Inhibition of SUMO-independent PML oligomerization by the human cytomegalovirus IE1 protein

Heejung Kang,1† Eui Tae Kim,1† Hye-Ra Lee,1† Jung-Jin Park,1 Yoon Young Go,2 Cheol Yong Choi2 and Jin-Hyun Ahn1

1Department of Molecular Cell Biology, Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, 300 Cheoncheondong, Jangangu, Suwon, Gyeonggido 440-746, Korea
2Department of Biological Science, Sungkyunkwan University, Suwon, Korea

Correspondence
Jin-Hyun Ahn
jahn@med.skku.ac.kr

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In human cytomegalovirus-infected cells, the immediate-early IE1 protein disrupts the subnuclear structures known as the PML oncogenic domains or PODs, via the induction of PML desumoylation. This activity correlates with the functions of IE1 in transcriptional regulation and in the stimulation of lytic infection. Here, the effects of IE1 in induction of desumoylation of PML were characterized. IE1 did not interfere with the formation of sumoylated forms of PML in vitro. In in vitro assays using the sumoylated proteins, a SUMO-specific protease SENP1 desumoylated both PML and IE1. However, the IE1 proteins generated from bacteria or insect cells were unable to desumoylate PML in the same conditions. Although both IE1 and SUMO proteases such as SENP1, Axam and SuPr-1 efficiently desumoylated PML in co-transfection assays, they exerted different effects on the localization of PML. In cells transfected with either SENP1 or SuPr-1, the number of PML foci was reduced significantly and these remnant PML foci were devoid of SUMO-1 signals. However, in cells co-transfected with both SUMO proteases and IE1, these SUMO-independent PML foci were also completely disrupted. Furthermore, IE1, but not SENP1, was shown to disrupt the PML foci generated via transfection of a sumoylation-deficient mutant of PML. These data suggest that IE1 exhibits neither an inhibitory effect on sumoylation of PML nor intrinsic SUMO protease activity against PML in vitro. The finding that IE1 is capable of disrupting SUMO-independent PML aggregates suggests that inhibition of PML oligomerization by IE1 may play an important role in inducing PML desumoylation in vivo.

INTRODUCTION

Human cytomegalovirus (HCMV), a member of the subfamily Betaherpesvirinae, is a ubiquitous pathogen that can cause congenital disease and also produces serious complications in immunocompromised individuals (Pass, 2001). Among the approximately 150 proteins encoded by HCMV, the 72 kDa major immediate-early (IE) protein IE1 is the first and most abundantly expressed in infected cells and is believed to perform key regulatory functions in the preparation of the cells for the production of progeny virions (Mocarski, 2001). Studies with an IE1-deleted mutant virus have shown that IE1 is required for virus growth in cell cultures at a low m.o.i., whereas it appears to be unnecessary at a high m.o.i. (Greaves & Mocarski, 1998; Mocarski et al., 1996). This growth defect of the IE1-deleted mutant virus has been attributed to a failure or delay in the accumulation of viral delayed-early gene products (Ahn & Hayward, 2000; Gawn & Greaves, 2002; Greaves & Mocarski, 1998; Mocarski et al., 1996).

During the early stages of infection, IE1 initially targets to subnuclear structures referred to as PML oncogenic domains (PODs) or nuclear domain 10 (ND10). This targeting of POD by IE1 is a very transient effect. The PODs are disrupted within 3–4 h after infection, leading to the relocation of both IE1 and POD components, including PML and Sp100, into the nucleoplasm (Ahn et al., 1998; Ahn & Hayward, 1997; Korioth et al., 1996; Wilkinson et al., 1998). The POD-disrupting activity exhibited by IE1 is well-conserved among the IE proteins of other herpesviruses, such as herpes simplex virus 1 (HSV-1) ICP0 (Everett & Maul, 1994; Maul & Everett, 1994; Maul et al., 1993) and Epstein–Barr virus (EBV) Zta (Adamson & Kenney, 2001; Bell et al., 2000), which suggests its importance in the progression of viral growth. Among betaherpesvirus subfamily members, this activity is also conserved in mouse cytomegalovirus (MCMV) (Tang & Maul, 2003). However, in

†These authors contributed equally to this work.
Human herpesvirus 6, the equivalent IE1 proteins also target to the PODs, but remain stably associated with them throughout lytic infection (Gravel et al., 2002; Stanton et al., 2002). PODs have also been suggested to be involved in numerous cellular functions, including tumour suppression, transcriptional regulation, apoptosis, antiviral responses and the maintenance of genome stability (for reviews, see Bernardi & Pandolfi, 2003; Dellaire & Bazett-Jones, 2004; Regad et al., 2001; Salomoni & Pandolfi, 2002; Zhong et al., 2000b). The PML protein has multiple forms and is often covalently modified by the small ubiquitin-like modifier (SUMO) (Johnson, 2004; Seeler & Dejean, 2003), which is required for the formation of mature PODs (Ishov et al., 1999; Lallemand-Breitenbach et al., 2001; Zhong et al., 2000a).

Recent studies have indicated that the disruption of PODs by both HCMV IE1 and HSV-1 ICP0 involves the loss of sumoylated forms of PML (Boutell et al., 2003; Lee et al., 2004; Müller & Dejean, 1999). However, these two viral proteins appear to exploit different mechanisms. Unlike the reaction observed with ICP0, the PML desumoylation induced by IE1 is relatively resistant to the proteasome inhibitor MG132 and also does not result in any reduction in the levels of unmodified PML (Lee et al., 2004). Recently, it has been shown that ICP0 does not have intrinsic SUMO protease activity in vitro, but that ICP0-mediated PML degradation requires lysine 160, which is one of the principal targets of PML for sumoylation (Boutell et al., 2003). IE1 has also been found to bind to the N-terminal region of PML containing the RING-finger domain (Ahn et al., 1998), which is involved in both protein oligomerization and in the formation of supramolecular structures (Kentsis et al., 2002). In addition, a physical interaction of IE1 with PML has been demonstrated to be required at least for IE1-induced PML desumoylation in vivo (Lee et al., 2004). However, how IE1 induces PML desumoylation and POD disruption has yet to be elucidated clearly.

In the present study, we characterized the effects of IE1 in induction of desumoylation of PML. We obtained evidence to suggest that IE1 neither inhibits sumoylation of PML nor possesses intrinsic SUMO protease activity against PML in vitro. Furthermore, we determined that IE1 is also capable of interfering with the formation of SUMO-independent PML aggregates, suggesting that inhibition of PML oligomerization by IE1 may play an important role in inducing PML desumoylation in vivo.

**METHODS**

**Cell culture.** Human fibroblast, Vero and 293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS). For insect cell cultures, Spodoptera frugiperda (SF) 9 cells were grown in Grace's insect cell culture medium supplemented with 10% heat-inactivated FBS. Trichoplusia ni (Hi-Five) cells were grown in SF-900 II serum-free medium. The HCMV (Towne strain) viral stocks used in this study were prepared and used according to a previously described method (Lee et al., 2004).

**Expression vectors and plasmids.** Plasmids encoding GST–SAE2/SAE1 and His–UbC9 were provided by Ronald T. Hay (University of St Andrews, UK) and Chin Ha Chung (Seoul National University, Korea), respectively. pHA354, which expresses GST–SUMO-1GG, (an active form), has been described previously (Ahn et al., 2001). pT-E1E2S1, which encodes the E1 and E2 enzymes of SUMO conjugation, as well as an active form of SUMO-1, was provided by Hisato Saitoh (University of Kumamoto, Japan) and was used to introduce a synthetic SUMO-1 conjugation pathway into Escherichia coli (Uchimura et al., 2004a). Plasmids encoding HA–PML IV (pUS109) and HA–PML VI (pUS112) have been described previously (Lee et al., 2004). Plasmids expressing FLAG–Axam (pHR16), FLAG–SauPr-1 (pHR28) and FLAG–SauPr-1(C4665) (pHR27) were generated on a background of pSG5 (Green et al., 1988), using Gateway Technology (Invitrogen). Plasmids expressing FLAG–SENPI and FLAG–SENPI(C6035) (Bailey & O'Hare, 2004) were provided by Peter O'Hare (Marie Curie Research Institute, UK). Plasmids expressing myc–IE1 (pRYK107), myc–PML IV (pHA481) and myc–SENPI (pHR52) were generated on a background of pC53MT (Roth et al., 1991), using Gateway Technology. The HA–PML IV proteins containing the K160R or K940R mutation, as well as the FLAG–PML VI-3M protein, which contains the K65/160/490R mutation, were generated according to the Stratagene QuickChange site-directed mutagenesis protocol.

**In vitro sumoylation assay.** Recombinant GST–SAE2/SAE1 and GST–SUMO-1GG were expressed and purified from *E. coli* as described previously (Tatham et al., 2001). His–UbC9 was expressed and purified using Ni-NTA columns (Invitrogen) in accordance with the manufacturer's instructions. Myc-tagged PML IV protein was prepared using the TNT Quick Coupled Transcription/Translation System (Promega). GST–IE1 protein was purified from *E. coli*. Sumoylation reactions were conducted in a 30 μl volume containing a substrate protein (in vitro-translated myc–PML IV, GST–IE1, or both), 600 ng GST–SAE2/SAE1, 150 ng His–UbC9 and 500 ng GST–SUMO-1GG in buffer [50 mM Tris/HCl (pH 7.5), 5 mM MgCl2, 3 mM ATP]. The reaction was incubated for 1 h at 37°C. After termination of the reaction using SDS sample buffer containing β-mercaptoethanol, the reaction products were fractionated by 8% SDS-PAGE. The sumoylated forms of myc–PML IV or GST–IE1 were detected by immunoblotting assays, using anti-myc or anti-IE1 antibodies.

**Generation of SUMO-1-modified proteins in *E. coli*.** To generate the SUMO-1-conjugated PML or IE1 protein in *E. coli*, both a plasmid (Am(R)) encoding GST–PML VI or GST–IE1 and a plasmid (Cm(R)) encoding SUMO-1 conjugation components (E1, E2 and SUMO-1GG) were introduced into *E. coli* BL21 (DE3) (DE3) and the GST fusion proteins were expressed and purified, as described previously (Uchimura et al., 2004a). For GST–PML VI, both the unmodified and the SUMO-1-modified forms of GST–PML VI often accumulated in insoluble inclusion bodies. To obtain the soluble forms of GST–PML VI, the cell pellets were suspended in 9 vols cell lysis buffer II [50 mM Tris/HCl (pH 8.0), 10 mM EDTA (pH 8.0), 10 mM NaCl, 0.5% Triton X-100] and incubated for 5 min at room temperature. After 15 min of centrifugation at 15000 r.p.m. (Beckman JA-25.50) at 4°C, the pellets were resuspended in inclusion body solubilization buffer I [50 mM Tris/HCl (pH 8.0), 1 mM EDTA (pH 8.0), 100 mM NaCl, 8 M urea, 0.1 M PMSF] and incubated for 1 h at room temperature. Nine volumes of inclusion body solubilization buffer II [50 mM KH2PO4 (pH 10.7), 1 mM EDTA (pH 8.0), 50 mM NaCl] was added and the reaction was incubated for 30 min at room temperature. After centrifugation, the supernatant containing the soluble GST–PML VI protein was collected. The sample was adjusted to a pH of 8.0 and then incubated for an additional 30 min at room temperature. Prior to use, samples were dialysed using PBS (pH 7.4).
Production of GST–IE1 in insect cells using recombinant baculovirus. GST–IE1 protein was also generated in insect cells, using the BAC-to-BAC baculovirus expression system (Invitrogen). In brief, the transfer vector pHJK35, which encodes recombinant GST–IE1, was generated on a backbone of pDEST20 (Invitrogen) using Gateway Technology and introduced into DH10Bac E. coli harbouring a baculovirus bacmid. Recombinant bacmid DNA containing the GST–IE1 expression cassette was then isolated and verified via PCR analysis. S9 cells were transfected with the recombinant bacmid using Cellfectin reagent (Invitrogen). Recombinant baculovirus particles were harvested from the transfected cells and amplified. To conduct the large-scale production of GST–IE1, Hi-Five cells were infected with recombinant baculovirus at an m.o.i. of 3. At 96 h post-infection, the cells were harvested. Cell pellets were resuspended in lysis buffer [50 mM Tris/HCl (pH 7.5), 100 mM NaCl, 5 mM MgCl2, 0-1 % NP-40, 10 % glycerol (v/v), 1 mM PMSF, 1 mM dithiothreitol, 1 x protease inhibitor cocktail] and the GST–IE1 protein was purified.

Transient DNA transfection. 293T cells were transfected via the N,N-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid-buffered saline version of the calcium phosphate method, as described previously (Lee et al., 2004). Vero cells were transfected using Metafectin reagent (Biontex).

Antibodies and indirect immunofluorescence assay (IFA). Mouse monoclonal antibody (mAb) 8131, which detects epitopes present in both IE1 and IE2 (exons 2 and 3), was obtained from Chemicon. Anti-HA rat mAb (3F10), either conjugated with peroxidase or labelled with fluorescein, and anti-myc mouse mAb 9E10 were purchased from Roche. Anti-FLAG mouse mAb M2 was obtained from Sigma. Rabbit anti-PML polyclonal Ab (pAb), referred to as PML(C), and anti-SUMO pAb have been described previously (Ahn et al., 2001).

For the IFA, cells were fixed with 1 % paraformaldehyde and permeabilized with 0.2 % Triton X-100. All of the subsequently performed procedures have been described previously (Lee et al., 2004). All slides were examined and photographed using a Zeiss Axiophot microscope.

Immunoblot analysis. Cells were washed with PBS and total extracts were prepared via incubation of the cells with RIPA buffer [50 mM Tris/HCl (pH 8-0), 150 mM NaCl, 1-0 % NP-40, 0-5 % sodium deoxycholate, 0-1 % SDS] containing protease cocktail. For the immunoblot assays, samples were prepared via direct boiling in SDS loading buffer. The clarified cell extracts were then separated by 8 % SDS-PAGE, followed by a standard procedure using an enhanced chemiluminescence system (Amersham).

RESULTS

Effects of IE1 on PML sumoylation in reactions involving the E1 and E2 enzymes of SUMO conjugation

To determine whether IE1 affects the formation of sumoylated forms of PML in vitro, we initially set up a series of in vitro sumoylation reactions. When either in vitro-translated myc–PML IV or bacterially produced GST–IE1 was incubated with bacterially purified GST–SAE2/SAE1 (E1), His–Ubc9 (E2) and GST–SUMO-1GG (active form of GST–SUMO-1), the sumoylated forms of PML or IE1 were generated efficiently (Fig. 1a). These findings indicated that both PML and IE1 could be modified efficiently by SUMO-1 in vitro, in an E3-independent manner.

We then investigated whether IE1 interferes with PML sumoylation when both proteins are incubated simultaneously in a sumoylation reaction. We found that both the PML and IE1 proteins were sumoylated efficiently and that the increasing amounts of IE1 had no significant effects on the levels of the sumoylated forms of PML (Fig. 1b). This suggested that IE1 neither interferes with PML sumoylation,
nor directly causes PML desumoylation. However, this result does not preclude the possibility that the presence of excess amounts of E1 and E2 enzymes for sumoylation renders it difficult to detect IE1-associated desumoylation activity.

**SENP1 desumoylates both PML and IE1 in vitro: the use of an E. coli sumoylation system to characterize desumoylation activity**

To address more directly the question of whether IE1 itself exerts a desumoylation effect on PML, we employed an improved assay system for the study of in vitro desumoylation activity. Recently, a binary vector system was established, which introduces a synthetic SUMO modification system into *E. coli* (Uchimura et al., 2004a, b). In this system, *E. coli* is transformed with two plasmids, one encoding the E1 and E2 enzymes for sumoylation as well as an active form of SUMO-1 (or SUMO-2) and the other encoding a substrate (Fig. 2a). With this system, we generated both unmodified and SUMO-1-modified forms of both GST–IE1 and GST–PML VI in *E. coli* and purified them. As controls, the wild-type and catalytically inactive C603S mutant forms of SENP1, a SUMO protease, were also purified from *E. coli* as GST fusion forms. When the SUMO-1-modified forms of GST–IE1 or GST–PML VI were incubated with SENP1, the wild-type SENP1 was found to completely desumoylate both IE1 and PML VI (Fig. 2b, lanes 2 and 5), whereas the C603S mutant did not (Fig. 2b, lanes 3 and 6). This result clearly showed that SENP1 is capable of desumoylating both PML VI and IE1 in vitro. Thus, the use of bacterially generated sumoylated substrate proteins and SUMO proteases provides an ideal experimental system for the study of desumoylation activity under cell- and SUMO conjugation enzyme-free conditions.

**IE1 does not have an intrinsic desumoylating activity against PML in vitro**

Using the sumoylated forms of PML VI generated in *E. coli*, we asked whether purified IE1 was able to desumoylate PML. When the sumoylated PML VI protein was incubated for 1 h with increasing amounts (0-1 to 0-8 pmole) of bacterially produced GST–IE1, IE1 did not desumoylate PML at all, whereas similar amounts of GST–SENP1 resulted in the complete desumoylation of PML under the same conditions (Fig. 3a, top and middle panels). To exclude potential problems inherent to the different post-translational modification systems used in *E. coli* compared with eukaryotic cells, we also utilized GST–IE1 generated in insect cells infected with a recombinant baculovirus that expressed GST–IE1. However, even this eukaryotic version of GST–IE1 did not desumoylate PML (Fig. 3a, bottom panel). Moreover, when eightfold higher amounts (6-4 pmole) of bacterially purified GST–SENP1 or GST–IE1 were employed, GST–SENP1 desumoylated PML almost completely within 10 min, whereas GST–IE1 did not do so even after 80 min (Fig. 3b). The use of comparable amounts of GST–SENP1 and GST–IE1 proteins in these assays was confirmed by Coomassie blue staining (Fig. 3c). Collectively, our results demonstrated that neither IE1 protein generated from *E. coli* nor IE1 generated from insect cells exhibits intrinsic PML-desumoylating properties in vitro.

**IE1 does not require lysine 160 of PML to induce PML desumoylation in co-transfection assays**

HSV-1 ICP0 requires lysine 160 of PML for POD disruption via induction of PML degradation. We investigated whether
or not IE1-induced PML desumoylation also required specific sumoylated PML lysine residues. Two mutant PML IV proteins, containing lysine-to-arginine substitutions at aa 160 or 490, both of which are primary sumoylation sites in PML, were generated and used to co-transfect cells with IE1 and SUMO-1. Immunoblot analysis indicated that IE1 also reduced the levels of the sumoylated forms of these mutant PML IV proteins (Fig. 4a). This result showed that, unlike ICP0, IE1 does not require lysine 160 to induce PML desumoylation and is consistent with the idea that HCMV and HSV-1 may exploit different mechanisms to reduce the levels of sumoylated PML.

**Differential effects of IE1 and SUMO proteases on the formation of PML foci**

We next compared the effects of expression of IE1 and SUMO proteases such as SENP1, Axam and SuPr-1 on the integrity of PODs and the sumoylation levels of PML. First, we conducted co-transfection assays with three cellular SUMO proteases: SENP1, Axam and SuPr-1, to determine their abilities to desumoylate PML. Our results indicated that, like IE1, all three of the tested SUMO proteases efficiently desumoylated PML VI or PML IV, whereas the C466S mutant SuPr-1 used as a control did not (Fig. 4b). These results also suggested that IE1-induced PML desumoylation is not PML isoform specific.

We then conducted a comparison of the effects of ectopic expression of IE1 and SUMO proteases, including SENP1 and SuPr-1, on the formation of PML foci. When Vero cells were transfected with SENP1, we observed that the number of PML foci decreased significantly. However, these few remnant PML foci became larger, exhibited brighter PML signals and were co-localized with SENP1 (Fig. 5a–c). Consistent with a previous observation (Nefkens et al., 2003), these novel PML foci (unlike standard PODs) did not contain detectable levels of SUMO-1 by IFA (Fig. 5d–f), which suggests that the PML foci found in the SENP1-transfected cells were comprised of non-sumoylated PML proteins. However, when the cells were transfected with both SENP1 and IE1, all PML foci were completely disrupted and both SENP1 and IE1 proteins were diffusely localized (Fig. 5g–l). We also observed a similar effect of SuPr-1 expression on PML (Fig. 5m–o) and SUMO-1 signals (data not shown), except that IE1 had no effect on SuPr-1 localization (Fig. 5p–r). These results suggested that, unlike SENP1 and SuPr-1, IE1 is still able to disrupt even those PML foci that are composed of non-sumoylated forms of PML.

**IE1 interferes with the formation of SUMO-independent PML aggregates**

The capacity of IE1 to disrupt the SUMO-independent PML foci was also investigated using the mutant PML-3M protein, which harbours lysine-to-arginine substitutions at all three amino acid positions (codons 65, 160 and 490) at which sumoylation is known to occur (Kamitani et al., 1998). A similar mutant PML-3M protein has been demonstrated to retain its ability to target to the nuclear matrix and to become localized as foci in PML−/− cells (Lallemand-Breitenbach et al., 2001), thereby indicating that the sumoylation-deficient PML is nevertheless able to form at least a limited number of POD-like aggregates via

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**Fig. 3.** IE1 does not desumoylate PML in vitro. (a) A total of 2 pmol GST–PML VI protein (GST–PML VI-S; about 1.5 pmol of the unmodified form and 0.5 pmol of the sumoylated form), which was generated using an *E. coli* sumoylation system, was incubated for 1 h with increasing amounts (0.1, 0.2, 0.4 and 0.8 pmol) of GST–SENP1 or GST–IE1 generated in *E. coli*, or with GST–IE1 produced in insect cells after recombinant baculovirus infection. The reaction products were separated by 8% SDS-PAGE, followed by immunoblot (IB) analysis using anti-PML rabbit pAb. The locations of the unmodified (closed arrowheads) and sumoylated forms (open arrowheads) of GST–PML VI are shown. (b) The sumoylated GST–PML VI protein (2 pmol) was incubated with 6.4 pmol GST–SENP1 or GST–IE1 generated from *E. coli* for the indicated times. The reaction products were analysed as in (a). (c) Increasing amounts (1.6, 3.2, 6.4 and 12.8 pmol) of the GST–SENP1 and GST–IE1 proteins used in (a) and (b) were fractionated by 8% SDS-PAGE, followed by Coomassie blue staining.
protein dimerization and oligomerization. In accordance with these observations, when Vero cells were transfected with the mutant PML-3M protein, this protein also became localized in nuclear foci (Fig. 6a–c). When the cells were co-transfected with PML-3M and SENP1, these PML foci were not affected by SENP1 (Fig. 6d–f). However, when the cells were co-transfected with PML-3M and IE1, all PML foci were disrupted by IE1 and both the PML-3M and IE1 proteins became diffusely localized within the nucleus (Fig. 6g–i). This result indicated that, unlike SENP1, IE1 also interferes with the formation of PML aggregates generated in a SUMO-independent manner.

**DISCUSSION**

In the present study, we have characterized the effects of IE1 in induction of desumoylation of PML. As PML sumoylation has been determined as a prerequisite for the formation of mature PODs (Ishov et al., 1999; Lallemand-Breitenbach et al., 2001; Zhong et al., 2000a), it has been suggested that IE1 might disrupt PODs via the induction of PML desumoylation. This suggestion is bolstered by our previous demonstration that IE1 is responsible for the induction of PML desumoylation in virus-infected cells and that the ability of IE1 mutants to desumoylate PML is, indeed, associated with their ability to disrupt PODs (Lee et al., 2004). However, the direct investigation of this IE1 activity has been impaired by an inability to purify the mature PODs or the sumoylated forms of PML, primarily because of the activity of desumoylating enzymes. In this regard, the use of an *E. coli* sumoylation system for the purification of sumoylated forms of PML allowed us to establish ideal experimental conditions in which the activity of IE1 against sumoylated forms of PML in vitro could be evaluated. In this study, we have shown that IE1 exhibits neither an inhibitory effect on sumoylation of PML nor desumoylating activity against PML in vitro and that IE1 can disrupt even SUMO-independent PML aggregates.

These findings provided useful information with regard to models to explain the ability of IE1 to induce POD disruption and PML desumoylation. Previously, we proposed several mechanisms that might be responsible for IE1-induced PML desumoylation (Lee et al., 2004). First, IE1 may prevent the formation of sumoylated forms of PML, either by competing with PML for SUMO or via the direct inhibition of PML sumoylation by conjugation enzymes. In particular, the fact that IE1 is also a SUMO substrate (Nevels et al., 2004; Sadanari et al., 2005; Spengler et al., 2002; Xu et al., 2001) appeared to support the SUMO competition hypothesis. However, this scenario now seems unlikely for HCMV. Previously, the characterization of two mutant IE1 proteins indicated that a sumoylation-deficient mutant IE1(K450R) protein still retained its ability to disrupt PODs (and desumoylate PML) (Lee et al., 2004; Nevels et al., 2004; Spengler et al., 2002) and that a deleted IE1(D290–320) protein could be sumoylated as efficiently as the wild-type protein, but did not retain the ability to induce PML desumoylation (Lee et al., 2004). The data presented in this study demonstrate conclusively that purified IE1 does not interfere with PML desumoylation in *in vitro* sumoylation reactions.
IE1 has also been suggested to actively promote the removal of SUMO from PML, either via its own direct intrinsic SUMO protease activity or by the ability to recruit SUMO proteases to PODs indirectly, thereby facilitating PML desumoylation. Importantly, the use of the recently developed *E. coli* sumoylation system enabled us to generate target sumoylated proteins without contamination from SUMO protease activity, which is absent in *E. coli*. By using the purified sumoylated substrates, we were able to prevent contamination from excess SUMO modification components, which can be problematic in assays for desumoylating activity. Using this assay system, we determined that

Fig. 5. Differential effects of the IE1 and SUMO proteases on the formation of PML foci. Vero cells were transfected with plasmids for HA–SENP1 (a–f) or HA–SuPr-1 (m–o) or were co-transfected with HA–SENP1 and IE1 (g–l) or with HA–SuPr-1 and IE1 (p–r). After 48 h, cells were fixed with paraformaldehyde and double-labelled IFA was carried out, as indicated. HA–SENP1 or HA–SuPr-1 was detected with fluorescein isothiocyanate-labelled anti-HA rat mAb (green). IE1 and endogenous PML or SUMO-1 were detected using anti-IE1 mouse mAb, anti-PML or anti-SUMO-1 rabbit pAbs, respectively, and rhodamine red X-coupled anti-mouse or anti-rabbit IgG (red). Two side-by-side panels of single-labelled IFA images and a third panel with a merged image are shown.
bacterially purified SENP1 efficiently desumoylates PML, whereas IE1, whether generated from *E. coli* or from insect cells, causes no PML desumoylation. As the PML protein used in this assay was not produced in eukaryotic cells, it cannot be excluded that modification of PML, such as phosphorylation, contributes to the induction of desumoylation by IE1. However, our results constitute clear evidence that IE1 itself does not possess a SUMO protease activity against bacterially produced PML in vitro.

The notion that IE1 may induce PML desumoylation indirectly via the recruitment of SUMO proteases to PODs originates with the observation that HSV-1 ICP0 co-localizes with SENP1 in HSV-1-infected cells and promotes the recruitment of SENP1 to PODs in co-transfection experiments, although this study did not address whether or not ICP0 interacted with SENP1 (Bailey & O’Hare, 2002). We were not able to demonstrate an interaction between IE1 and SUMO proteases, such as SENP1, Axam and SuPr-1, in either in vitro- or in vivo-binding assays (data not shown). Nevertheless, it remains possible that IE1 may interact with other SUMO proteases or that these interactions are too transient to be detected. However, several lines of experimental evidence now strongly suggest that HCMV IE1 employs a different mechanism to disrupt PODs than HSV-1 ICP0. Unlike the reaction observed with HSV-1 ICP0, the disruption of PODs in HCMV-infected cells occurs in a proteasome-independent manner (Xu et al., 2001). IE1 effected a reduction of the sumoylated forms of PML, but did not alter the levels of unmodified PML, whereas ICP0 caused a loss of both the sumoylated and the unmodified forms of PML (Boutell et al., 2003; Lee et al., 2004). Furthermore, as was demonstrated in this study, unlike ICP0-induced PML degradation (Boutell et al., 2003), IE1-induced PML desumoylation does not require PML lysine 160.

Finally, it is also possible that IE1 simply induces the disaggregation of the PODs (presumably through physical binding to PML), with subsequent exposure of sumoylated PML to cellular SUMO proteases. This idea is generally supported by the data in the present study. In our transfection assays, IE1 disrupted both the remaining endogenous SUMO-independent PML foci generated after

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**Fig. 6.** IE1 disrupts the PML foci generated by the sumoylation-deficient mutant PML (K65/160/490R) protein. Vero cells were transfected with a plasmid encoding FLAG–PML VI-3M, which harbours lysine-to-arginine substitutions at 65, 160 and 490 (a–c) or co-transfected with plasmids encoding FLAG–PML VI-3M and HA–SENP1 (d–f) or FLAG–PML VI-3M and HA–IE1 (g–i). At 48 h, the cells were fixed via the parafomaldehyde procedure, followed by double-labelled IFA. FLAG–PML VI-3M was detected by anti-FLAG mouse mAb and rhodamine red X-coupled anti-mouse IgG (red). HA–SENP1 and HA–IE1 were detected by anti-HA rat mAb labelled with fluorescein isothiocyanate (green). To stain the cell nucleus, we used mounting solution containing 4’,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). Two side-by-side panels of single-labelled IFA images and a third panel with a merged image are shown.

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transfection of SUMO proteases and the exogenous PML foci generated by transfection of the sumoylation-deficient mutant PML protein. This clearly shows that IE1 can interfere with the formation of PML aggregates, even in a SUMO-independent manner. We previously demonstrated that IE1 interacts with the N-terminal region of PML harbouring the RING-finger domain (Ahn et al., 1998). Considering that the RING-finger domain of PML is involved with both protein oligomerization and the formation of supramolecular structures (Kentsis et al., 2002), we propose that IE1 binding to PML interferes with both PML oligomerization and the formation of supramolecular structures. This activity of IE1 may play an important role in triggering the disassembly of PODs in a SUMO-independent manner and in inducing the desumoylation of PML by SUMO proteases.

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