Small ubiquitin-related modifier (SUMO) pathway-mediated enhancement of human cytomegalovirus replication correlates with a recruitment of SUMO-1/3 proteins to viral replication compartments

Myriam Scherer,1 Nina Reuter,1 Nadine Wagenknecht,1 Victoria Otto,1 Heinrich Sticht2 and Thomas Stamminger1

Correspondence
Thomas Stamminger
thomas.stamminger@viro.med.uni-erlangen.de

Received 21 December 2012
Accepted 12 February 2013

1Institute for Clinical and Molecular Virology, University of Erlangen-Nuremberg, Schlossgarten 4, 91054 Erlangen, Germany
2Institute of Biochemistry, University of Erlangen-Nuremberg, Fahrstr. 17, 91054 Erlangen, Germany

Recent studies have suggested that the small ubiquitin-related modifier (SUMO) conjugation pathway may play an important role in intrinsic antiviral resistance and thus for repression of herpesviral infections. In particular, it was shown that the herpes simplex virus type-1 regulatory protein ICP0 acts as a SUMO-targeted ubiquitin ligase (STUbL), inducing the widespread degradation of SUMO-conjugated proteins during infection. As the IE1 protein of human cytomegalovirus (HCMV) is known to mediate a de-SUMOylation of PML, we investigated whether HCMV uses a similar mechanism to counteract intrinsic antiviral resistance. We generated primary human fibroblasts stably expressing FLAG-SUMO-1 or FLAG-SUMO-3 and analysed the SUMOylation pattern after HCMV infection or isolated IE1 expression. However, Western blot experiments did not reveal a global loss of SUMO conjugates, either in HCMV-infected or in IE1-expressing cells, arguing against a function of IE1 as an STUbL. Interestingly, we observed that FLAG-SUMO-1 and FLAG-SUMO-3, subsequent to IE1-mediated promyelocytic leukemia protein (PML) de-SUMOylation and the consequent disruption of PML nuclear bodies, were recruited into viral replication compartments. This raised the question of whether FLAG-SUMO-1/3 might promote HCMV replication. Intriguingly, overexpression of FLAG-SUMO-1/3 enhanced accumulation of viral DNA, which correlated with an increase in viral replication and in virus particle release. Together, these data indicate that HCMV, in contrast to other herpesviruses, has evolved subtle mechanisms enabling it to utilize the SUMO conjugation pathway for its own benefit, resulting in an overall positive effect of SUMO conjugation for HCMV replication.

INTRODUCTION

Post-translational modification of cellular as well as viral proteins by members of the small ubiquitin-related modifier (SUMO) family is emerging as a major regulatory principle of protein function and many different pathogens have evolved mechanisms to exploit the SUMO modification system for their own advantage (for a review see Wimmer et al., 2012). In mammals, there are four reported SUMO paralogues named SUMO-1 to SUMO-4, although the relevance of SUMO-4 at the endogenous level remains of debate (Da Silva-Ferrada et al., 2012). While SUMO-2 and SUMO-3 share 95% sequence identity and, at present, are assumed to be functionally identical, they are only 50% identical to SUMO-1. The covalent attachment of the 11 kDa SUMO moiety to lysine residues in specific target proteins is controlled by an enzyme cascade, analogous to the ubiquitin pathway, and can alter various properties of the modified substrate such as its localization, activity, interaction partners or stability (Johnson, 2004; Müller et al., 2001; Wilkinson & Henley, 2010).

Recently, a bioinformatic study revealed that the ability to be SUMOylated appears to be a common feature of proteins associated with a subnuclear dot-like structure known as nuclear domain 10 (ND10) or PML nuclear bodies. The further observation that all components of the SUMO conjugation system are present at these dynamic multiprotein complexes led to the hypothesis that ND10 may function as a hotspot for SUMO modification (Van Damme et al., 2010). In fact, SUMOylation of the key component PML itself is regarded to constitute the basis for ND10 formation, as it was reported that only SUMOylated PML has the capacity to recruit ND10-associated proteins such as hDaxx and Sp100 (Ishov et al., 1999; Shen et al., 2006; Zhong et al., 2000).
In previous studies, the ND10 components PML, hDaxx and Sp100 were identified as cellular restriction factors that mediate intrinsic immunity against herpesviral infections [reviewed by Tavalai & Stamminger (2009)]. This antiviral activity, however, is counteracted by viral regulatory proteins, e.g. ICP0 of herpes simplex virus type-1 (HSV-1) and IE1 of human cytomegalovirus (HCMV). Both proteins were found to induce a disruption of ND10 structures, which involves the loss of SUMOylated forms of PML and Sp100 (Everett et al., 1998; Lee et al., 2004; Müller & Dejean, 1999; Tavalai et al., 2011). While ICP0 has recently been reported to act as a SUMO-targeted ubiquitin ligase (STUbL) that leads to a widespread proteasome-dependent degradation of SUMO-conjugated proteins in general (Boutell et al., 2011), the mechanism by which IE1 overcomes ND10-mediated repression remains unclear.

Besides affecting the SUMOylation status of cellular proteins, IE1 itself serves as a target of the SUMO modification pathway (Müller & Dejean, 1999). Contrasting effects of IE1 SUMOylation on HCMV infection have been described: on the one hand, a repressive effect on HCMV replication was suggested as SUMOylation of IE1 abrogates the binding of STAT2 and abolishes its interferon (IFN)-antagonistic activity (Huu et al., 2008); on the other hand, SUMO modification of IE1 was described to facilitate the expression of the immediate-early protein IE2 and to promote viral replication (Nevels et al., 2004). Similarly, the immediate-early protein IE2 is also SUMOylated, which was shown to be important for efficient replication of HCMV (Berndt et al., 2009). However, conflicting results on IE2 SUMOylation were provided from studies using alternative HCMV laboratory strains (Lee & Ahn, 2004). Thus, whereas a clear contribution of the SUMOylation pathway to intrinsic resistance against HSV-1 infection was demonstrated (Boutell et al., 2011), the importance of SUMO modification for HCMV infection remains unclear.

In this study, we used SUMO-overexpressing fibroblasts to characterize further the interplay between HCMV and the cellular SUMO conjugation system. We found that HCMV specifically influences the SUMOylation of only a small subset of proteins, e.g. PML, but does not affect SUMO-modified proteins in general. Furthermore, we made the interesting observation that, following PML de-SUMOylation and ND10 disruption, SUMO proteins are incorporated into viral replication centres and promote HCMV replication. This unexpected finding, together with the observation that viral proteins are major targets of SUMO modification during infection, suggests that HCMV has evolved mechanisms to exploit the cellular SUMOylation pathway for its own benefit.

RESULTS

Generation of primary human fibroblasts with stable expression of FLAG-tagged SUMO-1 or SUMO-3

To elucidate further the role of SUMO conjugation during HCMV infection, we generated cells that stably overexpress FLAG-tagged versions of SUMO-1 (F-SUMO-1) or SUMO-3 (F-SUMO-3). For this, the coding sequences for F-SUMO-1 and F-SUMO-3 were cloned into the lentiviral expression vector pLenti6.4/V5-DEST followed by lentiviral transduction of primary human foreskin fibroblasts (HFFs), which resulted in cell populations termed HFF/F-SUMO-1 and HFF/F-SUMO-3. As control, HFFs containing integrated copies of empty vector were generated. Subsequent selection of blasticidin-resistant cells yielded a transduction efficiency of more than 98%, as assessed by indirect immunofluorescence analysis (data not shown). To verify that the introduced F-SUMO proteins are conjugated to cellular targets, we next performed Western blot analysis to detect SUMOylated proteins. Numerous high-molecular-mass conjugates were observed with an anti-FLAG antibody, whereas comparatively small amounts of free F-SUMO-1 and F-SUMO-3 were present, confirming that F-SUMO proteins act as substrates for conjugation (Fig. 1a, left panel). In addition, by the use of SUMO-specific antibodies, we detected an overall increased SUMO-1 and SUMO-3 conjugation in lysates of HFF/F-SUMO-1 and HFF/F-SUMO-3, respectively (Fig. 1a, middle and right panels). Analysis of the subcellular localization by indirect immunofluorescence revealed that both F-SUMO-1 [Fig. 1b(i)–(iv)] and F-SUMO-3 [Fig. 1b(v)–(viii)] exhibited a nuclear diffuse distribution and accumulated in dot-like structures co-localizing with PML-associated ND10, thus showing the same localization pattern as endogenous SUMO proteins (Boddy et al., 1998). Furthermore, comparative staining of HFF/F-SUMO-1, HFF-SUMO-3 and control HFFs with antibodies against endogenous SUMO-1 or SUMO-2/3 excluded an aberrant localization pattern of overexpressed F-SUMO proteins (Fig. 1c).

HCMV infection does not result in a global loss of SUMO conjugates, but induces a subtle alteration of the SUMOylation pattern

The ICP0 protein of HSV-1 has recently been demonstrated to act as an STUbL, which induces a global degradation of SUMO-conjugated proteins, including PML and Sp100, and thereby counteracts the antiviral activity of ND10 (Boutell et al., 2011). Moreover, we and others have previously shown that IE1 not only de-SUMOylates PML but also directly modulates the SUMOylation status of Sp100 (Kim et al., 2011). As these data indicate that IE1 as well may have an extended spectrum of target proteins, we wanted to explore the effect of IE1 on the stability of SUMO-1 and SUMO-3 conjugates. To address this issue, HFF/F-SUMO-1 and HFF/F-SUMO-3 were infected with the HCMV laboratory strain AD169 at an m.o.i. of 3, and were harvested at different times after infection [24–72 h post-infection (p.i.)] for Western blot analysis of the SUMO conjugation pattern. As evident from Fig. 2(b, c), HCMV infection did not result in a general loss of SUMO-1 or SUMO-3 conjugates, as it was detected after infection with HSV-1 (Fig. 2a). This could also be observed.
after infection with the highly endotheliotropic HCMV strain TB40/E (Sinzger et al., 2008) (data not shown). A comparison of the SUMOylation pattern in mock- and HCMV-infected cells revealed that several additional bands appeared after HCMV infection. We noted two prominent bands at approximately 100 and 115 kDa (Fig. 2b, c, indicated by asterisks), which presumably represent SUMOylated species of the immediate-early proteins IE1 and IE2. As reported previously, these viral proteins can be modified by both SUMO-1 and SUMO-2/3 (Hofmann et al., 2000). In accordance, a distinct 100 kDa band corresponding to SUMOylated IE1 was detected with an anti-IE1 antibody in HFF/F-SUMO-1 and HFF/F-SUMO-3 (Fig. 2b, c, middle panels). Together, our data suggest that HCMV does not have the capacity to induce a widespread loss of SUMO conjugates. Furthermore, viral proteins themselves appear to be major targets of SUMO modification during infection.

HCMV induces de-SUMOylation of PML in F-SUMO-overexpressing cells

To rule out that the overexpression of F-SUMO hampers the detection of protein de-SUMOylation by HCMV, we next investigated whether there was a loss of SUMOylated PML during infection. Western blot experiments revealed that expression of F-SUMO-1 or F-SUMO-3 did not induce changes in the general pattern of PML variants, except that a double band of mono-SUMOylated PML arose from the modification by endogenous SUMO and...
**Fig. 2.** HCMV infection does not result in a general de-conjugation of F-SUMO-1 and F-SUMO-3. (a) SUMOylation pattern after infection with HSV-1. HFF/F-SUMO-1 and HFF/F-SUMO-3 were either not infected (mock) or infected with HSV-1-GFP-VP22 at an m.o.i. of 0.5 and harvested at 24 h p.i. for Western blotting. F-SUMO-conjugated proteins were stained with the anti-FLAG antibody M2. Detection of GFP-VP22 and actin was included as infection control and internal loading control, respectively. (b, c) SUMOylation pattern after infection with HCMV. HFF/F-SUMO-1 (b) and HFF/F-SUMO-3 (c) were either not infected (mock) or infected with AD169 at an m.o.i. of 3, and harvested at indicated times (24–72 h p.i.) for Western blotting. F-SUMO-conjugated proteins were detected with the anti-FLAG antibody M2. Detection of IE1 and β-actin was included as infection control and internal loading control, respectively.

**Fig. 3.** HCMV induces a de-SUMOylation of PML in F-SUMO-overexpressing cells. Control HFF (a), HFF/F-SUMO-1 (b) and HFF/F-SUMO-3 (c) were either not infected (mock) or infected with AD169 at an m.o.i. of 3 and harvested at the indicated times following infection (2–8 h p.i.). Western blotting was performed to analyse the abundances of SUMOylated and non-SUMOylated PML species using the monoclonal PML antibody 5E10. The localization of polySUMO species of PML that disappear as infection progresses is indicated by square brackets at the right side of each blot. IE1 and β-actin were included as infection control and internal loading control, respectively.
F-SUMO (Fig. 3, asterisks). Starting at 6 h after HCMV infection, a depletion of SUMOylated PML was detected in control cells and simultaneously in HFF/F-SUMO-1 and HFF/F-SUMO-3 (Fig. 3, lanes 6 and 8). Consistent with previous findings (Lee et al., 2004; Tavalai et al., 2011), this de-SUMOylation was restricted to poly-SUMOylated species of PML while the mono-SUMOylated forms remained unaffected at early stages after infection in HFF/F-SUMO and control cells. Therefore, we regard the F-SUMO-expressing HFFs as a suitable system for studying HCMV-induced alterations of protein SUMOylation.

**Expression of IE1 in the absence of other viral proteins does not induce a general reduction of SUMO conjugation, but negatively interferes with the SUMOylation of a small subset of proteins**

Next, we determined the influence of IE1 expression alone on SUMO conjugate abundance. HFF/F-SUMO-1 and HFF/F-SUMO-3 were transduced with lentiviruses expressing IE1 or control lentiviruses, and were subjected to Western blotting (Fig. 4). Similar to the situation during HCMV infection (Fig. 2b, c), expression of IE1 alone did not induce a widespread reduction of SUMO-1 and SUMO-3 conjugates, but resulted in a discrete alteration of the SUMOylation pattern. In particular, as observed before in infected cells, an additional 100 kDa band appeared in cells expressing IE1 due to the modification of IE1 itself by F-SUMO-1 or F-SUMO-3 (Fig. 4, upper panels). When we compared the pattern of PML and Sp100 variants in control HFFs and IE1-expressing HFFs, however, we found that IE1 expression led to a clear depletion of SUMOylated PML and Sp100 (Fig. 4, middle panels). We conclude that IE1 does not have STUbL-like properties, as it has been reported for ICP0, but uses a different strategy to interfere specifically with the SUMOylation of a small subset of proteins.

**F-SUMO-1 and F-SUMO-3 localize at ND10 upon HCMV infection and are subsequently recruited into viral replication centres**

To characterize further the HCMV-induced changes of protein SUMOylation, we analysed the intracellular localization of SUMO proteins during the time course of infection. In a first experiment, HFF/F-SUMO-1 and HFF/F-SUMO-3 were infected with HCMV, strain AD169, and harvested at early time points (2–6 h p.i.) followed by indirect immunofluorescence detection of F-SUMO and IE1. Upon infection, F-SUMO-1 and F-SUMO-3 were initially found at still intact ND10 structures [Fig. 5a(i)–(iv), b(i)–(iv)], as observed in uninfected cells (Fig. 1b). Here, they co-localized with IE1, which transiently accumulates at ND10 at very early times post-infection (Korioth et al., 1996). Starting at 6 h p.i., in parallel with the loss of poly-SUMOylated PML variants (Fig. 3), a disintegration of ND10 was induced by the continuous production of IE1, resulting in a dispersed localization of IE1 and in the release of ND10-associated proteins into the nucleoplasm (Ahn & Hayward, 1997). Intriguingly, F-SUMO-1 and F-SUMO-3 retained their punctate distribution, although there appeared to be fewer but enlarged dots, resembling early forms of viral replication compartments [Fig. 5a(ix)–(xii), b(ix)–(xii)]. These F-SUMO-containing dots partially co-localized with pre-replication compartments.
foci [Fig. 5c(i)–(iv), b(i)–(v)], which were visualized by detection of the major transactivator protein IE2 in a next experiment. At later times post-infection, this was followed by an accumulation of both F-SUMO-1 [Fig. 5c(v)–(xii)] and F-SUMO-3 [Fig. 5d(v)–(xii)] in viral replication compartments where they perfectly co-localized with IE2. This observation suggests that SUMO proteins may play a role in viral replication processes.

IE2 is not responsible for the recruitment of F-SUMO-1 and F-SUMO-3 into viral replication compartments

Given that IE2 is a major SUMO conjugation target (Fig. 2b, c) (Hofmann et al., 2000), we investigated whether the recruitment of F-SUMO-1 and F-SUMO-3 into replication compartments depends on IE2. We utilized the recombinant virus AD169ex5-ΔSM2 expressing a SUMOylation-negative mutant of IE2 (Fig. 6a), which harbours mutations of both the covalent SUMO attachment sites and the non-covalent SUMO interaction motif (Berndt et al., 2009). Infection of HFF/F-SUMO-1 and HFF/F-SUMO-3 with AD169ex5-ΔSM2, however, still resulted in efficient incorporation of F-SUMO-1 (Fig. 6b) and F-SUMO-3 (Fig. 6c) into viral replication centres which were detected by staining of the DNA polymerase subunit UL44. This suggests that not IE2 but another factor is responsible for the recruitment of SUMO proteins to sites of viral replication, and is in line with the observation that no perfect co-localization of IE2 and F-SUMO proteins was observed.

Fig. 5. F-SUMO-1 and F-SUMO-3 localize to viral replication compartments during HCMV infection. (a, b) Subnuclear localization of F-SUMO proteins at early times of HCMV infection. HFF/F-SUMO-1 (a) and HFF/F-SUMO-3 (b) were infected with wild-type AD169 at an m.o.i. of 3. Localization of F-SUMO and IE1 was detected at indicated time points (2–6 h p.i.) by immunostaining with a polyclonal anti-FLAG antibody and the anti-IE1 antibody p63-27, respectively. (c, d) Subnuclear localization of F-SUMO proteins at late times of HCMV infection. HFF/F-SUMO-1 (c) and HFF/F-SUMO-3 (d) were infected with recombinant AD169/IE2-EGFP at an m.o.i. of 1. Cells were harvested at indicated times post-infection (24–72 h p.i.) for immunostaining with the anti-FLAG antibody M2. Replication centres were detected via autofluorescence of IE2-EGFP. Cell nuclei were identified by detection of DAPI signals.
detected prior to the presence of fully formed replication centres [Fig. 5c(i)–(iv), d(i)–(iv)].

Expression of F-SUMO-1 or F-SUMO-3 promotes HCMV replication

The finding that F-SUMO-1 and F-SUMO-3 are efficiently incorporated into viral replication compartments raised the question of whether expression of F-SUMO proteins has any influence on HCMV replication efficacy. To test this, HFF/F-SUMO-1, HFF/F-SUMO-3 and control fibroblasts were infected with a recombinant HCMV expressing enhanced green fluorescent protein (AD169-GFP) at an m.o.i. of 0.01, followed by GFP quantification at day 9 after infection. Interestingly, we measured a three- to fourfold increase of GFP fluorescence in HFF/F-SUMO-1 and HFF/F-SUMO-3 compared with control cells, indicating enhanced replication of HCMV in the presence of F-SUMO proteins (Fig. 7a). To confirm this observation, we infected HFF/F-SUMO and control cells with wild-type HCMV, strain AD169, at an m.o.i. of 0.01 and after 4 days, we determined the yields of released infectious particles in the supernatants by titration on HFF cells. Consistent with the results obtained by GFP quantification, we observed that three- to fourfold more infectious particles were released from cells expressing F-SUMO-1 or F-SUMO-3 (Fig. 7b). Quantification of plaques in a standard plaque assay also revealed a modest but significant increase after infection of HFF/F-SUMO-1 and HFF/F-SUMO-3 (Fig. 7c). To investigate whether this results from an increased viral uptake, we again infected the cells with HCMV at an m.o.i. of 0.01. After 8 and 96 h p.i., DNA was extracted and used for real-time PCR quantification of HCMV DNA. We detected no significant differences in the viral load at 8 h p.i. in HFF/F-SUMO and control HFFs (Fig. 7d, left), indicating that viral uptake is not altered by F-SUMO expression, but we measured an enhanced accumulation of viral DNA at 4 days p.i. in HFF/F-SUMO (Fig. 7d, right). To confirm these data, we independently generated F-SUMO-expressing cells that were obtained by retroviral transduction of telomerase-immortalized primary HFFs. As shown in Fig. 7(e, f), infection of those cells, termed HFFi, HFFi/F-SUMO-1 and HFFi/F-SUMO-3, with either the clinical HCMV strain VR1814 or laboratory strain AD169 resulted in an enhanced accumulation of viral DNA at 4 days p.i., respectively. Furthermore, a recombinant AD169-derived virus, expressing a SUMOylation-negative

---

**Fig. 6.** Lack of IE2 SUMOylation does not affect the recruitment of F-SUMO-1 and F-SUMO-3 into viral replication compartments. (a) HFFs were infected with either wild-type AD169 or recombinant AD169ex5-ΔSM2 expressing SUMOylation-negative IE2. At 48 h p.i., the cells were harvested and subjected to Western blot analysis for detection of IE2 using the polyclonal anti-pHM178 antiserum (upper panel). β-Actin detection served as internal loading control (lower panel). (b, c) Subnuclear localization of F-SUMO proteins after infection with recombinant AD169 ex5-ASM2. HFF/F-SUMO-1 (b) and HFF/F-SUMO-3 (c) were infected with recombinant AD169ex5-ASM2 at an m.o.i. of 1. In subsequent immunofluorescence experiments, the localization of F-SUMO proteins was investigated at the indicated times after infection (24–72 h p.i.) using a polyclonal anti-FLAG antibody. Replication compartments were detected by co-staining of the viral polymerase processivity factor UL44 with the monoclonal antibody BS 510. Cell nuclei were identified by DAPI staining.
IE1 protein, was applied. As shown in Fig. 7(g), we observed a comparable increase in viral DNA at 4 days p.i. of F-SUMO cells, excluding that overexpression of SUMO enhances the previously described IFN-antagonistic function of IE1 (Huh et al., 2008). In conclusion, our data demonstrate that overexpression of SUMO proteins has an overall positive effect on HCMV replication, suggesting a beneficial role of the SUMO conjugation system during CMV infection.

DISCUSSION
SUMOylation is implicated in the regulation of a wide range of cellular processes. As such, it is not surprising that
viral proteins were among the first discovered to influence the host SUMO system (Boggio & Chiocca, 2006). For HSV-1, the SUMOylation pathway has been shown to mediate intrinsic antiviral resistance, but which is inactivated by the viral ubiquitin ligase ICP0. In a recent study, ICP0 was reported to have properties related to those of cellular STUbLs, as it non-covalently interacts with SUMO (Boutell et al., 2011). In this study, we investigated whether IE1 of HCMV likewise causes a global inhibition of protein SUMOylation. We found that HCMV infection influences the SUMOylation status of a small subset of proteins, e.g. PML, but does not affect SUMO-1 or SUMO-3 conjugates in general. This observation was confirmed by Western blot analysis of HFFs with expression of IE1 alone, indicating that IE1 does not act as a general STUbL, but has a more specific mode of target recognition. As a physical interaction between IE1 and its known targets PML and Sp100 was demonstrated and, moreover, binding to PML was shown to be necessary for de-SUMOylation by IE1 (Kim et al., 2011; Lee et al., 2004), it is reasonable to suppose that IE1 recognizes its target proteins via a direct interaction. Additionally, it was shown that PML de-SUMOylation by IE1, in contrast to ICP0, does not require proteasomal activity (Lee et al., 2004). Thus, we assume not only different targets of IE1 but also essential differences in the mechanism of de-SUMOylation, which will require further studies to be elucidated fully.

Comparison of the SUMOylation pattern in the absence and presence of HCMV infection furthermore revealed that several additional bands of SUMOylated proteins appeared in infected cells. Two of the prominent bands most probably represent SUMOylated species of IE1 and IE2, suggesting that viral proteins are main substrates of the SUMO conjugation machinery. Given that contrasting consequences of IE1 and IE2 SUMOylation on the progression of HCMV infection have been described (Berndt et al., 2008; Huh et al., 2008; Lee & Ahn, 2004; Nevels et al., 2004), the importance of the SUMO system for viral replication has remained controversial:

SUMOylation of viral proteins may either be part of a host defence mechanism or may be a viral strategy to exploit the cellular SUMO system for efficient replication. To define further the role of SUMO conjugation for HCMV infection, we investigated the distribution of SUMOylated proteins during a course of infection via immunofluorescence experiments. We observed that SUMO-1 and SUMO-3 initially localized to ND10 domains, as long as these were still intact. Following IE1-mediated de-SUMOylation of PML and dispersal of ND10 proteins, however, SUMO proteins were incorporated into viral replication compartments, which has already been reported for SUMO1 in a previous study (Lee & Ahn, 2004). As this suggested a possible role of SUMO proteins in viral replication processes, we next determined the impact of SUMO-1 and SUMO-3 overexpression on HCMV replication. Intriguingly, we found that expression of both of SUMO-1 and of SUMO-3 promotes HCMV replication, pointing to a beneficial role of protein SUMOylation during HCMV infection.

At present, IE1 and IE2 are the only HCMV proteins known to undergo SUMO conjugation. The fact that IE1 does not localize to sites of viral replication but exhibits a diffuse distribution at times after ND10 disruption, as well as the finding that the incorporation of SUMO-1 and SUMO-3 into replication compartments is independent of IE2, gave rise to the idea that SUMOylation of additional viral proteins positively influences HCMV replication. Alternatively, as suggested in a previous study, SUMO-modified cellular proteins such as p53 or PCNA might play a role (Lee & Ahn, 2004). As a first step towards the identification of possible viral candidates, an in silico prediction of SUMOylation motifs within HCMV protein sequences was performed. As illustrated in Table 1, we identified 26 HCMV proteins harbouring at least one putative SUMOylation site. Among them, we found several promising candidates that play an important role in viral replication, such as the DNA polymerase itself (UL54), the DNA polymerase processivity factor (UL44), the single-strand DNA-binding protein (UL57) or the helicase (UL105) (Table 1, dark grey panels). Interestingly, while this paper was under review, Sinigalia et al. (2012) noted
that UL44 indeed can serve as a target for SUMO modification. Thus, a further experimental confirmation of the predicted SUMOylation sites and determination of their importance for HCMV replication remain worthy subjects for future investigation as they may provide further insights into the mechanisms used by viruses to exploit the cellular SUMO pathway for their own benefit.

METHODS

Oligonucleotides and plasmid constructions. The oligonucleotide primers used for this study were purchased from Biomers GmbH. The following primers were used for cloning reactions: 5'attB1_FLAG, GGAGCACAATTTTGATACAAAAAAGCAGCTTGCAGACTGACAAAGACGATGA; 3'attB2_SUMO-1, GGGGACCACTTTGTACAAAGAAAGCTGGGTCTTACTGGTCAGCCTTGCTTC. The following primers were used for cloning reactions: 5'attB1_FLAG and 3'attB2_SUMO-1 together with plasmid FLAG-SUMO-1, GGGGACCACTTTGTACAAAGAAAGCTGGGTCTTACTGGTCAGCCTTGCTTC; 5'attB1_FLAG and 3'attB2_SUMO-3, GGGGACCACTTTGTACAAAGAAAGCTGGGTCTTACTGGTCAGCCTTGCTTC.

To construct plasmids utilized for generation of FLAG-SUMO-1, FLAG-SUMO-3- and IE1-expressing lentiviruses, the respective coding sequences were amplified using the following primers and templates: primer 5'attB1_FLAG and 3'attB2_SUMO-1 together with plasmid FLAG-SUMO-1-pcDNA3 (Hofmann et al., 2000) as template for amplification of FLAG-SUMO-1; primer 5'attB1_FLAG and 3'attB2_SUMO-1 together with plasmid FLAG-SUMO-1-pcDNA3 (Hofmann et al., 2000) as template for amplification of FLAG-SUMO-3; and primer 5'attB1/IE1 and 3'attB2/IE1 together with plasmid pHM494 (Hofmann et al., 2000) as template for amplification of IE1. The resulting PCR products were transferred into the intermediate vector pDONR221 and subsequently introduced, along with the cellular EF-1z promoter, into the lentiviral expression vector pLent6.4/R4R2/V5-DEST using the Gateway recombination technology (Invitrogen).

Cells and viruses. Primary HFFs were maintained in Eagle’s minimal essential medium (Gibco-BRL) supplemented with 5 % fetal calf serum. HFFs stably expressing FLAG-SUMO-1, FLAG-SUMO-3 (designated HFF/F-SUMO-1 and HFF/F-SUMO-3) and control HFFs were cultured in Dulbecco’s minimal essential medium (Gibco-BRL) supplemented with 10 % fetal calf serum and 2 μg bacitracin ml⁻¹.

Infection experiments were performed with either the HCMV laboratory strain AD169, the clinical isolates VR1814 (FIX-BAC-derived) (Hahn et al., 2002) or TB40/E (Sinzger et al., 2008), or the recombinant viruses: AD169-GFP (expressing GFP under control of the HCMV major immediate-early enhancer–promoter; the expression cassette inserted between US9 and US10 (Marschall et al., 2000), AD169/IE2-EGFP (expressing EGFP-tagged IE2) (Sourdinos et al., 2007), AD169ex5-ASM2 (expressing IE2 with mutated SIM and SUMOylation sites) (Berndt et al., 2009), AD169-IE1(K450R) (expressing IE1 with mutated SUMOylation site) or herpes simplex strain HSV-1-GFP-VP22 (expressing GFP fused to VP22) (Elliott & O’Hare, 1999) (kindly provided by Manfred Marschall, Erlangen, Germany). Viral stocks of wild-type and of all AD169-derived recombinant HCMVs were titrated by IE1p72 fluorescence (Lorz et al., 2006). For this, HFFs were infected with serial dilutions of viral supernatants. After 24 h of incubation, cells were fixed and stained with monoclonal antibody (mAb) p63-27 (Andreon et al., 1989). Subsequently, the number of IE1-positive cells was determined, and viral titres (IE units) were calculated. For titration of HSV-1-GFP, HFFs were infected with serial dilutions of viral supernatant. At 8 h p.i., the number of positive cells was determined by GFP autofluorescence, and viral titres (GFP units) were calculated.

For analysis of viral growth in transduced HFFs, the replication of HCMV AD169-GFP was quantified by automated fluorometry as described previously (Marschall et al., 2000). Determination of virus release was performed by infection of transduced HFFs (3 x 10⁵) with HCMV AD169 at an m.o.i. of 0.01. At 4 days after infection, the supernatant was harvested and yields of infectious virus were measured by titration on HFF cells as described above. Each experiment was performed in triplicate, and standard deviations were calculated.

Retroviral transduction and selection of stably transduced cells. For the generation of HFFs stably expressing FLAG-SUMO-1, FLAG-SUMO-3 as well as control cells, replication-deficient lentiviruses were prepared using the pLent6.4/R4R2/V5-DEST MultiSite Gateway vector kit from. For this, 293T cells were co-transfected with the respective pLent6.4/R4R2/V5-DEST vectors together with packaging plasmids pLP1 (encoding HIV-1 Gag and Pol), pLP2 (encoding HIV-1 Rev) and pSV-G (expressing the vesicular stomatitis virus envelope protein) using the Lipofectamine 2000 reagent (Invitrogen). Viral supernatants were harvested 48 h after transfection, clarified by centrifugation, filtered and stored in aliquots at −80 °C. HFFs were incubated for 24 h with lentivirus supernatants in the presence of Polybrene (7.5 μg ml⁻¹; Sigma-Aldrich). Then, G418 (2 μg ml⁻¹) was added to the cell culture medium to select a stably transduced cell population. A second, independent batch of HFFs stably expressing FLAG-SUMO-1 and FLAG-SUMO-3 was generated using telomerase-immortalized primary HFFs. The respective cells were termed HFFi, HFFi-SUMO-1 or HFFi-SUMO-3.

For the generation of HFF/F-SUMO-1 and HFF/F-SUMO-3 with additional expression of IE1 as well as control cells, lentiviruses were prepared as described above using the IE1 expression construct or the empty vector. HFF/F-SUMO-1 and HFF/F-SUMO-3 were incubated for 24 h with lentivirus supernatants in the presence of Polybrene (7.5 μg ml⁻¹; Sigma-Aldrich) and were harvested at 96 h post-transduction.

Real-time PCR. HFF/F-SUMO-1, HFF/F-SUMO-3 and control HFFs (3 x 10⁵ cells per well) were infected with AD169 at an m.o.i.

Table 1. Prediction of SUMOylation sites within HCMV proteins

<table>
<thead>
<tr>
<th>No.</th>
<th>Site</th>
<th>No.</th>
<th>Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CDS20</td>
<td>US27</td>
<td>14 CDS88</td>
</tr>
<tr>
<td>2</td>
<td>CDS28</td>
<td>US34A</td>
<td>15 CDS91</td>
</tr>
<tr>
<td>3</td>
<td>CDS30</td>
<td>RL10</td>
<td>16 CDS97</td>
</tr>
<tr>
<td>4</td>
<td>CDS32</td>
<td>RL12</td>
<td>17 CDS99</td>
</tr>
<tr>
<td>5</td>
<td>CDS53</td>
<td>UL20</td>
<td>18 CDS108</td>
</tr>
<tr>
<td>6</td>
<td>CDS57</td>
<td>UL24</td>
<td>19 CDS114</td>
</tr>
<tr>
<td>7</td>
<td>CDS60</td>
<td>UL27</td>
<td>20 CDS119</td>
</tr>
<tr>
<td>8</td>
<td>CDS68</td>
<td>UL35</td>
<td>21 CDS123</td>
</tr>
<tr>
<td>9</td>
<td>CDS76</td>
<td>UL43</td>
<td>22 CDS129</td>
</tr>
<tr>
<td>10</td>
<td>CDS77</td>
<td>UL44</td>
<td>23 CDS138</td>
</tr>
<tr>
<td>11</td>
<td>CDS79</td>
<td>UL46</td>
<td>24 CDS139</td>
</tr>
<tr>
<td>12</td>
<td>CDS81</td>
<td>UL48</td>
<td>25 CDS143</td>
</tr>
<tr>
<td>13</td>
<td>CDS83</td>
<td>UL49</td>
<td>26 CDS146</td>
</tr>
</tbody>
</table>

Downloaded from www.microbiologyresearch.org by
IP:  54.70.40.11
On: Sun, 06 Jan 2019 14:50:27

1382

Journal of General Virology 94

M. Scherer and others
of 0.01. At 8 or 96 h p.i., the DNA was extracted from virus-infected cells using a DNeasy tissue kit (Qiagen), followed by real-time PCR as described previously (Lorz et al., 2006; Tavalai et al., 2006). For each sample, DNA extracts were analysed in triplicate.

**Indirect immunofluorescence analysis and Western blotting.**

For indirect immunofluorescence analysis, which was performed as described previously, HFF/F-SUMO-1 and HFF/F-SUMO-3 were grown on coverslips at a density of 3 x 10⁵ cells per well (Tavalai et al., 2011). For Western blotting, extracts from transduced or infected cells were prepared in an SDS-PAGE loading buffer, separated on 8–17.5 % polyacrylamide gels and transferred to nitrocellulose membranes. For separation of SUMO conjugates, urea-containing polyacrylamide gels were used. Chemiluminescence was detected according to the manufacturer’s protocol (ECL Western blot detection kit; Amersham Pharmacia Biotech).

**Antibodies.** For the detection of FLAG-tagged versions of SUMO-1 and SUMO-3, the monoclonal anti-FLAG antibody M2 or a polyclonal anti-FLAG antibody was used (Sigma-Aldrich). Endogenous SUMO-1 was detected with a mouse mAb (kindly provided by Gerrit Praefcke, University of Cologne, Germany), and endogenous SUMO-2/3 was detected using a rabbit mAb from Epitomics. For detection of PML we used either the mAb 5E10 (kindly provided by Manfred Marschall, Erlangen, Germany), Gerrit Praefcke (Cologne, Germany) and Hans Will (Hamburg, Germany) for providing anti-FLAG antibody M2 or a monoclonal anti-FLAG antibody E7 (kindly provided by Dr Peter Hemmerich, Leibniz Institute for Age Research, Jena, Germany).

**ACKNOWLEDGEMENTS**

We thank Drs Roel van Driel (Amsterdam, The Netherlands), Peter Hemmerich (Jena, Germany), Bodo Plachter (Mainz, Germany), Manfred Marschall (Erlangen, Germany), Gerrit Praefcke (Cologne, Germany) and Hans Will (Hamburg, Germany) for providing reagents and Regina Müller for excellent technical assistance. This work was supported by the DFG (SFB796, subprojects A2 and B3) and the IZKF Erlangen.

**REFERENCES**


