and immunoprecipitation

The immunoprecipitation of MMA-modified proteins with the 19D4 antibody (see above) was performed as described previously [10, 71]. In short, 100 µl of protein G Sepharose beads (GE Healthcare, Freiburg, Germany) were washed three times with cold PBS buffer and incubated with 1.6 ml 19D4 (rat IgG2c) or 1.6 ml of isotype control antibody under rotation at 4 °C overnight. Coupled beads were then washed three times with cold PBS and once with lysis buffer (25 mM Tris-HCl, pH 7.4, 150 mM KCl, 2 mM EDTA, 0.5 % IGEPAL) and protease inhibitors (Complete mini, Roche). We washed 150 ml of Raji or BL41 cell lines at a density of 10^6 cells ml^{-1} three times in cold PBS before they were suspended in 1 ml of lysis buffer for 30 min on a vertical rotator at 4 °C and then centrifuged at 20.000xg for 30 min at 4 °C. We removed 20 µl of the cleared lysate for protein input analysis, while the remainder was divided into two equal volumes for 19D4 and isotype control precipitation. The coupled beads were incubated with the lysate on a vertical rotator at 4 °C overnight. The beads were washed three times with wash buffer (50 mM Tris-HCl, pH 7.4, 300 mM KCl, 1 mM $MgCl_2$, 0.5 % IGEPAL) and suspended in SDS gel buffer for further analysis. Immunoprecipitation for FLAG and HA-tag was performed using the same protocol as described above. The immunoprecipitation of MRE11 was performed with the following modification: 40 µl of protein A Sepharose beads (GE Healthcare) were used for coupling with either 16 µl of MRE11 antibody or 16 µl of rabbit pre-immune serum. Additional preclearing of lysate was performed using 40 µl of protein A Sepharose beads with 16 µl of rabbit pre-immune serum for 1 h on a vertical rotator at 4 °C. For immunoblotting, samples were pre-heated at 95 °C in 4× sample buffer for 8 min and subjected to SDS-PAGE followed by immunoblotting using ECL [12, 72]. Molecular mass marker proteins were either assembled from individually obtained purified proteins (Sigma; phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carboanhydrase, 30 kDa) or obtained from New England Biolabs (blue prestained protein standard, broad range, 11–190 kDa, # P7706S).

Mass spectrometry analysis

The gel slices were equilibrated with 50 mM NH4HCO3 for 10 min. For reduction and alkylation, each gel slice was incubated in 45 mM dithiothreitol (30 min, 55 °C), followed by two incubation steps (15 min, RT) in 100 mM iodoacetamide. After two further equilibration steps with 50 mM NH4HCO3, tryptic digestion was performed overnight at 37 °C with 70 ng porcine trypsin (Promega, WI, USA). Peptides were further extracted from the gel slices with 80 % acetonitrile. Liquid chromatography tandem mass spectrometry was performed on an EASY-nLC 1000 nano-chromatography system (Thermo Scientific, Waltham, MA, USA) coupled to an Orbitrap XL instrument (Thermo Scientific). Dried peptide samples were diluted in 10 µl 0.1 % formic acid (FA), injected on a trap column (PepMap100 C18, 75 µm×2 cm, 3 µm particles, Thermo Scientific) and separated at a flow rate of 200 nL min^{-1} (column: PepMap RSLC C18, 75 µm×50 cm, 2 µm particles; Thermo Scientific) using a 30 min linear gradient from 2 % to 50 % solvent B (0.1 % formic acid, 100 % ACN). For data acquisition, a top-five data-dependent CID method was used. Mass spectrometry data were processed with MASCOT version 2.1.03 (Matrix Science, London, UK) using the human and EBV subset of the UniProt database. the search criteria were as follows: (i) enzyme, trypsin; (ii) fixed modification, carbamidomethylation (C); (iii) variable modifications, oxidation (M), monomethyl (R) and dimethyl (R); (iv) peptide mass tolerance, 10 p.p.m.; (v) MS/MS mass tolerance, 0.8 Da; (vi) peptide charges, 1+, 2+ and 3+; (vii) instrument, ESI trap; and (viii) allow up to one missed cleavage. For proteins identified with more than 10 spectra, Scaffold version 2.4 (Proteome Software, Inc., Portland, OR, USA) was used for spectral count-based label-free quantification in order to detect proteins enriched in the immunoprecipitation samples compared to the control samples.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
This research did not involve human subjects. Experiments involving animals: monoclonal antibodies were generated using Lou/C rats in the laboratory of Dr Elisabeth Kremmer, Helmholtz Zentrum München, following an established method. The immunization of rats was approved by the Regierung von Oberbayern, Aktenzeichen (209.1/211-2531.6-4/99).

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