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Covalent conjugation of the equine infectious anemia virus Gag with SUMO

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ABSTRACT

The conjugation of small ubiquitin-like modifier (SUMO) to the target protein, namely, SUMOylation, is involved in the regulation of many important biological events including host-pathogen interaction. Some viruses have evolved to exploit the host SUMOylation machinery to modify their own protein. Retroviral Gag protein plays critical roles in the viral life cycle. The HIV-1 p6 and the Moloney murine leukemia virus CA have been reported to be conjugated with SUMO. In this study, we report for the first time, to our knowledge, the covalent conjugation of equine infectious anemia virus (EIAV) Gag with SUMO. The *C*-terminal p9 domain of Gag is a main target for SUMOylation and SUMO is attached to multiple sites of p9, including K30 whose mutation abolished p9 SUMOylation completely. The SUMOylation of p9, but not the p9-K30 mutant, was also detected in equine fibroblastic cells ATCC® CCL-57™. Ubc9 and its C93 residue are indispensable for the SUMOylation of p9. Using confocal microscopy, it is found that EIAV Gag localizes primarily, if not exclusively, in the cytoplasm of the cell and the co-localization of EIAV Gag with Ubc9 was observed. Our findings that EIAV Gag is SUMOylated at p9-K30, together with previous findings on the defects of p9-K30 mutant in viral DNA translocation from cytoplasm to the nucleus, suggests that SUMOylation of Gag may be involved in such functions.

1. Introduction

Group-specific antigen (Gag) of retroviruses is synthesized as a polyprotein precursor containing four major domains: matrix (MA), capsid (CA), nucleocapsid (NC) and a *C*-terminal peptide, which is p6 in human immunodeficiency virus (HIV) and p9 in equine infectious anemia virus (EIAV). Gag polyprotein functions in various stages of the viral replicative cycle. It includes the MA domain playing a role in targeting the Gag to the plasma membrane [1], the CA domain in Gag multimerization [2], the NC domain in viral genomic RNA incorporation [3], and the p6/p9 domain in releasing budding virions from the plasma membrane [4]. Upon virion maturation, Gag is processed by virus-encoded protease to yield major structure proteins. The MA is associated with the inner leaflet of the lipid bilayer and CA reassembles to form a conical shell around the viral RNA in complex with NC and other viral proteins [5]. Gag proteins are also critical at the early stages of viral infection [6,7].

Gag can be co-translationally myristylated [8], post-translationally ubiquitinated [9–11] and phosphorylated [12]. Among retroviral Gag proteins, HIV-1 p6 and the Moloney murine leukemia virus CA were also found to be conjugated with small ubiquitin-like modifier (SUMO) [13,14].

SUMO is a member of ubiquitin-like protein superfamily that is covalently attached to target proteins to alter their localization, stability and activity [15,16], and is involved in the regulation of many important biological events including host-pathogen interaction [17–19]. Many target proteins contain a consensus motif ψKXE and SUMO is attached to the ε-amino group of the lysine residue by an isopeptide bond [20]. SUMOylation is an ATP-

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dependent enzymatic cascade involving the SUMO-activating enzyme (E1, Aos1 and Uba2) [21], SUMO-conjugating enzyme (E2, Ubc9) [22], and SUMO ligase (E3) [23]. For many target proteins, only E1 and E2 are sufficient for *in vitro* conjugation [24]. SUMO-specific protease is required for the cleavage of SUMO from its substrate [25].

Unlike ubiquitination that all Gags from HIV [9,10], simian immunodeficiency virus [9], feline immunodeficiency virus [26] and EIAV [11] have been demonstrated, among lentiviral Gag proteins, SUMOylation was only proved in HIV-1 p6 [13]. It is interesting to investigate whether Gag SUMOylation is unique to HIV or whether it also occurs in other lentiviruses.

EIAV causes a persistent infection in equids that is characterized by recurring febrile episodes associated with viraemia, fever, thrombocytopaenia and wasting symptoms [27]. Compared with other lentiviruses, EIAV has the smallest genome size, encodes fewer regulatory proteins, and the first successful lentiviral vaccine was developed for EIAV [28]. Therefore, in this study, the SUMOylation of EIAV Gag was investigated and the covalent conjugation of SUMO to the EIAV Gag mainly at the K30 residue of p9 was found. Our finding that EIAV Gag is SUMOylated at p9-K30, together with previous findings on the defects of p9-K30 mutant in producing replication-competent virus, suggests a potential mechanism of how Gag SUMOylation regulates viral replication.

2. Materials and methods

2.1. Plasmid

The EIAVuk proviral clone was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. Ronald Montelaro [29]. The Gag, MA, CA, NC and p9 fragments were amplified from the EIAVuk, using primers listed in the supplementary, and cloned into pET-28 (Novagen), pET-28a-HF [30] or pEGFP-C3-linker vector (Clontech), respectively. Site-directed mutagenesis was conducted to generate p9-K5R, p9-K10R, p9-K30R, p9-K31R, p9-K36R and p9-K38R mutants and the Ubc9-C93S mutant, using primers listed in the supplementary. Other plasmids were described previously [31]. All the plasmids constructed in this study were verified by sequence analysis.

2.2. E. coli expression/modification system, in vitro SUMOylation assay and Western blot

E. coli BL21 (DE3) was co-transformed with p-TE1E2S1 [32] or p-TE1E2S1△E2 [31], together with Gag or p9 prokaryotic expression plasmid, selected with appropriate antibiotics. Protein expression and purification were conducted as described [31] except that the isopropylthio-β-D-galactoside (IPTG) induction time was 6 h.

The *in vitro* SUMOylation reaction was performed in a 20 μl mixture containing 20 mM HEPES, pH 7.4, 5 mM magnesium chloride, 2 mM ATP, 0.1 mg/mL bovine serum albumin, 25 μg/mL Aos1/Uba2, 20 μg/mL Ubc9, 50 μg/mL SUMO1 and 25 μg/mL purified substrate protein. The reaction mixture was incubated at 37 °C for 2 h before detected by Western blots.

SDS-PAGE and Western blots were performed as described [31]. Polyclonal antibodies against SUMO1, Ubc9 and EGFP were generated in rabbits using purified recombinant proteins. Monoclonal antibodies against His_6 and FLAG were purchased from Clontech and Stratagene, respectively.

2.3. Mass spectrometry analysis

E. coli BL21 (DE3) was co-transformed with pET28a-HF-p9 and p-TE1E2S1. After IPTG induction, soluble protein was applied to HIS-Select® Nickel Affinity Gel and the His_6-tagged protein was purified by affinity chromatography. The 30 kDa protein band was excised from a coomassie blue-stained SDS-PAGE gel, digested with trypsin and subjected to MALDI-TOF MS analysis using Applied Biosystems 4700 Proteomics Analyzer.

2.4. Cell culture and transfection

The equine fibroblastic cell line derived from dermal cells CCL-57 (E. Derm, NBL6, ATCC® CCL-57™) and the human embryonic kidney cell line 293T were cultured in DMEM (HyClone) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37 °C in 5% CO_2. Transfection was conducted with the polyethylenimine reagent (Polysciences, Inc.). At 48 h post-transfection, cells were harvested for Western blots or fixed for confocal microscopy.

2.5. Immunofluorescence assay and confocal microscopy

The 293T cells were fixed with 2.5% glutaraldehyde for 45 min and washed twice with phosphate-buffered saline (PBS) for 10 min each in the dark. For DNA staining, 4′,6-diamidino-2-phenylindole (DAPI) in PBS was used at a 0.1 μg/mL final concentration for 45 min. For immunofluorescence assay, the anti-Ubc9 primary antibody and the Alexa Fluor® 594 goat anti-rabbit IgG (Molecular Probes) were diluted 1:500 and 1:200, respectively. Image acquisition and analysis was performed with Leica TCS SP5 laser-scanning confocal microscope at 405 nm (DAPI), 488 nm (EGFP) and 543 nm (Alexa Fluor® 594) wavelengths.

3. Results and discussion

3.1. EIAV Gag is covalently conjugated with SUMO, mainly at the C-terminal p9 domain

EIAV Gag (Fig. 1A) shares a high degree of sequence and structure homology with other lentiviral Gags. The existence of potential SUMOylation sites was indicated when EIAV Gag was analyzed using SUMOplot Analysis Program to predict and score SUMOylation sites (Fig. 1B). Therefore, we examined whether EIAV Gag can be SUMOylated. A 55 kDa band was detected when *E. coli* BL21 (DE3) was transformed with pET28-Gag (lane 1, Fig. 1C). When *E. coli* was co-transformed with pT-E1E2S1, a plasmid that contains genetically engineered genes encoding mouse E1, *Xenopus laevis* E2 and human SUMO1 [32], not only the 55 kDa Gag was produced, a more intense band at about 70 kDa and two additional bands at 85 kDa and 100 kDa were also detected (lane 2, Fig. 1C). The size differences between the adjacent two bands corresponded with the expected size of an additional SUMO molecule. Moreover, removal of the E2 gene from the pT-E1E2S1 plasmid [31] eliminated the ability of *E. coli* BL21 (DE3) to produce the higher molecular weight bands (lane 3, Fig. 1C), indicating that the EIAV Gag was covalently modified by one or more SUMO.

We next asked which domain of the EIAV Gag is the target for SUMOylation. *In vitro* SUMOylation assays were performed using purified E1 (Aos1+Uba2), E2 (Ubc9), and SUMO1. A known SUMO substrate RanGap1 [32] was used as a control (lanes 1 and 2, Fig. 1D). No higher molecular weight band was detected when purified MA or CA were tested (lanes 3 and 5, Fig. 1D). However, besides the expected size protein, higher molecular weight proteins were detected for NC and p9 (lanes 7 and 9, Fig. 1D). The size differences between the adjacent two bands represented the addition of a SUMO molecule. Moreover, all the shifted bands were detected only when SUMO1 was included (compare odd and even lanes of Fig. 1D), suggesting that both NC and p9 were covalently modified

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