Covalent modification by SUMO is required for efficient disruption of PML oncogenic domains by Kaposi’s sarcoma-associated herpesvirus latent protein LANA2

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The multifunctional Kaposi’s sarcoma-associated herpesvirus (KSHV) latent protein latency-associated nuclear antigen 2 (LANA2) has a critical role in KSHV-induced B-cell malignancies. LANA2 increases the level of small ubiquitin-like modifier (SUMO)2-ubiquitin-modified PML and induces the disruption of PML oncogenic domains (PODs) by a process that requires a non-covalent SUMO interaction domain (SIM) in LANA2. We now demonstrate that LANA2 is covalently conjugated to SUMO1 and SUMO2 both in vitro and in latently KSHV-infected B-cells. We show that a LANA2 SIM mutant exhibits a slightly altered sumoylation pattern, which suggests that non-covalent SUMO interactions represent a mechanism for determining SUMO substrate recognition and modification. In addition, several lysine residues were mapped as SUMO conjugation sites. A sumoylation-deficient mutant shows impaired ability to induce disruption of PODs, which suggests that either directly bound or covalently conjugated SUMO moieties may act as a bridge for interaction between LANA2 and other SUMO-modified or SUMO-interacting proteins required for disruption of PODs.

Latency-associated nuclear antigen 2 (LANA2; also known as viral interferon regulatory factor 3) is a Kaposi’s sarcoma-associated herpesvirus (KSHV) latent protein encoded by ORFK10.5 and is detected exclusively in B-cells infected with the virus (Lubyova & Pitha, 2000; Rivas et al., 2001). LANA2 has been suggested to play an important role in KSHV-mediated tumorigenesis, because of its ability to inhibit p53 function (Rivas et al., 2001), dsRNA-activated protein kinase R (PKR)-dependent apoptosis (Esteban et al., 2003), nuclear factor κB activity (Seo et al., 2004), interferon regulatory factor 7-mediated interferon signal transduction (Joo et al., 2007) and virus-mediated transcriptional activation of the α-interferon promoter (Lubyova & Pitha, 2000). LANA2 also stabilizes and induces the transcriptional activity of hypoxia-inducible factor-1α (Shin et al., 2008) and disrupts PML oncogenic domains (PODs) (Marcos-Villar et al., 2009). Moreover, LANA2 has been shown to be required for the survival of cells infected with KSHV alone or dually infected by Epstein–Barr virus and KSHV (Wies et al., 2008). The multifunctional nature of LANA2 suggests that post-translational modifications may modulate its activities.

Small ubiquitin-like modifier (SUMO) is an 11.5 kDa protein that is conjugated to multiple proteins and has been reported to exhibit multiple effects, including modulation of protein stability, subcellular localization and activity (Zhao, 2007). Since the discovery of SUMO1 in 1996, the list of proteins that have been reported to be modified by SUMO1 has grown. Viral proteins were among the first substrates found to be post-translationally modified by SUMO, and sumoylation seems to facilitate viral infection of host cells (Boggio & Chiocca, 2006).
To determine whether LANA2 could be modified by SUMO conjugation, in vitro sumoylation assays using $^{35}$S-methionine-labelled in vitro-translated LANA2 protein were performed as described previously by Campagna et al. (2010). Incubation of the in vitro sumoylation reaction with SUMO1 revealed a broad additional band approximately 20 kDa larger than the LANA2 band corresponding to LANA2–SUMO1 protein (Fig. 1a, left panel). In addition, when the reaction was incubated in the presence of SUMO2 multiple bands corresponding to LANA2–SUMO2 protein were detected, which is indicative of the attachment of polymeric chains (Fig. 1a, left panel). To further demonstrate that this modification was due to sumoylation, SUMO2-modified LANA2 was incubated with 2 μg glutathione S-transferase (GST) or GST–SENP1 (Biomol), a SUMO-specific proteinase containing the catalytic domain of SENP1, described recently by Li et al. (2008). Addition of GST–SENP1 deconjugated LANA2–SUMO2 (Fig. 1a, right panel). These results demonstrated that LANA2 is sumoylated in vitro.

In order to analyse whether LANA2 is modified by SUMO in vivo, HEK-293 cells were co-transfected with the LANA2 expression plasmid together with Ubc9 and pcDNA vector (Invitrogen), His6–SUMO1 or His6–SUMO2 (Desterro et al., 1998; Vertegaal et al., 2006), and cell extracts were analysed by Western blotting and probing with anti-LANA2 antibody (Novus Biologicals). Immunoblot analysis revealed an additional band of the expected size only in those cells transfected with SUMO2 (Fig. 1b, upper-left panel). To confirm that the slower migrating form corresponds to SUMO2-modified LANA2 protein, cells transfected as described above were lysed in 6 M guanidine hydrochloride and histidine-tagged proteins were isolated on nickel beads.

**Fig. 1.** Covalent modification of LANA2 by SUMO. (a) In vitro modification of LANA2 by SUMO1 and SUMO2 (left panel) and LANA2–SUMO2 de-sumoylation by SENP1 (right panel). (b) Western blots (WB) of HEK-293 cells transfected with the indicated plasmids and probed with anti-LANA2 antibody (upper-left panel). Histidine-tagged proteins purified from cells co-transfected with LANA2, Ubc9 and His6–SUMO1 (lower-left panel) or His6–SUMO2 (right panel) were analysed by Western blotting and probed with the indicated antibodies. (c) Western blots of BC-1 cell lysates immunoprecipitated with anti-LANA2 antibody and probed with anti-LANA2 (left panel), anti-SUMO1 (middle panel) or anti-SUMO2 (right panel) antibodies. Immunoprecipitation with mouse anti-PKR mAb (Santa Cruz Biotechnology) served as a negative control. The major sumoylated bands are indicated with arrowheads. Arrows indicate the position of unmodified LANA2 in (a) and (b).
as described previously by Campagna et al. (2010). When the nickel-bound fraction was analysed by Western blotting and probed with anti-LANA2 antibody, sumoylated forms of LANA2 were clearly visible after expression of SUMO2 (Fig. 1b, right panel). These additional bands were clearly recognized by the anti-SUMO2 antibody (Zymed Laboratories), demonstrating that LANA2 is modified in vivo by SUMO2. However, we could not detect any additional band corresponding to LANA2–SUMO1 conjugates after transfection with the SUMO1 expression plasmid (Fig. 1b, lower-left panel), although experiments done in parallel with PML revealed a clear sumoylation of the target protein (Supplementary Fig. S1, available in JGV Online). We also noted that LANA2 can interact with immobilized nickel in its non-sumoylated form (Fig. 1b, lower-left and right panels) as well as with cobalt resin (not shown). This interaction is probably due to lower-affinity ionic interactions and made the purification of the sumoylated fraction of the LANA2 protein difficult. Taken together, these results demonstrated that LANA2 is sumoylated by SUMO1 and SUMO2 in vitro and by SUMO2 in vivo.

To confirm that endogenous LANA2 protein is sumoylated in KSHV-infected cells, protein extracts from BC-1 cells prepared in the presence of N-ethylmaleimide, which has been previously shown to stabilize SUMO-modified proteins (Suzuki et al., 1999), were immunoprecipitated with anti-LANA2 antibody. Western-blot analysis of the immunoprecipitates using anti-LANA2 antibody revealed a major band of approximately 75 kDa, corresponding to LANA2, and two additional slower-migrating bands: one broad band of about 95–100 kDa that might correspond to the band detected in the in vitro sumoylation assay after incubation with SUMO1 and a light band of about 120 kDa (Fig. 1c, left panel). Western blots of BC-1 cell lysates immunoprecipitated with anti-LANA2 antibody and probed with anti-SUMO1 antibody (Santa Cruz Biotechnology) revealed two main bands of about 95 and 100 kDa and one higher-molecular-mass LANA2–SUMO1 species (Fig. 1c, middle panel). Similar additional bands and a smear of higher molecular mass LANA2–SUMO2 species were detected when the same immunocomplexes were incubated with anti-SUMO2 antibody (Fig. 1c, right panel). These data indicated that LANA2 is a SUMO substrate in latently KSHV-infected BC-1 cells.

It has been shown recently that, for some proteins, the presence of a SUMO interaction domain (SIM) within the substrate itself can increase sumoylation by facilitating the recruitment of SUMO-loaded Ubc9 via non-covalent interaction of the Ubc9-bound SUMO with the SIM (Kerscher, 2007; Lin et al., 2006; Meulmeester & Melchior, 2008; Minty et al., 2000; Takahashi et al., 2005; Zhu et al., 2008). We showed previously that LANA2 could interact in a non-covalent fashion with SUMO1 through a SIM (Marcos-Villar et al., 2009). To examine the effect of a LANA2 SIM mutant on the covalent modification of LANA2 by SUMO, in vitro sumoylation assays using the LANA2 SIM mutant (LANA2ASIM) as a substrate were carried out. Small but statistically significant differences were observed between SUMO1 conjugation (Fig. 2a, upper and right panels) or SUMO2 conjugation (Fig. 2a, lower and right panels) from wild-type (wt) LANA2 and LANA2ASIM (Student’s t-test). In addition, conjugation of LANA2ASIM with SUMO2 was severely decreased in comparison with LANA2 wt in vivo (Supplementary Fig. S2). These results indicated that the LANA2–SUMO non-covalent interaction modulates LANA2 sumoylation.

We then sought to identify the region of LANA2 involved in this modification. Since SUMO1 can be covalently attached to a single lysine only as a monomer (Meluh & Koshland, 1995; Tatham et al., 2001), our in vitro sumoylation results in the presence of SUMO1 indicated that either only a particular lysine residue in LANA2 was sumoylated or LANA2 was singly SUMOylated at different lysine residues in LANA2. Initially we tested a series of LANA2 deletion mutants using the in vitro conjugation assay. Three of four LANA2 deletion constructs were susceptible to sumoylation in vitro (Fig. 2b and not shown), which suggests that LANA2 might be modified at several lysine residues. Typically, lysine residues subject to SUMO modification are found among a SUMO modification consensus motif (V/I/L/M/F)KX(E/D) (Gill, 2004; Hay, 2005; Johnson, 2004). Inspection of the LANA2 sequence revealed the presence of the tetrapeptide FKRE at lysine 57, thus suggesting a potential site of SUMO modification. To test the possibility that K57 was involved in SUMO conjugation, it was changed to arginine which is unable to act as an acceptor for SUMO proteins. Next, the SUMO modification potentials of LANA2 wt and of mutant LANA2K57R proteins were compared in vitro. Mutation of this domain revealed a slight but statistically significant reduction of in vitro sumoylation with both SUMO1 and SUMO2 (Student’s t-test) (Fig. 2c), suggesting that other lysine residues are also implicated in conjugation to SUMO. Several proteins have been found to be conjugated to SUMO via lysine residues that are not situated in sumoylation consensus sites (Adamson & Kenney, 2001; Chakrabarti et al., 2000; Hoege et al., 2002; Kamitani et al., 1998; Lin et al., 2003; Rui et al., 2002). Moreover, covalent binding of SUMO to lysine residues is dynamic; lysine to arginine substitutions at preferred SUMO modification sites can result in enhanced modification at secondary sites (Eladad et al., 2005; Lin et al., 2006). Of the 19 lysines in LANA2, 17 are located in the LANA2 deletion fragments shown to be substrates for SUMO conjugation. Thus, we decided to mutate other lysine residues in LANA2 based on their positions in the protein and the score for sumoylation after being analysed in silico using a combination of the programs SUMOsp and SUMOplot. A plasmid encoding the LANA2 protein containing all of these mutations (LANA2–SUMOMUT) (Supplementary Table S2, available in JGV Online) was then assayed in an in vitro sumoylation assay. A clear reduction in SUMO1 or SUMO2 conjugation was observed after mutation of the chosen lysines (Fig. 2d, left and middle panels) and the additional introduction of
Fig. 2. Mapping the sites of SUMO modification of LANA2. (a) SIM domain contributes to sumoylation in LANA2. LANA2 wt and LANA2ΔSIM were tested for in vitro sumoylation with different amounts of SUMO1 (upper panel) and SUMO2 (lower panel). The area corresponding to LANA2–SUMO1 or LANA2–SUMO2 conjugates (indicated on the right of the panels) in each lane was cropped, analysed by densitometry and the result was divided by that of the unmodified protein band and this ratio was normalized to that of the wt protein. The data presented are means ± SD of three (SUMO1) and five (SUMO2) independent experiments (right panel). *, P<0.05; **, P<0.005; by Student's t-test compared with LANA2 wt. (b) In vitro sumoylation assays using LANA2 deletion constructs as the substrate. The major sumoylated bands are indicated with arrowheads. (c) In vitro-translated LANA2 wt and LANA2K57R were tested for sumoylation in vitro with SUMO1 (left panel) and SUMO2 (middle panel). The area corresponding to LANA2–SUMO1 or LANA2–SUMO2 conjugates (indicated on the right of the panels) in each lane was cropped, analysed by densitometry and the result was divided by that of the unmodified protein band and this ratio was normalized to that of the wt protein. The data presented are means ± SD of three independent experiments for SUMO1 or SUMO2 (right panel). *, P<0.05; **, P<0.005; by Student's t-test compared with LANA2 wt. (d) LANA2 wt and LANA2–SUMOMUT were tested for in vitro sumoylation with SUMO1 (left panel) and SUMO2 (middle panel) or transfected into HEK-293 cells with the indicated plasmids and after 36 h of expression the purified histidine-tagged proteins were analysed by Western blotting probed with anti-LANA2 antibody (right panel).
the SIM mutation did not alter LANA2 sumoylation further (not shown). Histidine-tagged proteins were then purified from lysates of cells transiently co-transfected with LANA2 wt or LANA2–SUMOMUT together with Ubc9 and His6–SUMO2 plasmids and analysed by Western blotting probed with anti-LANA2 antibody. While a SUMO2-modified form was clearly evident in those cells transfected with LANA2 wt, no evidence of SUMO2

Fig. 3. SUMO modification is involved in disruption of PODs. MCF-7 cells transfected with pcDNA, LANA2 wt or LANA2–SUMOMUT were analysed by immunofluorescence using the indicated antibodies. Arrows indicate cells that are positively expressing LANA2. The percentage of transfected cells with fewer than ten PML or Sp100 dots is represented in the bar charts.
modification could be detected in the LANA2–SUMOMUT-transfected cells (Fig. 2d, right panel).

At a molecular level, sumoylation alters protein functions by masking and/or adding interaction surfaces or by inducing conformational changes that result in altered interactions (Ulrich, 2009). As a result, a wide variety of consequences have been observed downstream, including changes in localization, activity and/or stability (Desterro et al., 1998; Weger et al., 2004). No appreciable differences in the half-life of LANA2 and LANA2–SUMOMUT proteins were observed (Supplementary Fig. S3), suggesting that sumoylation does not affect the overall stability of LANA2.

Covalent and non-covalent SUMO interactions have recently been demonstrated to affect the recruitment of proteins into subnuclear structures (Matunis et al., 2006). The SIM domain in LANA2 is required for disruption of PODs, suggesting that the association of LANA2 with the subnuclear structures through SUMO proteins is required for the control of PODs by LANA2. Consequently, we speculated that covalent interaction of SUMO with LANA2 might also be required for LANA2 activity at the PODs. MCF-7 cells were transfected with pcDNA, LANA2 wt or LANA2–SUMOMUT, double immunostained with anti-LANA2 and anti-PML (Santa Cruz Biotechnology) or anti-Sp100 (Chemicon International) antibodies and then analysed by confocal microscopy. Cells expressing LANA2 showed fewer PML or Sp100 dots than those cells that did not express the protein (Fig. 3), as previously reported (Marcos-Villar et al., 2009). In contrast, the majority of the cells expressing LANA2–SUMOMUT, in which subcellular localization was similar to that of LANA2 wt, did not show a clear alteration in PML or Sp100 staining (Fig. 3). Quantification of the PML or Sp100 dots in more than 200 cells confirmed that the percentage of cells with fewer than ten PML foci per nucleus was clearly reduced in those cells expressing LANA2–SUMOMUT in comparison with those expressing LANA2. In addition, PML protein stability was determined after transfection of MCF-7 cells with a plasmid encoding HA–PMLIV together with pcDNA, LANA2 wt or LANA2–SUMOMUT. The short half-life of PML observed after LANA2 expression was clearly prolonged in those cells expressing LANA2–SUMOMUT (Supplementary Fig. S4). Therefore, we can conclude that SUMO modification of LANA2 is needed for efficient disruption of PODs, which is mediated by the viral protein.

Dynamic sumoylation of host and viral proteins is thought to play an important role in herpesvirus replication. In the case of KSHV, the existence of at least two viral proteins capable of exploiting the cellular sumoylation machinery supports the relevance of SUMO modification in viral replication. Here, we demonstrate that LANA2 is sumoylated and that this SUMO–LANA2 covalent interaction, as we demonstrated previously for non-covalent SUMO binding to LANA2, is required for efficient disruption of PODs by LANA2. Recently, it has also been demonstrated that the K-bZIP protein encoded by KSHV is a SUMO E3 ligase that catalyses its own sumoylation, as well as that of its interacting partners, in a SIM-dependent manner (Chang et al., 2010). It is tempting to speculate about a possible interplay between these two viral proteins in achieving efficient viral replication. However, further studies will be required in order to prove this hypothesis.

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