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Effects of SUMO-1 upon Epstein-Barr virus BZLF1 function and BMRF1 expression

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Abstract

Epstein-Barr virus (EBV) is a human herpesvirus that has infected at least 90% of the world population. This very successful virus causes infectious mononucleosis and is associated with many different types of cancer. The EBV BZLF1 protein is a transcription factor that has also been shown to interact with many host cell proteins and pathways. BZLF1 (Z) is tagged by the small ubiquitin-related modifier-1 (SUMO-1) protein. Here, we present studies of the functional consequences of SUMO-1 modification of Z. We found that SUMO-1 modification of Z has no apparent effect upon the stability and localization of the Z protein. We did find, however, that SUMO-1 modification decreases the transactivation activity of Z on specific promoters. In addition, when SUMO-1 is supplied to cells when lytic replication is induced, EBV BMRF1 levels greatly increase, suggesting that SUMO-1 enhances EBV lytic replication. Therefore, SUMO-1 modification of proteins appears to have an important role in EBV lytic replication.

Keywords: Epstein-Barr virus; BZLF1; BRLF1; BMRF1; SUMO-1

Epstein-Barr virus (EBV) is a human herpesvirus that has infected a majority of the world's population. EBV is the causative agent of infectious mononucleosis and is associated with several cancers, including lymphomas and nasopharyngeal carcinoma [1]. Upon infection of a host, EBV infects two cell types: epithelial cells, where the virus replicates in a lytic manner, and B cells, where the virus enters a latent state [1–4]. During lytic replication, the majority of EBV genes are expressed, which include the immediate-early, early, and late genes [2]. The EBV immediate-early genes, BZLF1 and BRLF1, are both nuclear proteins and function as transcriptional activators of the EBV early gene promoters, including the BMRF1 gene [2,5–12]. BMRF1 encodes a polymerase processivity factor [13]. BZLF1 also transactivates the BRLF1 promoter, while BRLF1 transactivates the BZLF1 promoter [14,15]. Expression of either of the immediate-early genes in latently infected EBV-positive cells will initiate lytic replication [2]. BZLF1 is also required for replication of the EBV genome, by binding to and activating transcription from the origin of lytic replication, ori-lyt [16,17].

Viruses have evolved mechanisms to manipulate the host cell environment in order to evade an immune response and replicate efficiently. Such mechanisms often involve binding to and inactivating key cellular proteins. EBV manipulates its environment during lytic replication through the immediate-early proteins, BZLF1 (Z) and BRLF1 (R). Z binds to and interferes with the function of CREB-binding protein (CBP), activates the MAP kinases p38 and c-Jun N-terminal kinase (JNK), arrests cell cycle, interferes with interferon-γ signaling, inactivates p53 function, and disrupts promyelocytic leukemia (PML) bodies [18–25]. R binds to CBP, activates p38 and JNK, and activates the cell cycle [19,26,27].

Viral proteins have also gained the ability to be post-translationally modified by host cellular proteins. The

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cytomegalovirus (CMV) IE1, vaccinia virus E3L, bovine papillomavirus E1, and EBV Z and R proteins all become SUMO-1 modified [24,28–31]. SUMO-1 (small ubiquitin-related modifier 1) is a small protein that is covalently attached to proteins on specific lysine residues. SUMO-1 modification of several cellular proteins, including PML, Ran GAP1, c-Jun, STAT1, and p53, has been shown to be required for their proper cellular localization and/or function (reviewed in [32]). Likewise, SUMO-1 modification of viral proteins plays an important role in viral protein function. For example, SUMO-1 modification enhances the transcriptional activation ability of CMV IE2 [32,33].

We previously determined that the EBV Z protein is SUMO-1 modified [24]. This conjugation occurs at lysine 12 of Z. A Z mutant, referred to as Zm12/13, contains a lysine to alanine switch at position 12 of Z and no longer becomes SUMO-1 modified [24]. However, the consequences of SUMO-1 modification upon Z function and EBV replication have remained unclear. In this report, we show the effects of SUMO-1 upon Z protein stability and Z transactivation function. We also demonstrate SUMO-1's effect upon disruption of EBV latency and subsequent levels of BMRF1 protein produced.

Materials and methods

Cell lines. HeLa is a cervical carcinoma cell line. D98-HE-R-1 is an EBV-positive epithelial cell line formed by the fusion of a HeLa subclone (D98) with the EBV-positive Burkitt's lymphoma cell line P3HR/1. Epithelial cell lines were maintained in Dulbecco's modified Eagle's medium H supplemented with 10% fetal calf serum. Raji is an EBV-positive B cell line and was maintained in RPMI supplemented with 10% fetal calf serum.

Plasmids. The Z expression vector contains wild-type Z in the pcDL-SRα 296 vector (gift of Diane Hayward). Zta (m12/13), referred to as Zm12/13 in this paper, contains mutations at amino acids 12 and 13 (from KF to AA) (gift of Diane Hayward). The HA-SUMO-1 vector contains the SUMO-1 gene, HA-tagged, downstream of the CMV promoter, in the pHSS10B vector. The R expression vector contains the R gene in the RTS-15 vector.

EapBS-CAT contains the early EBV BMRF1 promoter sequences from −331 to +1 linked to the chloramphenicol acetyltransferase (CAT) gene. ZpBS-CAT contains EBV Z promoter sequences from −552 to +1 linked to the CAT gene. RpBS-CAT contains the EBV R promoter sequences from −962 to +5 linked to the CAT gene.

DNA purification. Plasmid DNA was purified through Qiagen columns as described by the manufacturer (Qiagen).

DNA transfection. DNA was transfected into cells by the calcium-phosphate method [34]. Briefly, 10–20 μg of DNA was added to 0.1× TE (1 mM Tris, pH 8, 0.1 mM EDTA, pH 8) to a final volume of 220–440 μl. 250–500 μl of 2× HBS, pH 7.05 (280 mM NaCl, 10 mM KCl, 1.5 mM $Na_2HPO_4{\cdot}7H_2O$, 12 mM dextrose, and 50 mM Hepes), was added to the DNA, mixed, and followed by the slow addition of 31–62 μl of 2 M $CaCl_2$. The mixture was incubated at room temperature for 20 min and added dropwise to 1×10^5–1×10^6 cells.

Mitotic cell isolation. Cells grown in flasks were rapped to release mitotic cells, which round up off the flask. These mitotic cells were collected separately from the remaining interphase cells.

CAT assays. Cell extracts were prepared 48 h post-transfection and incubated at 37° with [^{14}C]chloramphenicol in the presence of acetyl coenzyme A as described previously [35]. The percent acetylation of chloramphenicol was quantified by thin-layer chromatography followed by Phosphorimager analysis.

Protein preparation. Cells were washed twice with PBS, resuspended in ELB buffer (0.25 M NaCl, 0.1% NP40, 50 mM Hepes, pH 7, 5 mM EDTA, and protease inhibitors), and freeze/thawed twice. The lysed cells were centrifuged and the supernatant was used for immunoblot analysis.

For SUMO-1 modified proteins, the cells were washed twice with PBS and resuspended in a 1:3 mixture of buffer I (5% SDS, 0.15 M Tris/HCl, pH 6.8, and 30% glycerol) and buffer II (25 mM Tris/HCl, pH 8.3, 50 mM NaCl, 0.5% NP40, 0.5% deoxycholate, 0.1% SDS, and protease inhibitors). The cells were briefly sonicated, centrifuged, and the resulting supernatant was used for immunoblot analysis.

Immunoblot analysis. Immunoblot analysis was performed for the detection of the BMRF1 and Z proteins as follows: briefly, 40 μg of protein was loaded in each lane and SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed. The proteins were transferred onto nitrocellulose, blocked in 1× phosphate-buffered saline (PBS)/5% milk/0.1% Tween 20, and incubated in primary antibody for 1–2 h at room temperature or overnight at 4 °C [monoclonal anti-EBV Zebra (1:100), from Argene; monoclonal EAD (1:100), from Capricorn]. The membrane was washed in PBS/0.1% Tween 20, incubated in secondary antibody for 30–60 min at room temperature [goat-anti-mouse-horseradish peroxidase (GAM-HRP; 1:10,000), from Jackson Labs], washed, and the results were visualized with the ECL chemiluminescent kit (Amersham) according to the manufacturer's instructions.

Results

SUMO-1 does not alter the stability or localization of the Z protein

The Z protein has been previously shown to become SUMO-1 modified. Z can be observed in three abundant protein bands of 35, 46, and 66 kDa (see [24] and Fig. 1A). By examining the sumoylation state of Z in interphase versus mitotic cells, we have also found that Z is more highly sumoylated in mitotic cells (Fig. 1B). Western blot analysis with an anti-Z antibody indicated that mitotic cells had higher levels of the 46 and 66 kDa Z proteins (Fig. 1B).

SUMO-1 has been shown to increase the life-span of proteins such as PML (reviewed in [36]). To examine whether SUMO-1 may increase the stability of Z, we transfected HeLa cells with wild-type Z and mutant Zm12/13 expression plasmids, each with or without SUMO-1 expression plasmid. Western blot analysis was performed with an anti-Z antibody (Fig. 1C). The presence of SUMO-1 did not have any effect upon the levels of Z protein, such that there were equal levels of Z protein both with or without SUMO-1. In addition, the levels of Zm12/13 protein were identical to those of the wild-type Z protein, indicating that the loss of the SUMO-1 site had no effect upon protein stability. Therefore, SUMO-1 conjugation does not appear to affect Z protein stability.

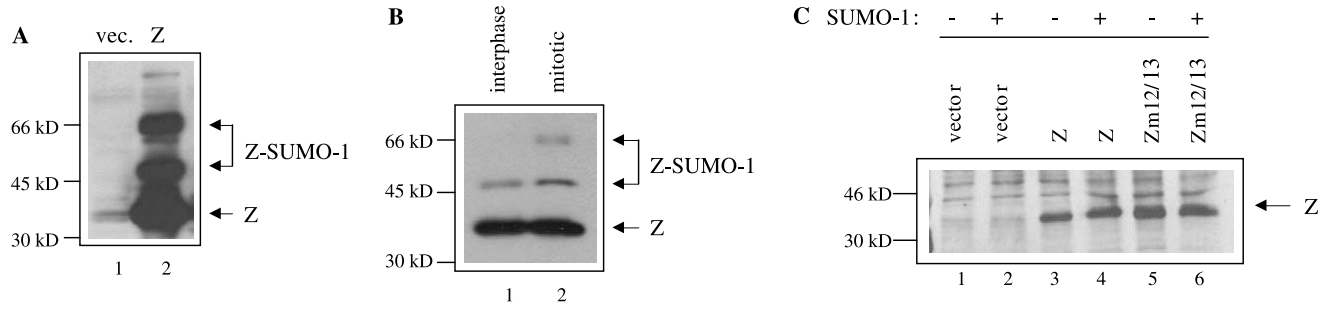

Fig. 1. SUMO-1 modification of Z does not alter the stability of the Z protein. (A) HeLa cells were transfected with either 5 μg of vector or 5 μg of Z expression vector. Forty-eight hour post-transfection, cells were harvested for SUMO-1 proteins and Western blot analysis was performed with an anti-Z antibody. (B) HeLa cells were transfected with 10 μg of Z expression vector. Forty-eight hour post-transfection, mitotic cells were separated from interphase cells, harvested for SUMO-1 proteins, and Western blot analysis was performed with an anti-Z antibody. (C) HeLa cells were transfected with either 5 μg of vector, 5 μg of Z expression vector, or 5 μg of Zm12/13 expression vector (mutated at amino acids 12 and 13), along with either 0 or 6 μg of SUMO-1 expression vector. Cells were harvested 32 h post-transfection. Western blot analysis was performed with an anti-Z antibody.

SUMO-1 has also been shown to confer specific protein localization [32]. Therefore, we investigated whether sumoylation contributed to the cellular localization of Z. We transfected HeLa cells with the Z and Zm12/13 expression vectors, each with or without SUMO-1 expression vector. The cells were fixed and immunostained with anti-Z antibody. The even-spread nuclear localization noted for Z was not altered by the addition of SUMO-1, nor was it altered in Zm12/13 expressing cells (data not shown). Therefore, SUMO-1 does not appear to influence Z localization.

SUMO-1 modification of Z decreases the transactivation activity of Z on the EBV BMRF1 and R promoters

Ahn et al. [33] have shown that the SUMO-1 modification of the CMV IE2 protein increased the transactivation activity of IE2. To examine whether the SUMO-1 modification of Z also affected Z's transactivation function, we transfected HeLa cells with three reporter constructs that contained Z-responsive promoters: the BMRF1 promoter, the R promoter, and the Z promoter. Each reporter plasmid was co-trans-

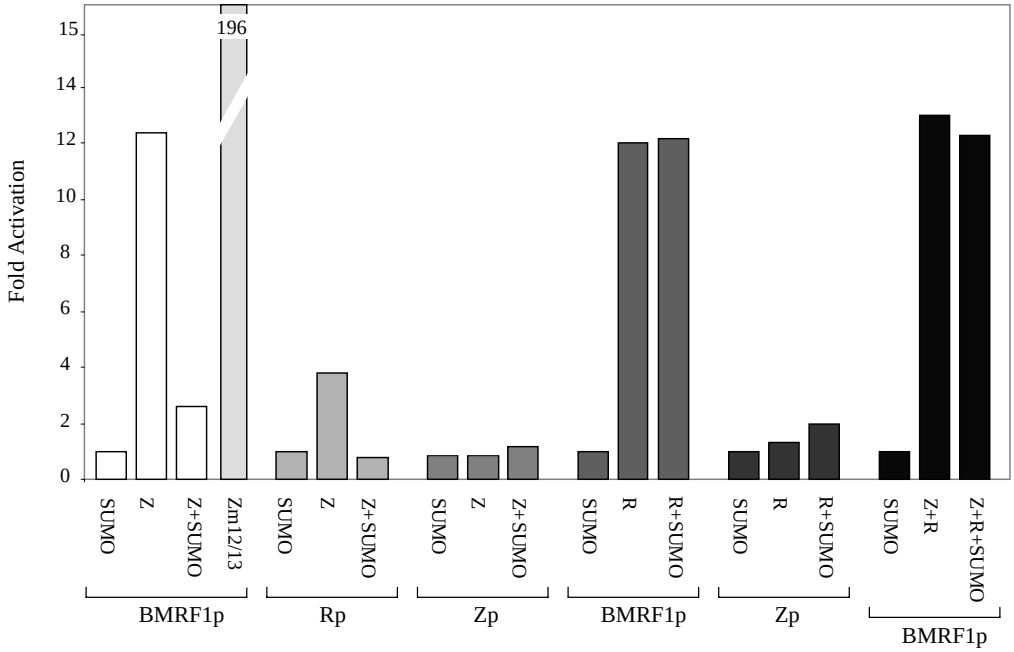

Fig. 2. SUMO-1 decreases Z transcriptional activity of the BMRF1 and R promoters. HeLa cells were transfected with 4 μg of either BMRF1-CAT, R-CAT, or Z-CAT reporter plasmids, plus either 2 μg of vector, 2 μg of SUMO-1 expression vector, 2 μg of transactivator (Z, R, or Zm12/13) expression vector, or transactivator plus SUMO-1 expression vectors together. CAT assays were performed. The results shown are derived from three separate experiments. The percent acetylation was converted to fold activation, and the results were normalized such that the SUMO-1 alone activation equaled 1-fold. p, promoter.

fected with either a control plasmid, SUMO-1 expression plasmid, Z expression plasmid, or Z plus SUMO-1 expression plasmids. CAT assays were performed and the results are presented in Fig. 2. We found that while Z transactivated the BMRF1 promoter to 12.4-fold, the addition of SUMO-1 to Z decreased this transactivation to 2.6-fold. Similarly, Z transactivated the R promoter to 3.8-fold, and the addition of SUMO-1 to Z decreased the transactivation to 0.8-fold. In contrast, we did not see a decrease in Z transactivation of the Z promoter upon SUMO-1 addition. While Z did not effectively transactivate its own promoter in this set of experiments (1-fold), the addition of SUMO-1 to Z led to a modest increase in transactivation, to 1.4-fold.

As the Zm12/13 mutant is not SUMO-1 modified [24], we compared the ability of this mutant versus wild-type Z to transactivate the BMRF1 promoter (Fig. 2). Amazingly, Zm12/13 transactivated the BMRF1 promoter to 196-fold (16 times higher than wild-type Z). Thus, removing Z's SUMO-1 site appears to relieve repression and leads to a great increase in Z transactivation, confirming a negative role of SUMO-1 on Z transactivation (at least for the BMRF1 promoter).

Since R has also been shown to become SUMO-1 modified [31], we examined whether the transactivation activity of R would be altered by SUMO-1 addition. The BMRF1 and Z promoters are transactivated by R and were used in this study (Fig. 2). R transactivated the BMRF1 promoter to 12-fold. The addition of SUMO-1 to R had no effect upon this transactivation (12.2-fold). Likewise, SUMO-1 had no negative effect upon R's transactivation of the Z promoter, and in fact slightly increased transactivation of the Z promoter (from 1.3- to 2-fold).

Given that in the context of actual EBV lytic replication both Z and R would be present, we co-transfected the Z and R expression vectors together, with or without SUMO-1, to examine BMRF1 transactivation (Fig. 2). Co-transfection of Z and R together slightly increased the transactivation of BMRF1 (to 13.3-fold) in relation to Z alone or R alone. Interestingly, the addition of SUMO-1 to Z and R had little effect upon the transactivation of the BMRF1 promoter, slightly decreasing it to 12.3-fold. This indicates that while Z transactivation activity is attenuated by SUMO-1, the combination of Z and R together, as found during lytic replication, still promotes abundant BMRF1 gene expression.

SUMO-1 increases the ability of EBV to disrupt viral latency

To examine how SUMO-1 affected lytic replication in EBV-positive cells, we transfected either vector DNA or a Z expression vector with either 0, 2, 4, or 6 μg of SUMO-1 expression vector into latently infected, EBV-positive (D98-HE-R1) cells, and performed immunoblot analysis with an anti-BMRF1 antibody (Fig. 3A). Whereas SUMO-1 expression alone had no effect upon the disruption of viral latency, SUMO-1 and Z co-expression resulted in high levels of BMRF1 protein (Fig. 3A, lanes 7 and 8), suggesting that the presence of SUMO-1 increases the efficiency of Z-mediated viral disruption of latency. Similarly, we examined the ability of SUMO-1 to augment R-mediated disruption of viral latency (Fig. 3B). The addition of SUMO-1 to R-expressing cells led to an increase in BMRF1 production over R alone (Fig. 3B, lanes 4–6).

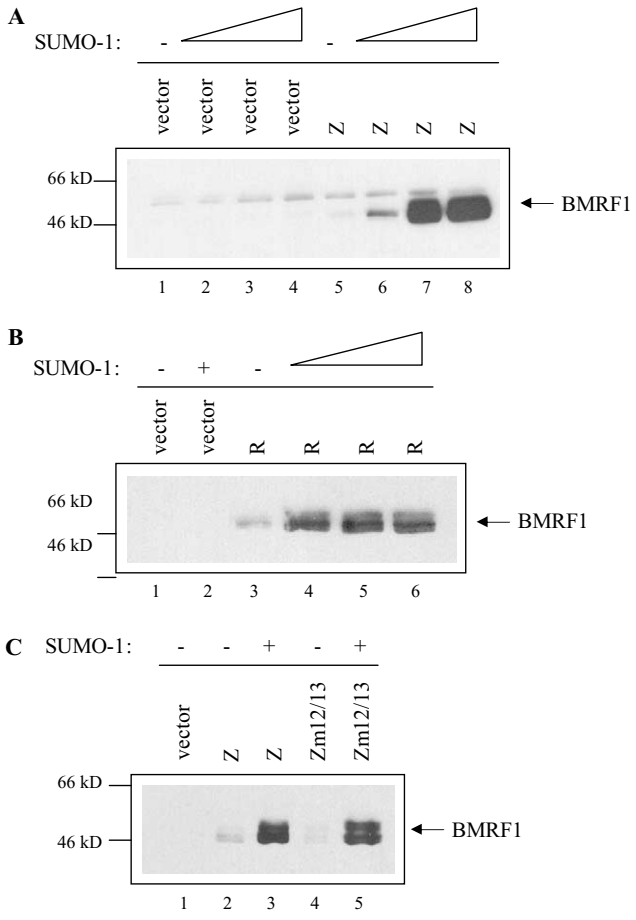

Fig. 3. SUMO-1 increases the ability of Z and R to disrupt EBV latency. (A) D98-HE-R-1 cells were transfected with either 5 μg of vector (lanes 1–4) or 5 μg of Z expression vector (lanes 5–8), along with increasing amounts (0, 2, 4, or 6 μg) of SUMO-1 expression vector. Cells were harvested 46 h post-transfection. Western blot analysis was performed with an anti-BMRF1 antibody. (B) D98-HE-R-1 cells were transfected with either 5 μg of vector (lanes 1 and 2) or 5 μg of R expression vector (lanes 3–6), along with increasing amounts (0, 2, 4, or 6 μg) of SUMO-1 expression vector. Cells were harvested 48 h post-transfection. Western blot analysis was performed with an anti-BMRF1 antibody. (C) D98-HE-R-1 cells were transfected with either 5 μg of vector (lane 1), 5 μg of Z expression vector (lanes 2 and 3), or 5 μg of Zm12/13 expression vector (lanes 4 and 5) along with either 0 or 6 μg of SUMO-1 expression vector. Cells were harvested 30 h post-transfection. Western blot analysis was performed with an anti-BMRF1 antibody.

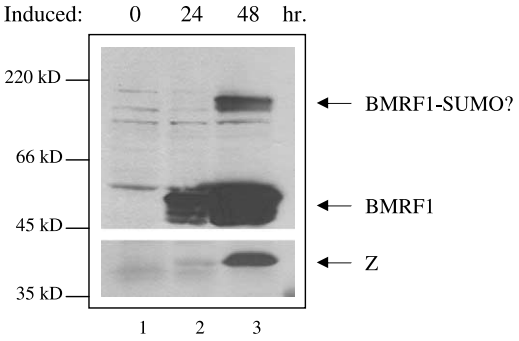

Fig. 4. BMRF1 may be SUMO modified. Raji cells were induced into lytic replication by addition of 20 ng/ml TPA and 3 mM sodium butyrate for 0, 24, or 48 h. SUMO-1 proteins were prepared and Western blot analysis was performed with anti-BMRF1 antibody (top panel). The blot was reprobed with anti-Z antibody (lower panel).

To examine whether the increase of BMRF1 production was dependent upon the SUMO-1 site of Z, we transfected EBV-positive (D98-HE-R1) cells with Z or Zm12/13 expression vectors, each with or without SUMO-1 expression vector, and performed immunoblot analysis with an anti-BMRF1 antibody (Fig. 3C). We found that the Zm12/13-SUMO-1 combination induced BMRF1 production as well as wild-type Z. Thus, the large increase of BMRF1 production seen with SUMO-1 addition does not appear to be dependent upon SUMO-1 modification of Z.

BMRF1 may be SUMO-modified

The traditional protein preparation method used for Western blot analysis of BMRF1 (as in Fig. 3) yields a stack of about three protein bands clustered around 50 kDa (there are different phosphorylated forms of BMRF1). To examine whether BMRF1 itself may be SUMO-modified, we examined the BMRF1 protein using our method to preserve SUMO-1 modified proteins. We induced EBV-positive Raji cells into lytic replication with the addition of TPA and sodium butyrate and harvested cells at 0, 24, and 48 h post-induction. Western blot analysis was performed with anti-BMRF1 and anti-Z antibodies (Fig. 4). Interestingly, at 48 h post-induction, additional, higher molecular weight BMRF1 bands appeared (Fig. 4, lane 3). Such bands did not appear when protein lysates were not prepared with the SUMO-preservation method (data not shown). Thus, the BMRF1 protein may be another EBV target for SUMO-1 modification, and this may contribute to the increase in BMRF1 protein noted when SUMO-1 was co-transfected with Z or R to disrupt EBV latency (Fig. 3).

Discussion

SUMO modification occurs on a variety of proteins and elicits varying effects. The EBV Z protein, which is necessary for EBV to undergo lytic replication, also becomes SUMO-1 modified [24]. To establish a functional consequence for SUMO-1 modification of Z, we studied the effects of SUMO-1 modification on various aspects of the Z protein. We found that SUMO-1 appears to have no effect upon Z protein stability or Z protein localization within the nucleus. We did find, however, that SUMO-1 does elicit an effect upon the transactivation function of Z. In reporter assays, SUMO-1 inhibited Z transactivation of both the EBV BMRF1 and R promoters. Furthermore, mutating the SUMO-1 site such that Z could no longer become SUMO-1 modified led to a great increase in Z's transactivation activity. It is yet unknown whether SUMO-1 alters Z's ability to interact with transcriptional machinery or whether SUMO-1 alters Z's DNA binding ability.

The EBV R protein has also been shown to become SUMO-1 modified [31]. We did not find, however, that sumoylation had any effect upon R's transactivation activity. Thus, while Z and R function in a similar manner to activate EBV promoters, sumoylation preferentially downregulates Z, but not R, transactivation functions. When Z and R were co-expressed in EBV-negative cells to mimic early EBV lytic replication, SUMO-1 had little effect upon the resulting BMRF1 expression, presumably because R was able to make up for the SUMO-1 effects on Z function. The negative effects of SUMO-1 on Z transactivation function may come into play on other promoters, however, such as the R promoter, and may act to fine-tune Z's transactivation activity.

A very striking result of SUMO-1 modification was the high levels of BMRF1 expression that were brought about when SUMO-1 was added to EBV-positive cells along with either Z or R. Z and R are both individually capable of disrupting EBV latency, since the expression of one leads to expression of the other. The addition of SUMO-1 dramatically increased the levels of BMRF1 produced, especially when co-transfected with Z. Yet, the effect may have nothing to do with the sumoylation of Z or R, since the Z mutant Zm12/13, which cannot be SUMO-1 modified, yielded the same high levels of BMRF1 when SUMO-1 was added. The level of BMRF1 protein is often used to gauge the level of EBV lytic replication that occurs after disruption of viral latency. Thus, the addition of SUMO-1 may greatly enhance the entire cycle of EBV lytic replication. Another explanation, however, is that SUMO-1 directly affects the BMRF1 protein. We show that BMRF1 may be SUMO modified since we found higher molecular weight BMRF1 proteins when the proteins were prepared with a SUMO-specific protocol. These higher molecular weight proteins are in the 100 kDa range, and since BMRF1 is approximately 50 kDa, this would most likely mean that several SUMO-1 proteins are conjugated into

a chain onto the BMRF1 protein. Since SUMO-1 has been shown to promote stability of proteins, it is possible that SUMO-1 modified BMRF1 is more stable and accumulates when extra SUMO-1 is provided. This may account for the increased level of BMRF1 noted when SUMO-1 was co-transfected with either Z or R into EBV-positive cells.

Another explanation for the high levels of BMRF1 produced when SUMO-1 is added to EBV-positive cells is that another protein, cellular or viral, is SUMO-1 modified and acts to greatly enhance EBV lytic replication. In any case, it is very interesting that SUMO-1 has such a large impact on EBV lytic replication. Further studies in this area will help elucidate the mechanisms behind SUMO-1's effects upon EBV biology.

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