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PIAS1 enhances SUMO-1 modification and the transactivation activity of the major immediate–early IE2 protein of human cytomegalovirus

Jang-Mi Lee[a], Hee-Jung Kang[a], Hye-Ra Lee[a,b], Cheol Yong Choi[c], Won-Jong Jang[d], Jin-Hyun Ahn[a,*]

[a] *Department of Molecular Cell Biology, Sungkyunkwan University, School of Medicine, Samsung Biomedical Research Institute, 300 Chunchundong, Jangangu, Kyonggido 440-746, South Korea*
[b] *School of Life Sciences and Biotechnology, Korea University, Anamdong 5-1, Sungbukku, Seoul 136-701, South Korea*
[c] *Division of Biology, Sungkyunkwan University, 300 Chunchundong, Jangangu, Kyonggido 440-746, South Korea*
[d] *Department of Microbiology, Kunkuk University School of Medicine, Chungju, Chungbuk 380-701, South Korea*

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Abstract The protein inhibitor of activated STAT1 (PIAS1), known to be a small ubiquitin-like modifier (SUMO) E3 ligase, was found to interact with the human cytomegalovirus IE2 protein. We found that the sumoylation of IE2 was markedly enhanced by wild-type PIAS1 but not by a mutant containing a Cys to Ser substitution at position 351 (C351S) within the RING finger-like domain. In target reporter gene assays, wild-type PIAS1, but not the C351S mutant, enhanced the IE2-mediated transactivations of viral polymerase promoter and cellular cyclin E promoter and this augmentation required the intact sumoylation sites of IE2. Our results suggest that PIAS1 acts as a SUMO E3 ligase toward IE2 and that it may regulate the transactivation function of IE2. To our knowledge, IE2 is the first viral target found to be regulated by a SUMO E3 ligase.

Key words: Human cytomegalovirus; Immediate–early protein; Protein inhibitor of activated STAT1; Small ubiquitin-like modifier-1

1. Introduction

The human cytomegalovirus (HCMV) can cause severe disease complications and pathogenesis on infection of newborns or immunocompromised individuals, whereas the infection of immunocompetent individuals is usually asymptomatic. During the lytic-cycle infection of HCMV, a cascade of immediate–early, delayed–early, and late gene expression occurs. The major immediate–early (MIE) proteins, 72-kDa IE1 (or IE72) and 86-kDa IE2 (or IE86), are nuclear phosphoproteins that are synthesized from the MIE locus by differential splicing, and are thought to act in concert to govern all downstream gene expression [1,2].

The IE2 protein acts as a powerful non-specific transactivator of both viral and cellular genes as well as a repressor of its own MIE promoter through direct DNA binding to the MIE *cis*-repression signal near the 5′ cap site ([3] and references therein). IE2 interacts with components of the basal transcription complex including TBP, TFIIB and TFIID, and with TBP-associated factors such as TAFII110 and TAFII130, suggesting that IE2 has a TAF-like function [4]. IE2 also interacts with numerous transcription factors such as, Ap-1, Egr-1, CREB, CBP, SP1-1/Pu.1, Tef-1, and P/CAF as well as cell cycle modulators such as RB and p53 ([3] and references therein). A recent study using a recombinant viral genome confirmed that IE2 is essential for all subsequent lytic-cycle viral gene expression in cell culture [5]. IE2 has been shown to target adjacent to PML-associated nuclear bodies, which are also known as PML oncogenic domains (PODs) or as nuclear domain 10, to form immediate transcription domains, and to accumulate in viral DNA replication compartments during infection [6,7].

IE2 is covalently modified by a small ubiquitin-like modifier (SUMO), and sumoylation of IE2 appears to be required for the transactivation function of IE2 [8,9]. Recently, analysis of amino acid variations of IE2 from different HCMV strains suggested a correlation between transactivation activity of IE2 and its sumoylation level [10]. However, the exact role of IE2 sumoylation in virus infection is not understood. SUMO is covalently conjugated to the lysine residue of target protein through a pathway distinct from but analogous to the ubiquitin conjugation system [11–14]. SUMO is activated by E1 enzyme (a heterodimer of SAE1/SAE2), then transferred to an E2 conjugation enzyme (Ubc9), and attached to the ε-amino group of specific lysine residues of protein substrates in an either E3 ligase-dependent or -independent manner. To date, three different types of E3 ligase (members of the PIAS family, RanBP2, and Pc2) have been reported in mammalian cells [15–18].

The protein inhibitor of activated STAT1 (PIAS1) was originally identified as a STAT1 binding partner, which inhibits the transactivational activity of STAT1 [19], and which is almost identical to the Gu/RNA helicase II-binding protein [20]. PIAS1 also acts as a cofactor of nuclear hormone receptors, such as the androgen and glucocorticoid receptors [21–24]. Recently, PIAS1 was shown to enhance the sumoylation of androgen receptor [25]. In mammals, five members of the PIAS family (PIAS1, PIAS3, PIASxα, PIASxβ and PIASy) have been identified and all members of the PIAS family and a yeast homolog, Siz1, have been shown to have SUMO ligase activity [26,27]. In the present study, we identified PIAS1 as a cellular interacting partner of HCMV IE2 and

*Corresponding author. Fax: (82)-31-299 6435.
E-mail address: jahn@med.skku.ac.kr (J.-H. Ahn).

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evaluated their interaction. Our results suggest that PIAS1 has SUMO E3 ligase activity toward IE2 and that it may regulate the transactivation function of IE2.

2. Materials and methods

2.1. Cell culture, transfection, and virus infection

Vero and 293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. U373-SUMO-1 cells, which express SUMO-1 [28], was maintained in the same medium in the presence of 0.4 mg per ml of G418. For immunoblot analysis, 293T cells were seeded into six-well plates and DNA transfection was carried out using the *N*,*N*-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid-buffered saline (BBS) version of the calcium phosphate procedure. Vero cells were seeded into two-well slide chambers [for immunofluorescence assay (IFA)] or 12-well plates (for luciferase assays) and DNA mixtures were introduced into cells with FuGene 6 reagents (Roche). U373-SUMO-1 cells were transfected with the BBS reagent as above in six-well plates and super-infected with a recombinant virus, HCMV(Pol-LUC), containing the extragenic Pol-LUC fusion reporter gene as previously described [29].

2.2. Plasmid construction

All IE2 expression vectors in this study were generated with the IE2 cDNA derived from the Towne strain. Yeast plasmids expressing the GAL4-DB domain (pAS1-CYH2), GAL4-DB/IE2(87–542) (pWJ1), GAL4-DB/IE2(87–542, K175/180R) (pJHA339), and GAL4-DB/IE2(290–542) (pCJC441) were previously described [9,30]. Plasmids expressing GAL4-DB/IE2(S203A) (pYX141) and GAL4-DB/IE2(K175/180R, S203A) (pYX142) were generated in a pWJ1 background using the Stratagene QuickChange site-directed mutagenesis protocol. Plasmids expressing GAL4-DB/IE2(313–542) (pJHA211) and GAL4-DB/IE2(346–542) (pJHA225) were generated by subcloning the PCR fragment into pAS1-CYH2, and plasmids expressing the GAL4-A domain (pACTII) and GAL4-A/SUMO-1 (pJHA313) were previously described [9]. Plasmids expressing GAL4-A/PIAS1(wt) (pSAN19) were generated by placing intact mouse PIAS1 cDNA into pACTII, and plasmids expressing GST-IE2(290–542) (pHJK10), GST-IE2(313–542) (pHJK11), and GST-IE2(346–542) (pHJK13) were generated on pGEX-3X-derived vector, using Gateway technology (Invitrogen). pGST-SUMO-1 was a gift from Masahiro Fujimuro (Johns Hopkins University).

pSG5-driven expression plasmids for IE2(wt) (pJHA124), IE2(K175/180R) (pYX104), and flag-SUMO-1 (pJHA312) have been described previously [9]. Plasmids expressing flag-PIAS1(wt) (pWJ17) and HA-IE2 (pDJK171) were generated in a pSG5 background. Plasmids expressing the SRT-tagged PIAS1(wt) (pSAN22) and PIAS1(C351S) (pDJK158) were also generated using Gateway technology on pSG5-SRT which contains an SRT tag [31]. Reporter plasmids pLA12 containing the HCMV UL54(Pol)-luciferase reporter gene and pE(-207)-LUC containing the cyclin E-luciferase reporter gene were previously described [9].

2.3. Yeast two-hybrid interaction assays

The yeast strain Y190 was used as the host for rapid assays for lacZ expression using a X-Gal filter assay, and for the quantitation of the interaction between IE2 and PIAS1 using a β-galactosidase assay. Both assays have been described previously [30].

2.4. Antibodies, IFA, and immunoblot analysis

Mouse monoclonal antibody (MAb) M2 against flag epitope was purchased from Sigma. Mouse MAb 8131, which detects epitopes present in both IE1 and IE2 (exons-2 and -3), was purchased from Chemicon (Temecula, CA, USA). Anti-HA MAb 3F10 either conjugated with peroxidase or labeled with fluorescein and anti-myc mouse MAb 9E10 were purchased from Roche. Rabbit antipeptide polyclonal Ab (PAb) referred to as PML(C) was as described previously [29]. Mouse MAb against SRT epitope [31] was purchased from Ahram Biosystems Inc. (Seoul, Korea).

For IFA, cells were fixed with 1% paraformaldehyde and permeabilized with 0.2% Triton X-100. All subsequent procedures were as

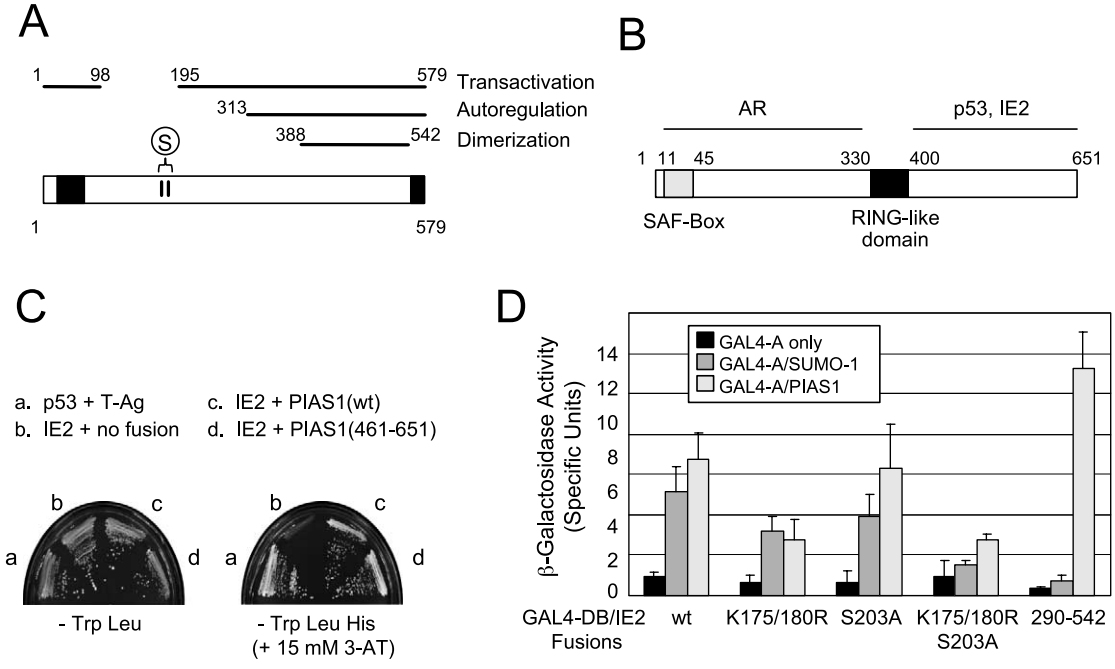

Fig. 1. Interaction of IE2 with PIAS1 in yeast. A: A diagram showing the domains of IE2 required for transactivation, autoregulation, and dimerization. Two transactivation domains and two major sumoylation sites (Lys residues at positions 175 and 180) are denoted as black boxes and encircled S, respectively. The numbers are protein amino acid positions. B: Structure of PIAS1. SAF (scaffold-associated factor)-box and RING finger-like domain of the protein are denoted as gray and black boxes, respectively. Binding regions for the androgen receptor, p53 and IE2(in this work) are also indicated. C: Yeast transformants containing the two plasmids encoding GAL4-DB and GAL4-A domain fusions were streaked on a growth plate lacking Trp and Leu or on a plate lacking Trp, Leu, and His but containing 15 mM of 3-amino triazole (3-AT), an inhibitor of the *HIS3* gene product, and grown at 30°C. IE2(87–542) lacking the two activation domains was used in this experiment. D: Binding of wild-type or mutant IE2 with SUMO-1 or PIAS1. Yeast transformants containing the indicated GAL4-DB/IE2 fusions and either GAL4-A only, GAL4-A/SUMO-1, or GAL4-A/PIAS1 were grown and the β-galactosidase activities of the transformants were measured as described in Section 2.

previously described [9]. Slides were examined and photographed under a Zeiss Axiopgoto2 microscope.

For immunoblot analysis, DNA-transfected cells were washed with phosphate-buffered saline and total extracts were prepared by boiling the cell pellets in sodium dodecyl sulfate (SDS) loading buffer. Equal amounts of the clarified cell extracts were separated on a SDS–8% polyacrylamide gel, and subjected to the standard enhanced chemiluminescence system procedure (Amersham).

2.5. Luciferase reporter assay

For luciferase reporter assays, transfected cells were collected and lysed using three freeze–thaw steps in 200 μl of 0.25 M Tris–HCl (pH 7.9) plus 1 mM dithiothreitol. Subsequent procedures were as previously described [9]. A TD-20/20 luminometer (Turner Designs) was used for the 10-s assay of the photons produced (measured in relative light units).

3. Results and discussion

3.1. Interaction of PIAS1 with HCMV IE2

In an attempt to identify cellular proteins interacting with HCMV IE2 protein, we screened a cDNA library prepared from human B lymphocyte using the yeast two-hybrid system [9]. Although HCMV does not infect B lymphocyte, this library was successfully used to isolate IE2-binding cellular partners [8,9]. A region of the IE2 protein spanning amino acid position 87–542, lacking both the N- and C-terminal transactivation domains was used as bait (Fig. 1A). One of the positive clones contained the C-terminal domain of PIAS1 (amino acids 416–651) (Fig. 1B). When the full length PIAS1 protein (amino acids 1–651) was tested for IE2 binding, it was found to bind to IE2 with the same strength as the C-terminal domain (461–651) of PIAS1, as judged by the ability of yeast cells to grow in selection plates (Fig. 1C). The interaction between p53 and SV40 large T-Ag was used as a positive control. This result suggests that the C-terminal domain of PIAS1 (from 461 to 651) participates in IE2 binding.

IE2 was shown to bind to both SUMO-1 and Ubc9 by yeast two-hybrid interaction assays, and to be covalently modified by SUMO-1 [8,9]. Two major sumoylation sites of IE2 were mapped to Lys residues at amino acid positions 175 and 180, and IE2 was also shown to bind directly to SUMO-1 moieties in in vitro binding assays. The interaction of IE2 with Ubc9 in yeast was mainly indirect because the sumoylation-defective mutant IE2(K175/180R) containing Lys to Arg substitutions at residues 175 and 180 almost lost its ability to bind to Ubc9 [9]. To investigate whether sumoylation of IE2 is also involved in PIAS1 binding, two-hybrid interaction assays of PIAS1 with the sumoylation-defective mutant IE2 proteins were conducted (Fig. 1D). Interactions of SUMO-1 with the mutant IE2 proteins were used as controls. We found that the interaction of PIAS1 with IE2(K175/180R) was reduced to 50% of wild-type IE2, suggesting that the sumoylation of IE2 is required for efficient PIAS1 binding. A motif that is involved in direct SUMO contact lies between positions 200 and 208 of IE2 [9]. Substitution mutations of Ser at 203 to Ala in both intact IE2 and IE2(K175/180R) backgrounds did not affect the interaction with PIAS1, though they slightly lost the ability to bind to SUMO-1. We also found that, unlike SUMO-1, PIAS1 interacted with the C-terminal domain of IE2(290–542), which is involved in both self-interaction and in interactions with many cellular proteins (Fig. 1D).

We further investigated whether the smaller C-terminal regions of IE2 bind to PIAS1. As was found in yeast interaction assays, both IE2(313–542) and IE2(346–542) bound to PIAS1 at around the same strength as they bound to IE2(290–542) (Fig. 2A). We confirmed the interaction of PIAS1 with the C-terminal domains of IE2 by using in vitro binding assays with glutathione-*S*-transferase fusion proteins. When the GST fusion proteins containing IE2(290–542), IE2(313–542) or IE2(346–542) were purified from *Escherichia coli* and bound to in vitro [^{35}S]-labeled PIAS1 protein, all GST-IE2 proteins bound to PIAS1 with a strength similar to the interaction of

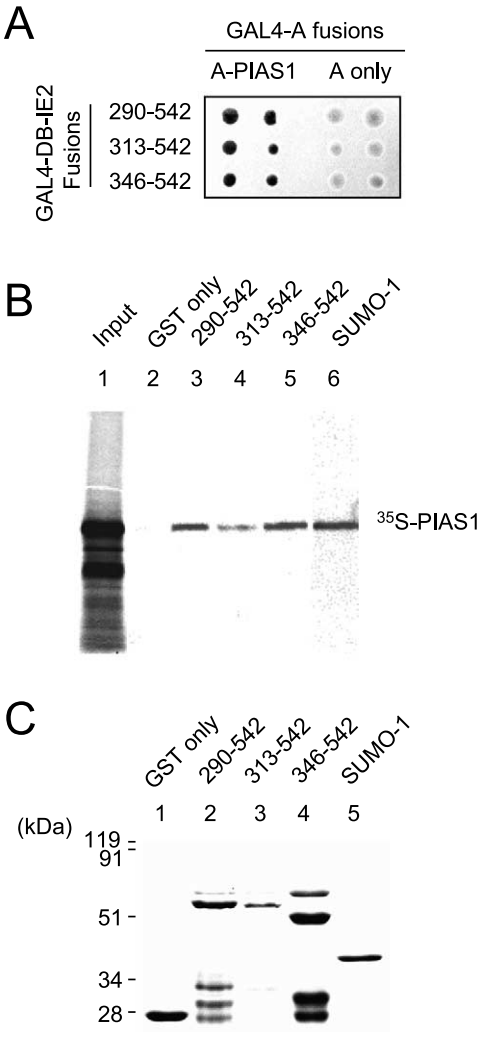

Fig. 2. Interaction of the C-terminal domains of IE2 with PIAS1. A: Interaction of the C-terminal domains of IE2 with PIAS1 in yeast two-hybrid assays. β-Galactosidase production by yeast transformants containing plasmids encoding GAL4-DB/IE2 fusion, and GAL4-A/PIAS1 or GAL4-A alone was determined by using X-gal filter assays, as described in Section 2. Two independent yeast transformants were assayed for each combination of interaction. B: In vitro binding assay of PIAS1 with GST fusion proteins. The GST or GST fusion proteins immobilized to glutathione-Sepharose beads were incubated with [^{35}S]methionine-labeled full length PIAS1. One-fifth of the labeled PIAS1 proteins used in each binding reaction were loaded as an input control. The bound proteins were fractionated and visualized by autoradiography. C: The relative sizes and amounts of the GST or of GST fusion proteins used in the assay. One-tenth of the GST or GST fusion proteins used in B were fractionated and stained with Coomassie blue. Note that the less efficient pull-down of ^{35}S-PIAS1 by GST-IE2(312–542) was due to the use of lower amount of GST-IE2(312–542) than other GST-IE2 fusion proteins.

GST-SUMO-1 with PIAS1 (used as a control) (Fig. 2B,C). Although we could not test whether the full length IE2 also binds to PIAS1 in this assay due to the low expression level of the GST-IE2(full length) fusion protein in *E. coli*, our results suggest that IE2 may bind to PIAS1 through the C-terminal domain of IE2, which is well conserved among equivalent proteins of betaherpesviruses [32].

3.2. PIAS1 is colocalized with IE2

The nuclear dot-like distribution pattern of exogenous PIAS1 in U2OS cell line has been previously reported [33]. We also found that flag-PIAS1 was distributed as a mixture of nuclear diffuse and punctate forms in Vero cells (Fig. 3a,d). In double-labeled IFA, the punctate forms of PIAS1 were localized adjacent to endogenous PML in PODs, suggesting that, like IE2 [6,7], a subset of PIAS1 is localized in the vicinity of PODs (Fig. 3a–f).

We further investigated whether PIAS1 colocalizes with IE2 in cotransfected cells. In double-labeled IFA of Vero cells cotransfected with flag-PIAS1 and IE2, both proteins were efficiently colocalized in nuclear punctate forms (Fig. 3g–l). Although we could not detect the colocalization of IE2 with endogenous PIAS1 in virus-infected cells due to the lack of antibody that detects endogenous PIAS1 in permissive HF cells, this IFA result suggests that IE2 may be associated with PIAS1 within the nucleus.

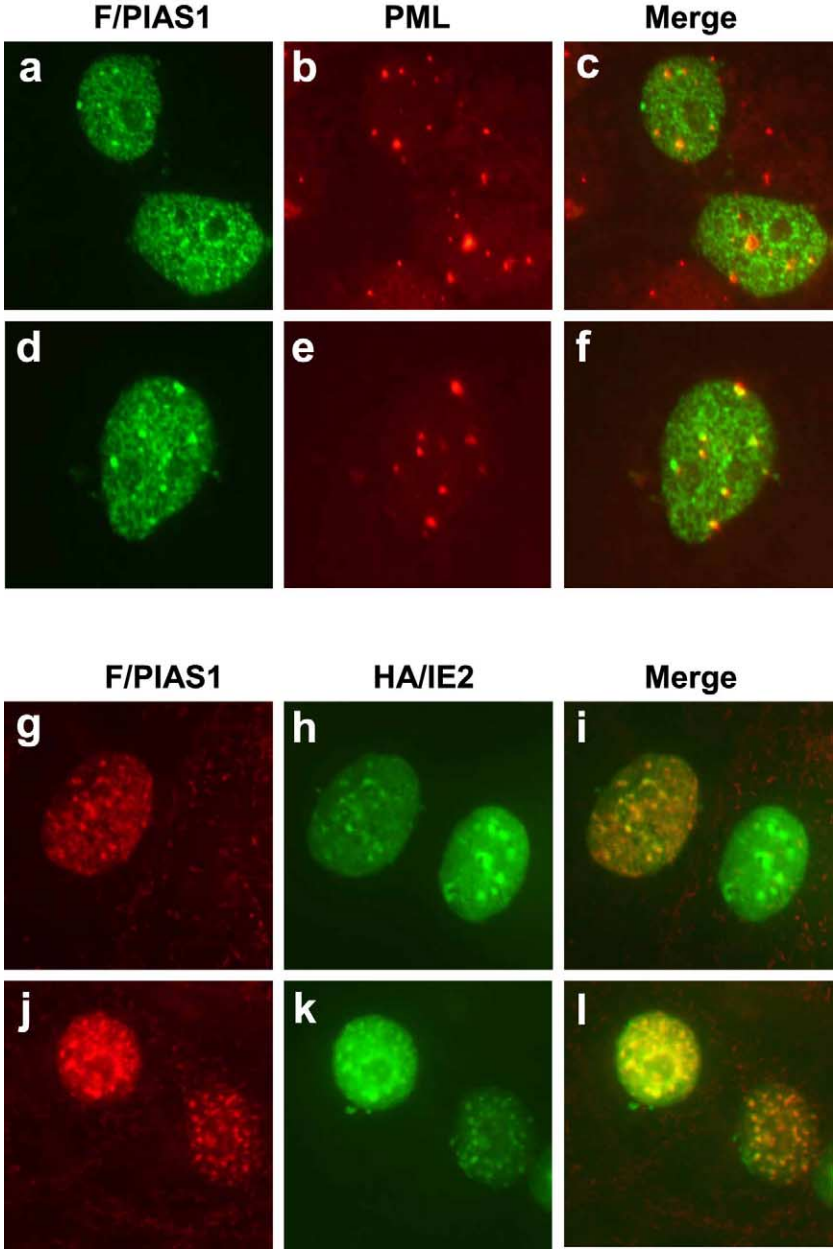

Fig. 3. Colocalization of PIAS1 with IE2. Vero cells were transfected with a plasmid encoding flag-PIAS1 alone (a–f) or cotransfected with plasmids encoding flag-PIAS1 and HA-IE2 (g–l). At 36 h after transfection, cells were fixed with formaldehyde and doubl-labeled IFA was carried out with mouse MAb M2 for flag-PIAS1 and either rabbit PAb PML(C) for endogenous PML (a–f) or FITC-labeled rat MAb (3F10) against HA-IE2 (g–l). Two different images were shown for each IFA. FITC-labeled or rhodamine-red X-conjugated anti-mouse IgG and rhodamine-red X-conjugated anti-rabbit IgG were used for visualization.

3.3. PIAS1 enhances the sumoylation of IE2 in cotransfected cells

Because members of the PIAS protein family are known to act as SUMO E3 ligases, we investigated whether PIAS1 enhances the sumoylation of IE2. In a preliminary experiment, 293T cells were transfected with either wild-type IE2 or sumoylation-defective IE2(K175/180R) alone, or cotransfected together with flag-SUMO-1, and immunoblot analysis of total extracts was carried out using mouse MAb8131 against IE2 (Fig. 4A). The results showed that the expression of wild-type IE2 alone produced a slowly migrating single SUMO-modified IE2 form as well as the normal 86-kDa form and, when coexpressed with SUMO-1, the levels of the single or double SUMO-modified forms of wild-type IE2 were markedly increased. However, as expected IE2(K175/180R) did not produce any sumoylated forms, confirming that Lys residues at positions 175 and 180 are the only sumoylation sites in DNA-transfected 293T cells.

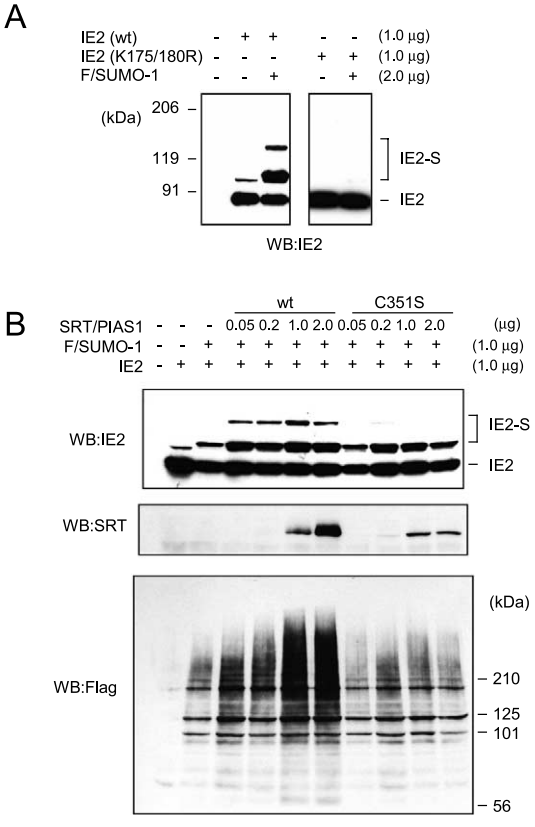

Fig. 4. Effect of PIAS1 on IE2 sumoylation in cotransfection assays. A: Sumoylation of IE2 in transiently DNA-cotransfected cells. 293T cells were cotransfected with plasmids encoding wild-type IE2, IE2(K175/180R), or flag-SUMO-1 as indicated. At 48 h after transfection extracts were prepared as described in Section 2. Equal amounts of cell extracts were separated on a SDS–8% polyacrylamide gel and immunoblot analysis was performed with MAb 8131 for IE2. B: Effect of PIAS1 on the sumoylation levels of IE2 and cellular proteins. 293T cells were cotransfected with plasmids encoding SRT-tagged wild-type or C351S mutant PIAS1, flag-SUMO-1, or IE2 as indicated. Forty-eight hours after transfection extracts were prepared and immunoblot analysis was carried out as described in A with MAb 8131 for IE2 (top panel). The same blot was stripped and the second and third immunoblot analyses were carried out with MAb against SRT-PIAS1 (middle panel) and with MAb M2 for flag-SUMO-1-conjugated proteins (bottom panel), respectively.

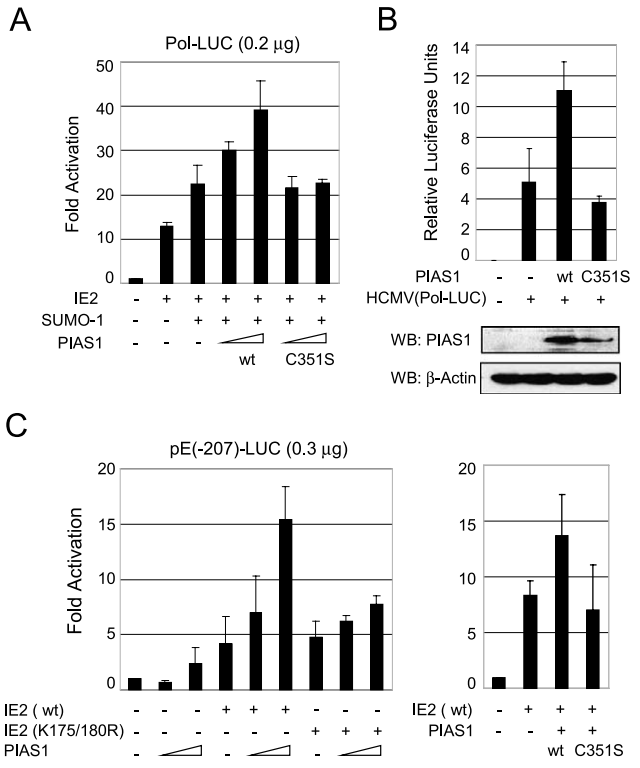

Fig. 5. Effects of PIAS1 on IE2-mediated transactivation. A: Effects of wild-type or C351S mutant PIAS1 proteins on the IE2-mediated transactivation of the HCMV polymerase promoter. Vero cells were cotransfected with 0.2 μg of reporter plasmid (pPol-LUC) containing a HCMV UL54(Pol)-luciferase reporter gene and 0.2 μg of plasmid encoding IE2, 0.2 μg of plasmid encoding SUMO-1, and 0.02 or 1.0 μg of plasmid encoding either wild-type or the C351S mutant PIAS1 proteins. All samples for reporter assays were made up to the same total amount of DNA with empty vectors. At 30 h after transfection, total cell extracts were prepared and assayed for luciferase activity. Luciferase activities are indicated as fold activation over the basal level of each reporter gene. The results shown are the mean values and standard errors of three independent experiments. B: U373-SUMO-1 cells were initially transfected with 5 μg of empty vector or plasmid encoding either wild-type or C351S mutant PIAS1. At 24 h after transfection, the cells were mock-infected or super-infected with recombinant HCMV(Pol-Luc) at an M.O.I. of 1.0. After further incubation for 24 h, cells were harvested for the assay of luciferase activity. The results shown are the average of two independent experiments (top). To see the expression levels of transfected PIAS1 proteins in the cell extracts assay, equal amounts of the same cell extracts from an experiment were separated on a SDS–8% polyacrylamide gel and immunoblot analysis was performed with anti-SRT antibody. Loading of the equal amounts of proteins was also verified by immunoblot analysis with anti-β-actin antibody (bottom). C: Effect of IE2 sumoylation sites on the cooperative augmentation of the IE2-mediated transactivation of the cellular cyclin E promoter by PIAS1. Vero cells were cotransfected with 0.3 μg of reporter plasmid, pE(−207)-LUC, containing a cellular cyclin E-luciferase reporter gene and 0.05 or 0.2 μg of plasmid encoding PIAS1 and 0.05 μg of plasmid encoding wild-type or K175/180R mutant IE2 proteins (left), or with 0.3 μg of reporter plasmid and 0.05 μg of IE2 and 0.2 μg of wild-type or C351S mutant PIAS1 (right). At 30 h after transfection, total cell extracts were prepared and assayed for luciferase activity as described in A.

To investigate effect of PIAS1 on the sumoylation of IE2, we carried out similar cotransfection assays in the presence of SRT-tagged PIAS1. When IE2 was coexpressed with both flag-SUMO-1 and SRT-PIAS1, the sumoylation level of IE2 was markedly increased by PIAS1 (Fig. 4B, upper panel). To investigate whether the positive effect of PIAS1 on the sumoy-

lation of IE2 is indeed due to its E3 ligase activity, we used a mutant PIAS1(C351S) that contained a Cys to Ser substitution at position 351, which disrupts the RING finger-like domain of PIAS1 and lacks SUMO E3 ligase activity [16]. The results showed that PIAS1(C351S) failed to enhance the sumoylation of IE2, thus demonstrating that the sumoylation level of IE2 is enhanced by the E3 ligase activity of PIAS1. The expressions of both wild-type and mutant PIAS1 were confirmed by immunoblot analysis of the same blot with anti-SRT antibody, although PIAS1(C351S) appeared to be less stable than the wild-type protein (Fig. 4B, middle panel). The general SUMO E3 ligase activity of the wild-type PIAS1, but not of PIAS(C351S), on cellular proteins were confirmed by immunoblotting of the same blot with anti-flag antibody, which detects all cellular proteins modified by ectopically expressed flag-SUMO-1 (Fig. 4B, bottom panel).

3.4. Effect of PIAS1 on IE2-mediated transactivation

To investigate whether the interaction of PIAS1 with IE2 affects the transactivation function of IE2, we carried out target reporter gene assays. The transcactivation activity of IE2 was well demonstrated in Vero cells [9,34]. Vero cells were cotransfected with a target reporter plasmid expressing luciferase under the control of the HCMV UL54 (polymerase) promoter (Pol-LUC) and effector plasmids expressing IE2, SUMO-1 and either wild-type PIAS1 or the C351S mutant PIAS1 (Fig. 5A). IE2 alone activated the Pol promoter by 13-fold, and the coexpression of IE2 with SUMO-1 enhanced the activation of Pol promoter by up to 23-fold. When IE2 was coexpressed with both SUMO1 and PIAS1, IE2-mediated transactivation was increased by up to 40-fold by wild-type PIAS1, but not by the C351S mutant, in a dose-dependent manner (Fig. 5A). This result suggests that PIAS1 may enhance the transactivation activity of IE2 by increasing the sumoylation level of IE2.

To address whether PIAS1 enhances the IE2-mediated transactivation of viral Pol promoter in virus-infected cells, the permissive U373 cells, which constitutively express SUMO-1 (U373-SUMO-1) [28], were initially transfected with wild-type or C351S mutant PIAS1, and subsequently super-infected with a recombinant HCMV(Pol-LUC), containing the extragenic Pol-LUC reporter gene [29]. The result of luciferase assay showed that wild-type PIAS1, but not C351S mutant, enhanced the activation of Pol promoter by two-fold in HCMV-infected cells (Fig. 5B, top). Although immunoblot analysis of the same extracts with anti-SRT antibody showed lower expression level of C351S mutant than the wild-type protein (Fig. 5B, bottom), the Pol-LUC activity in cells that received C351S protein was not increased at all compared to control cells that did not receive any PIAS1 protein. This indicates that the lack of positive effect by C351S mutant is not due to its low expression level. Because PIAS1 did not activate the Pol promoter at all in assays with Pol-LUC reporter gene in cotransfected U373 cells (data not shown), this result suggests that PIAS1 may enhance the IE2-mediated transactivation of Pol promoter in virus-infected cells.

IE2 has been shown to directly bind to and activate the cyclin E promoter in transient reporter assays [35]. We previously showed that the coexpression of both SUMO and Ubc9 enhances the IE2-mediated activation of the cyclin E promoter and that this process requires the sumoylation sites of IE2 [9]. To further investigate whether PIAS1 has a similar effect, target reporter gene assays were carried out with a plasmid expressing luciferase under the control of the cyclin E promoter. The results obtained showed that PIAS1 enhanced the IE2-mediated transactivation of the cyclin E promoter in a dose-dependent manner and this augmentation requires the intact sumoylation sites of IE2, and that the C351S mutant PIAS1 did not affect the activation of cyclin E promoter by IE2 (Fig. 5C). These results suggest that PIAS1 regulates the transcriptional activation function of IE2 through sumoylation.

In domain mapping experiments, two major sumoylation sites (Lys residues) of IE2 at position 175 and 180 were required intact for efficient PIAS1 binding, although the C-terminal domain that lacks these sumoylation sites supported a binding to PIAS1 in vitro. This result suggests that both indirect binding through SUMO moieties conjugated to IE2 and direct binding through the C-terminal domain may contribute to IE2:PIAS1 interaction. The former is consistent with the finding that PIAS1 has an ability to tether other sumoylated proteins in a non-covalent fashion [27]. In addition to the apparent SUMO E3 ligase activity toward IE2, this SUMO tethering activity of PIAS1 may regulate the intracellular localization or stability of IE2. Indeed, our results show that both IE2 and PIAS1 colocalized in punctate forms and that the steady state levels of IE2 appear to be increased in the presence of PIAS1 in cotransfected cells.

Our study shows that PIAS enhanced the IE2-mediated transactivation of both the viral polymerase and the cellular cyclin E promoters in a SUMO E3 ligase-dependent manner. Although one report questioned the role of IE2 on cyclin E level increases in HCMV-infected cells [36], several reports have demonstrated that IE2 transfection upregulates the cyclin E promoter, cyclin E mRNA levels, and cyclin E-associated kinase activity [35,37,38]. The activation of cyclin E appears to be a viral strategy to force cells to enter the S-phase, which facilitates a more favorable environment for HCMV replication [39]. Our results suggest that the enhanced sumoylation of IE2 by PIAS1 may contribute in part to the dysregulation of the cell cycle to facilitate the progress of HCMV infection. Given that IE2 was found to be very efficiently sumoylated in an in vitro system containing both E1 and E2, but not E3 [9], the strong interaction between IE2 and PIAS1 may reflect more than binding between an enzyme and a substrate. However, whether IE2 regulates the function of PIAS1 as a SUMO E3 ligase or as a transcriptional coactivator, remains to be further investigated.

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