Regulation of Vaccinia Virus E3 Protein by Small Ubiquitin-Like Modifier Proteins

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The vaccinia virus (VACV) E3L gene encodes two proteins of about 20 and 25 kDa (referred to as E3) that are expressed early in infection (71) and are present in both the nucleus and cytoplasm of infected and transfected cells. E3 is a double-stranded RNA (dsRNA)-binding protein that inhibits the activation of interferon (IFN)-induced double-stranded RNA-dependent protein kinase (PKR) (14) and acts as an inhibitor of the IFN-induced 2-5A-synthetase enzyme (52). The E3L gene has been described as being necessary for the VACV IFN-resistant phenotype in cultured cells (3, 14), host resistance to pathogens in transgenic mice (17), and the inhibition of apoptosis (39). E3L is also a host range gene necessary for efficient VACV replication in several cell lines (2). In addition to these functions, E3 acts as a transcriptional regulator of several genes related to apoptosis, the immune response, and viral pathogenesis (9, 34, 36). Posttranslational modifications are common mechanisms for the regulation of multifunctional proteins. Thus far, no posttranslational modifications of E3 have been described.

One type of virus-host interaction that is well established and widespread is the modulation of viral protein function by posttranslational modification systems, which include SUMOylation and ubiquitination. Viral proteins were among the first substrates found to be posttranslationally modified by the small ubiquitin-like modifier (SUMO) protein, and SUMOylation seems to facilitate viral infections of the host cells (8). SUMO is a member of the larger family of ubiquitin-like proteins, which shares about 18% sequence identity to ubiquitin and is structurally quite similar (31). Posttranslational modification with ubiquitin and ubiquitin-like proteins of the SUMO family involves isopeptide bond formation between the carboxyl group of the modifier and the epsilon amino group of a lysine residue in the target. Like ubiquitin, SUMO is covalently attached to lysine residues within the target protein, although in the majority of cases, SUMO is attached to a lysine within the target protein, and it was previously shown to be destabilized if the SUMO-interacting motif (SIM) upon SUMOylation (22). To date, a single conserved SIM has been identified, which consists of a hydrophobic core (L/V/I)x(L/V/I)(L/V/I) (5, 55). In mammals, there are four SUMO isoforms, including SUMO1, which most closely resembles the single yeast Smt3. SUMO2 and SUMO3, which are very similar to each other, contain an internal SUMOylation consensus site and more readily form polySUMO chains (44, 61), and SUMO4 has been linked to diabetes (26). SUMOylation regulates a wide range of processes, including transcriptional activity, protein stability, and nucleocytoplasmic transport. In addition, SUMOylation often promotes interactions between modified proteins and downstream factors containing SUMO-interacting motifs (SIMs) (22). To date, a single conserved SIM has been identified, which consists of a hydrophobic core (L/V/I)x(L/V/I)(L/V/I). This SIM has been detected in several cellular and viral proteins known to be modified by SUMO, and it was previously shown to be important for the formation of SUMO-dependent protein networks (41, 58).

There are viruses belonging to several different families that also utilize or modulate the ubiquitin-proteasome system to their advantage. Proteins can be monoubiquitinated, or the
initial ubiquitin monomer may itself act as a target, generating polyubiquitin chains. Distinct polyubiquitin signals that act in different cellular processes can be created by a variation in the choice of lysine linkage between ubiquitin monomers or in the length of the ubiquitin chain used.

The present investigation demonstrates that VACV protein E3 interacts with SUMO1 and can be covalently modified by either SUMO1 or SUMO2. The SUMO modification takes place on the lysine residues at positions 40 and 99 and negatively regulates E3 transcriptional transactivation on the p53-upregulated modulator of apoptosis (PUMA) and APAF-1 genes. We demonstrate that the presence of an intact SIM in E3 is required for its own SUMOylation and for the stability of the viral protein. We also demonstrate the conjugation of E3 to ubiquitin, a modification that does not induce the degradation of wild-type E3 (WT-E3) but triggers the proteasome-mediated degradation of the E3-ΔSIM mutant. E3 is the first example of a poxvirus protein regulated by covalent modification by ubiquitin, by the SUMO1 and SUMO2 proteins, as well as by noncovalent SUMO interactions. These results provide evidence that ubiquitin and ubiquitin-like proteins are important for the full biological activity of E3.

RESULTS

E3 protein colocalizes with SUMO1 in VACV-infected cells.

SUMO1 was previously isolated as an E3-interacting protein in a yeast two-hybrid assay (53). Based on these observations, we decided to study the putative colocalization between SUMO1 and E3 in the viral factories. First, MCF-7 cells were infected with VACV, and at 4 h after infection, cells were immunostained by using anti-vaccinia virus and anti-SUMO1 antibodies. SUMO1 was localized mainly in a nuclear speckled pattern in mock-infected cells, as expected (Fig. 1A). In contrast, in those cells infected with VACV, SUMO1 was recruited to the viral factories (Fig. 1A). When infected cells, as described above, were stained with anti-E3 and anti-SUMO1 antibodies, confocal analysis demonstrated a clear colocalization of both the E3 and SUMO1 proteins in the viral factories (Fig. 1B).

E3 protein is covalently modified by SUMO. To further analyze whether E3 can be covalently modified by SUMO, an in vitro SUMOylation assay was then carried out by using [35S]methionine-labeled E3 protein as a substrate. As expected, in vitro-translated E3 was detected as two bands of around 15 and 22 kDa (Fig. 2A). The incubation of the SUMOylation reaction mixture with SUMO1 led to the appearance of additional higher-molecular-mass bands that corresponded to E3-SUMO1 (Fig. 2A, left, arrowheads). In addition, the in vitro SUMOylation reaction mixture was incubated in the presence of SUMO2, a ladder of higher-molecular-mass bands was observed (Fig. 2A, left). To further prove that E3 is in fact SUMOylated in vitro, E3-SUMO2 obtained as described above was incubated in a deSUMOylation reaction mixture in the presence of GST or GST-SENP1 (Biomol), a SUMO-specific protease containing the catalytic domain of SENP1. The incubation of E3-SUMO2 in the presence of SENP1 led to the disappearance of the higher-molecular-mass bands corresponding to E3-SUMO2 (Fig. 2A, right), demonstrating that in vitro-synthesized E3 is covalently conjugated to SUMO. In order to analyze whether E3 is also SUMOylated in vivo, HEK-293 cells were cotransfected with a plasmid encoding E3 together with pcDNA, UbC9 and pcDNA-His6-SUMO1, or UbC9 and pcDNA-His6-SUMO2, and whole-protein extracts or nickel column-purified histidine-tagged proteins were then analyzed by Western blotting using an anti-E3 antibody. Cells cotransfected with pcCINEO-E3 and pcDNA showed two bands of around 15 and 22 kDa, as ex-
expected (Fig. 2B). However, when His$_6$-SUMO1 was cotransfected with E3, higher-molecular-mass bands of the expected size were detected in the purified extracts (Fig. 2B, left, arrowheads). Analysis of extracts from the His$_6$-SUMO2-transfected cells also revealed additional bands already visible in crude extracts that corresponded to E3-SUMO2, as demonstrated after Western blot analysis of the purified extracts (Fig. 2B, right). Note that since the levels of SUMO1-modified E3 were lower than the levels of SUMO2-modified E3, we required larger amounts of purified protein and longer exposure times to detect the E3-SUMO1 bands. Moreover, to demonstrate that E3 is also SUMOylated when produced by the virus, HEK-293 cells transfected with pcDNA, Ubc9 and pcDNA-His6-SUMO1, or Ubc9 and pcDNA-His6-SUMO2 were infected with VACV. Whole-cell extracts and histidine-tagged proteins recovered on nickel beads were analyzed by Western blotting with an anti-E3 antibody. Upon the purification of His-tagged complexes, two major E3-SUMO1 conjugates and several E3-SUMO2 bands were detected (Fig. 2C). Consistent with the data obtained after the analysis of E3 following transfection, SUMO2 more readily modified E3 than SUMO1. Altogether, these data demonstrate that the VACV E3 protein is SUMOylated by SUMO1 and SUMO2 in vitro and in vivo.

**SUMO conjugation modulates the transactivation activity of E3 on the PUMA and APAF-1 genes.** Having determined that VACV E3 is SUMO modified, we sought to identify the region of E3 involved in this modification. Many SUMO-modified proteins characterized to date are modified at lysine residues that exist within the $\psi$HXKxE consensus sequence (where $\psi$ is a hydrophobic residue, K is the modified lysine, x is any amino acid, and E is glutamic acid). Analysis of the amino acid sequence using SUMOylation prediction programs revealed a consensus sequence for SUMOylation, 39EKRE42, that is highly conserved among distantly related poxviruses (see Fig. S1 in the supplemental material) and at least one other putative nonconsensus SUMO conjugation domain located in position K99 as putative SUMO acceptor sites. To investigate whether these were the sites for SUMO modification, we constructed an E3 point mutant in which lysine 40 or 99 was replaced with arginine (E3-K40R and E3-K99R). In addition, an E3 mutant with a mutation in both lysine residues was generated (E3-K40RK99R), and in vitro SUMO conjugation
assays using the different E3 mutants as substrates were then carried out. The conjugation of SUMO1 was clearly reduced in the E3-K40R mutant, as revealed by the lower levels of the main E3-SUMO1 bands detected in the assay (Fig. 3A, left). E3 SUMOylation was not affected by the substitution of lysine 99. However, the double mutation of K40 and K99 to arginine dramatically reduced the SUMOylation of E3 (Fig. 3A, left).

Results observed after in vitro SUMO2 modification assays with the E3 mutants revealed similar results. The conjugation of SUMO2 to the E3-K40R mutant was clearly reduced in comparison with that observed with WT-E3, and although the modification of E3-K99R with SUMO2 was quite similar to that observed with WT-E3, the mutation of both lysines showed a more significant reduction in SUMO2 conjugation (Fig. 3A, right). To determine whether these two lysines are also implicated in SUMOylation in vivo, HEK-293 cells were cotransfected with pCINEO-E3-WT or pCINEO-E3-K40RK99R together with pcDNA or Ubc9 and pcDNA-His6-SUMO2, and analyses of the whole-protein extracts and of the nickel-purified histidine-tagged proteins using an anti-E3 antibody were carried out. A clear reduction in the E3-K40RK99R SUMO2 modification in comparison with that of WT-E3 was observed (Fig. 3B). In addition, the main E3-SUMO1 band detected in the cells cotransfected with SUMO1 and WT-E3 was not detected in those cells transfected with the E3-K40RK99R mutant (see Fig. S2 in the supplemental material). Altogether, these results indicated that E3 can be modified by SUMO at both residues. Covalent SUMO conjugation may regulate the subcellular localization or stability of the target proteins. In order to analyze if an E3 mutant with a reduced ability to be SUMOylated has an altered subcellular localization, MCF-7 cells were transfected with the plasmid encoding WT-E3 or the E3-K40RK99R mutant, and 36 h after transfection, cells were stained with an anti-E3 antibody. We could not detect significant differences in the subcellular localizations of both proteins (see Fig. S3 in the supplemental material). In addition, we did not observe any alteration in the subcellular localization of E3 after the cotransfection of pCINEO-E3-WT with SUMO1 or SUMO2 (data not shown). The effect of SUMOylation on the stability of E3 was then studied. MCF-7 cells were cotransfected with plasmids encoding WT-E3 or E3-K40RK99R together with pcDNA, Ubc9 and SUMO1, or Ubc9 and SUMO2 and treated with cycloheximide, and at different times after treatment, levels of E3 were analyzed. We could not detect differences in the stabilities of WT-E3 and the E3-K40RK99R mutant or after the cotransfection of SUMO1 or SUMO2 (see Fig. S4 in the supplemental material). Furthermore, the stability of E3-SUMO2 was similar to that of the unmodified E3 protein (Fig. S4), indicating that SUMOylation does not modulate E3 stability. VACV with E3L deleted (VACV-/H9004E3L) cannot replicate in HeLa cells, but this host-range phenotype could be complemented by an E3L gene expressed transiently from a plasmid (13). In addition, we also observed that VACV-ΔE3L has a replication defect in HEK-293 cells (see Fig. S5 in the supplemental material). Hence, the requirement of E3 SUMO conjugation for VACV-ΔE3L reconstitution in HeLa or HEK-293 cells was analyzed. Cells were transfected with pcDNA, WT-E3, or E3-K40RK99R, and 48 h after transfection, cells were infected with VACV at a multiplicity of infection (MOI) of 7.5 PFU per cell. The synthesis of viral proteins 16 h after infection and VACV production 24 h after infection were determined. The VACV titer recovered from E3-K40RK99R-transfected HeLa or HEK-293 cells was significantly higher than that recovered from pcDNA-transfected cells and similar to the viral titer obtained from WT-E3-transfected cells (Fig. S5). As expected, no VACV protein synthesis was observed in cells...
transfected with pcDNA. However, the synthesis of viral proteins in VACV-ΔE3L-infected cells was restored after the expression of WT-E3 from the transfected plasmid, and viral protein synthesis was similarly observed after the expression of the E3-K40RK99R mutant in HeLa or HEK-293 cells (Fig. S5). Altogether, these results suggest that the SUMO modification of E3 is not required for efficient VACV replication. SUMO conjugation may also alter the transactivation activity...
of the target proteins. Therefore, we were interested in testing whether a covalent modification by SUMO had any effect on the transactivation activity of E3. The N terminus of E3 was suggested previously to stabilize Z-DNA in the promoter regions in some genes, such as the p53 gene, resulting in the transactivation of the gene (34). Consequently, we decided to analyze whether an E3 mutant with a reduced ability to be SUMOylated has an altered transactivation activity. Initially, transactivation assays were performed with MCF-7 cells that were transfected with reporter plasmid p53-regulated PUMA-luc together with increasing amounts of pCINEO-E3-WT. As shown in Fig. 3C, the expression of WT-E3 induced the transactivation of the reporter in a dose-responsive manner. Importantly, the transactivation levels achieved in our experiments were similar to those previously reported (34). Next, the transactivation activity of WT-E3 or E3-K40RK99R after the cotransfection of SENP1 on PUMA-luc was analyzed. The cotransfection of SENP1 significantly increased the transactivation of the promoter induced by WT-E3 (Fig. 3D). The transfection of the E3-K40RK99R mutant induced a slightly higher but statistically significant transactivation of the promoter than WT-E3, and this activity was not altered after the cotransfection of SENP1 (Fig. 3D), suggesting that SUMOylation has a negative effect on the transactivation activity of E3. To reinforce these data, we analyzed the transcriptional activity of E3 on a different reporter. We observed that E3 induced the transactivation of the p53-regulated APAF-1-luc reporter in a dose-responsive manner (Fig. 3E). The cotransfection of SENP1 significantly increased the transactivation of the APAF-1-luc reporter induced by WT-E3 but did not alter the transcriptional activity of the E3-K40RK99R mutant (Fig. 3F). Altogether, these results indicate that SUMO conjugation negatively regulates the transcriptional activity of E3 at the PUMA and APAF-1 genes.

The integrity of the SIM domain in E3 is required for its stability, nuclear localization, and SUMOylation. Recently, it was demonstrated that several cellular and viral SUMO targets can also interact noncovalently with SUMO through a SUMO-interacting motif (SIM). The best-studied SIM contains a hydrophobic core sequence consisting of stretches of three or four hydrophobic valine, leucine, or isoleucine residues plus one acidic/polar residue at position 2 or 3 (27, 59). The E3 protein presents a domain that resembles a SIM at position 119VTVI122. A putative noncovalent interaction between SUMO1 and E3-SIM mutants was then carried out in the presence of MG132. HEK-293 cells were cotransfected with a plasmid encoding WT-E3 or E3L-SIM together with pcDNA or with Ubc9 and pcDNA-His6-SUMO2, and whole-protein extracts and nickel column-purified histidine-tagged proteins were then analyzed by Western blotting using an anti-E3 antibody. E3-SUMO2 bands detected in those cells transfected with pCINEO-E3-WT and pcDNA-His6-SUMO2 were not observed for the cells transfected with the pCINEO-E3-SIM mutant, indicating that the SIM domain in E3 is implicated in its SUMOylation in vivo. In vivo SUMOylation assays of WT-E3 or E3-SIM were then carried out in the promoter of the gene (34). Consequently, we decided to test the effect of the SIM domain on E3 SUMOylation in vitro. In vitro SUMOylation of E3-SIM mutants was retained into the cytoplasm, suggesting that the SIM domain is also required for nuclear translocation (Fig. 4E and see Fig. S7 in the supplemental material). Genetic and biochemical experiments suggested that the biological activity of E3 is derived primarily from its capacity to bind to dsRNA (12–14, 69), an interaction that also mediates its binding to ISG15 (25). Therefore, we analyzed the ability of the E3-SIM mutant to bind to dsRNA. This was assayed by a poly(I:C)-agarose-binding assay as previously described (37). Both the 35S-labeled in vitro-synthesized WT-E3 and E3-SIM proteins bound to dsRNA-agarose but not to agarose alone (Fig. 4F), indicating that the SIM domain is not required for the interaction of E3 and dsRNA. In addition, we studied whether the SIM domain is required for the interaction between E3 and ISG15. We detected the commmunoprecipitation of E3-SIM and ISG15 (see Fig. S8 in the supplemental material), indicating that the SIM domain is not required for the interaction between E3 and ISG15.
FIG. 4. The SIM domain in E3 is required for its interaction with SUMO, its stability, SUMOylation, and nuclear localization, but it is not essential to bind dsRNA. (A) HEK-293 cells were transfected with WT-E3 or E3-ΔSIM expression plasmids and treated or not with MG132 for 24 h. Analyses of the protein extracts by Western blotting with an anti-E3 antibody were then carried out. (B) Protein extracts from HEK-293 cells transfected with WT-E3 or E3-ΔSIM expression constructs and treated with MG132 for 24 h were incubated with GST or GST-SUMO1. Beads were washed, and the bound proteins were eluted and subjected to electrophoresis and Western blot analysis with an anti-E3 antibody. The input, representing 10% of the protein extracts, is shown. (C) [35S]methionine-labeled WT-E3 or E3-ΔSIM proteins were used as substrates in an in vitro...
The E3 protein is differentially ubiquitinated depending on the integrity of a SIM domain. The degradation of the E3-ΔSIM mutant by a proteasome-mediated mechanism suggested that E3 might also be modified by ubiquitin. In order to analyze whether E3 can be ubiquitinated, MCF-7 cells were transfected with pCINEO-E3-WT in the presence or absence of pcDNA-His6-ubiquitin as indicated. Analysis of the protein extracts by Western blotting with an anti-E3 antibody 48 h after transfection was carried out. (B) MCF-7 cells were transfected as indicated above (left) or transfected with pcDNA-His6-ubiquitin and then infected with VACV for 6 h (right). Whole-protein extracts and histidine-tagged proteins purified by use of nickel columns were then analyzed by Western blotting with an anti-E3 antibody. Arrowheads refer to ubiquitinated E3. (C) MCF-7 cells were transfected with WT-E3- or E3L-ΔSIM-expressing plasmids in the presence or absence of pcDNA-His6-ubiquitin and treated with MG132 for 24 h. Analysis of the whole-protein extracts and the histidine-tagged proteins purified by use of nickel columns was then carried out by Western blotting with an anti-E3 antibody.

The E3 protein is differentially ubiquitinated depending on the integrity of a SIM domain. The degradation of the E3-ΔSIM mutant by a proteasome-mediated mechanism suggested that E3 might also be modified by ubiquitin. In order to analyze whether E3 can be ubiquitinated, MCF-7 cells were transfected with pCINEO-E3-WT in the presence or absence of pcDNA-His6-ubiquitin, and at 36 h posttransfection, Western blot analysis of E3 was carried out. As shown in Fig. 5A, additional higher-molecular-mass bands were detected in those cells cotransfected with ubiquitin. Importantly, the Western blot analysis also revealed that the levels of the E3 protein were not significantly affected by the expression of ubiquitin, suggesting that E3 ubiquitination does not induce WT-E3 degradation (Fig. 5A). In order to demonstrate that the higher-molecular-mass bands correspond to E3-ubiquitin, His-binding proteins were purified from MCF-7 cells transfected as described above. Western blot analysis of the purified extracts using an anti-E3 antibody revealed that the additional bands detected after the transfection of ubiquitin corresponded with E3-ubiquitin (Fig. 5B, left). A broad band of the expected molecular mass that was recognized by the anti-E3 antibody was also observed after Western blot analysis of histidine-tagged proteins obtained from cells transfected with His6-ubiquitin and then infected with VACV (Fig. 5B, right), indicating that the E3 produced by the virus can also be modified by ubiquitin. Recent studies provided evidence that ubiquitin chains with different linkages or lengths may work as specific signals to control distinct molecular processes. To further char-

SUMOylation assay in the presence of SUMO1 (top) or SUMO2 (bottom). The reaction products were visualized by autoradiography. (D) Total extracts or histidine-tagged purified proteins were prepared from HEK-293 cells cotransfected with pCINEO-E3-WT or pCINEO-E3-ΔSIM together with pcDNA or pcDNA-Ube9 and pcDNA-His6-SUMO2, and an immunoblot analysis with an anti-E3 antibody was carried out. (E) MCF-7 cells were transfected with WT-E3 or E3-ΔSIM expression plasmids and treated with MG132 for 24 h. Cells were then stained with DAPI and an anti-E3 antibody, and the localization of E3 was detected by confocal microscopy. (F) [35S]methionine-labeled WT-E3 or E3-ΔSIM proteins were incubated with agarose or poly(LC)-agarose. Beads were washed, and the bound proteins were eluted and subjected to electrophoresis and autoradiography. The input, representing 10% of the quantity of protein used for the binding reaction, is shown.
acterize E3 ubiquitination, we cotransfected MCF-7 cells with pCINEO-E3-WT or pCINEO-E3-ASIM and pcDNA-His6-ubiquitin and treated the cells with MG132, and 36 h after transfection, purified histidine-tagged proteins were analyzed by Western blotting with an anti-E3 antibody. A high-mobility ubiquitin-positive smear was observed for the cells transfected with ubiquitin and the E3-ASIM mutant in comparison with the bands detected for those transfected with WT-E3 expression constructs (Fig. 5C).

**DISCUSSION**

The VACV E3L gene product is known as an IFN resistance protein, as a determinant of host range and viral pathogenesis, and as a modulator of cellular apoptotic and signal transduction pathways (35, 36, 42). The E3 protein binds to dsRNA, and this interaction mediates or enhances its binding to ISG15 and PKR, respectively (25, 57). In addition, E3 can bind to SUMO1 in a yeast two-hybrid assay, although this interaction was not confirmed at the biochemical level (53). This finding, together with the observation that SUMO1 is recruited to viral factories in response to VACV infection (49), prompted us to investigate the biochemical nature of the interactions of SUMO and E3. Our results demonstrate that E3 colocalizes with SUMO1 in the viral factories and that the E3 protein is modified by the covalent attachment of SUMO1 or SUMO2 in vivo and in vitro. Importantly, E3 SUMOylation was demonstrated for transfected as well as for infected cells, indicating that the SUMOylation of E3 is independent of VACV infection. Although SUMOylated lysine residues are often found within (L/V/I)x(L/V/I)(L/V/I) SIM consensus at the C terminus of the protein. Furthermore, we still detected colocalization between E3-K40RK99R and SUMO1 in the infected cells, suggesting that additional motifs in E3 may mediate the interaction with SUMO1. Although the mutation of the SIM domain did not alter the ability of E3 to interact with dsRNA or ISG15, analysis of the SIM E3 mutant showed impaired SUMOylation in vivo and in vitro that correlated with the noncovalent SUMO-binding properties. These results demonstrate that the SIM domain is not required for the interaction of E3 and dsRNA but that the SUMO-SIM interface is required for efficient E3 SUMOylation, as observed for an increasing number of target proteins (32, 41, 47, 60).

Recently, the existence of known SUMO targets that interact noncovalently with SUMO and contain SUMO-interacting motifs (SIMs) was demonstrated (32). Intriguingly, E3 contains a hydrophobic region with a sequence conforming to the (L/V/I)x(L/V/I)(L/V/I) SIM consensus at the C terminus of the protein. Furthermore, we still detected colocalization between E3-K40RK99R and SUMO1 in the infected cells, suggesting that additional motifs in E3 may mediate the interaction with SUMO1. Although the mutation of the SIM domain did not alter the ability of E3 to interact with dsRNA or ISG15, analysis of the SIM E3 mutant showed impaired SUMOylation in vitro and in vivo that correlated with the noncovalent SUMO-binding properties. These results demonstrate that the SIM domain is not required for the interaction of E3 and dsRNA but that the SUMO-SIM interface is required for efficient E3 SUMOylation, as observed for an increasing number of target proteins (32, 41, 47, 60). Importantly, the mutation of the E3 SIM domain led to an unstable protein that was degraded by a proteasome-mediated mechanism. Analysis of the subcellular localization in the presence of a proteasome inhibitor also demonstrated that the SIM mutant showed an exclusively cytoplasmic localization, in contrast to the nuclear/cytoplasmic distribution of the wild-type protein. Although there is a correlation between the cytoplasmic localization and proteasome-mediated degradation of E3-ASIM, a cytoplasmic E3 mutant reported previously was not described to be an unstable protein (13). In addition, the overexpression of ubiquitin-specific protease 7 (USP7/HAUSP), an enzyme that prevents the degradation of different cellular and viral proteins (11, 19, 40, 46, 65), partially rescues E3-ASIM from degradation without altering its cytoplasmic localization (see Fig. S9 in the supplemental material). Altogether, these results suggest that the degradation of the E3-ASIM protein is not a consequence of its cytoplasmic localization. A mutation of the SIM domain in the E3-K40RK99R mutant also led to an alteration of the SUMOylation, stability, and subcellular localization of the protein, supporting the idea that a noncovalent SUMO interaction but not SUMOylation modulates these E3 properties. Noncovalent SUMO interactions have been shown to affect the recruitment of proteins into subnuclear structures (45, 58) or their transcriptional activity (33, 54). Thus far, this is the first time that the integrity of a SIM domain within a protein has been demonstrated to be required for its stability.

In a classical model, ubiquitination was associated with proteasome-mediated degradation. We then decided to analyze whether E3 can be ubiquitinated. Viruses are connected to ubiquitin in a variety of ways. A role of ubiquitin in the degradation of viral proteins has been demonstrated in the cases
of the Epstein-Barr virus (EBV) LMP2a, human papillomavirus (HPV) E7, and HPV E2 proteins (30). However, some viral functions require ubiquitin modification for purposes other than protein turnover, as exemplified by the role of the ubiquitination of viral proteins in virion budding and release for the retroviral (HIV) Gag protein (15, 21), the Ebola virus Vp40 protein (70), or the rhadinovirus M protein (27). Recently, it was reported that poxviruses exploit the ubiquitination machinery by expressing viral proteins that function as ubiquitin ligases or regulate cellular ubiquitin ligases (48, 56, 64). In addition, it was also demonstrated that the proteasome and ubiquitin are required for poxvirus infection (62). Our results demonstrate that E3 can be modified by ubiquitin but that this ubiquitination does not induce wild-type E3 degradation. Ubiquitin itself possesses several lysines that can be used for the attachment of another ubiquitin molecule, allowing substrates to be modified with different types of ubiquitin chains (50, 51). In this sense, the consequences of polyubiquitination appear to depend on the length of the ubiquitin chain and on the type of linkage used. It is then tempting to speculate that the different stabilities observed between the WT-E3 and the E3-ΔSIM proteins are due to differences in ubiquitin chain lengths. An analysis of the conjugation of ubiquitin to WT-E3 or E3-ΔSIM revealed that whereas the WT-E3 ubiquitin modification was quite limited, very long ubiquitin chains were conjugated to the E3-ΔSIM mutant, reinforcing this hypothesis. Different evidences reveal the existence of cross talk mechanisms between the SUMOylation and ubiquitination systems (23). This is a new example of how SUMO may have a proapoptotic activity of CREB-binding protein via the recruitment of Daxx. Through SUMO-interacting motifs. EMBO J. 16:1510–1530.


