Communication

Pellino-1, an Adaptor Protein of Interleukin-1 Receptor/Toll-like Receptor Signaling, Is Sumoylated by Ubc9

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Covalent modifications of the Pellino-1 protein are essential for transmitting innate immune response signals downstream, as the phosphorylation and polyubiquitination of Pellino-1 mediated by the IRAK proteins appear to have roles in regulating Pellino-1 function. In this study, we demonstrate that the Pellino-1 protein is post-translationally modified by small-ubiquitin-related modifier-1 (SUMO-1). Sumoylation assays with Pellino-1 and SUMO-1 expression plasmids reveal that the Pellino-1 protein is sumoylated *in vitro* and *in vivo*. Treatment of SUMO-1 specific protease 1 (SEN1) inhibited the sumoylation of the Pellino-1 protein and a GST pull-down assay as well as a yeast two hybrid assay showed that Pellino-1 binds to the SUMO-conjugating enzyme, Ubc9. Furthermore, we identified the five lysine residues of the Pellino-1 protein where SUMO-1 covalently attaches. Some of the sumoylated sites overlap with previously identified ubiquitination sites, suggesting competition between sumoylation and ubiquitination, as well as suggesting that the sumoylated Pellino-1 protein may have a cellular function distinct from previously identified functions.

INTRODUCTION

Interleukin-1 receptor/Toll-like receptors (IL-1R/TLR) are important receptors that regulate immunity and inflammation (O'Neill, 2008; O'Neill and Dinarello, 2000). IL-1R and TLR have a similar cytoplasmic TIR domain, and share downstream signaling components (Akira et al., 2006; O'Neill and Bowie, 2007). Upon specific ligand binding, IL-1R and most TLRs initially interact with MyD88, which subsequently recruits proteins of the IL-1 receptor-associated kinase (IRAK) family, IRAK1 and IRAK4 (Akira et al., 2006; Kawai and Akira, 2007; O'Neill, 2008). IRAK4 rapidly phosphorylates IRAK1, which induces IRAK1 auto-phosphorylation (Cao et al., 1996; Li et al., 2002). Hyperphosphorylated IRAK1 binds to TRAF6 and transmits the signal to downstream signaling complexes, resulting in the activation of the NF- κ B transcription factor which modulates pro-inflammatory gene expression (Akira et al., 2006; Kawai and Akira,

2007; Moynagh, 2009).

Pellino-1 was initially identified as an adaptor protein interacting with *Drosophila* Pelle, a protein highly homologous with the IRAKs (Grosshans et al., 1999). Three homologous Pellino proteins, Pellino-1, Pellino-2 and Pellino-3, have been identified in mammals (Jensen and Whitehead, 2003; Jiang et al., 2003; Yu et al., 2002). In IL-1R/TLR signaling, Pellino-1 is involved in NF- κ B activation through the association and subsequent mutual modification by IRAK1 (Butler et al., 2007; Jiang et al., 2003; Moynagh, 2009; Ordureau et al., 2008; Smith et al., 2009). IRAK1 phosphorylates the Pellino-1 protein, resulting in activation of the E3 ligase activity of the RING-like domain at the Pellino-1 C-terminus (Smith et al., 2009). This activation of Pellino-1 promotes Lys63-linked polyubiquitination of IRAK1, which leads to the activation of downstream signaling components (Butler et al., 2007; Ordureau et al., 2008) by an as yet unknown mechanism. Recently, three lysine residues of Pellino-1, Lys-169, Lys-202, and Lys-266, were identified to be conjugated to ubiquitin *in vitro* (Smith et al., 2009). However, the phenotype of the Pellino-1 knock-out mouse model indicates that Pellino-1 is more important for TLR3 signaling than TLR4 signaling (Chang et al., 2009). Although the reasons for the discrepancies between the knock-out mice and cell line results are unknown, it is obvious that complicated modifications of IRAK and Pellino-1, including phosphorylation and ubiquitination, play an important role in mediating the signal transduction involved in inflammation and innate immunity.

The small ubiquitin-like modifier (SUMO) is a ubiquitin-like protein that controls diverse cellular functions, including nucleocytoplasmic trafficking, cell-cycle regulation, maintenance of genomic integrity, and transcription (Hay, 2005; Johnson, 2004; Kim and Baek, 2006; Melchior, 2000). Similar to the ubiquitination process, SUMO modification is a dynamic process that is mediated by activating (E1), conjugating (E2), and ligating (E3) enzymes, and is readily reversed by the sentrin/SUMO-specific proteases (SENPs) (Yeh, 2009). Ubc9 protein is the only conjugating E2 enzyme found to be involved in SUMO modification so far (Gong et al., 1997).

In this study, we demonstrate a novel modification of Pellino-1 protein by SUMO-1. We also identify five lysine residues in

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Received September 29, 2010; accepted October 4, 2010; published online November 25, 2010

Keywords: IL-1R/TLR signaling, pellino-1, SUMO modification, Ubc9

Pellino-1 which are conjugated with SUMO-1.

MATERIALS AND METHODS

Cell culture and transfection

HeLa cells were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum. All plasmids were transfected using jetPEI™ (Polyplus transfection).

Plasmid construction and site-directed mutagenesis

Myc-Pellino-1 was amplified from full-length Pellino-1 cDNA (Choi et al., 2006) by a polymerase chain reaction and sub-cloned into the *EcoRI* and *SalI* sites of pCS3MT vector. The lysine to arginine point mutants of Pellino-1 were generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The SUMO-1 gene was amplified from GFP-SUMO-1 (Ahn et al., 2009) by a polymerase chain reaction and cloned into the *SalI* and *SmaI* sites of the pCI-neo vector. The open reading frame encoding wild type SENP1 (HA-SENP1) was constructed into pcDNA-HA by a polymerase chain reaction as described (Bailey and O'Hare, 2002). The dominant negative SENP1 (HA-SENP1-C603S), with cysteine 603 changed into serine (Bailey and O'Hare, 2004), was constructed from wild type SENP1 (HA-SENP1) using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The amplified fragments and the sequences of the point mutants were verified by sequencing.

Protein preparation for *in vitro* sumoylation

Preparation of the SUMO-1 mixture consisting of the E1 enzyme, E2 enzyme, and SUMO-1 protein was previously described (Ahn et al., 2009). Briefly, BL21(DE3) cells were transformed with pGEX-SAE1/SAE2, pGEX-Ubc9, and pGEX-SUMO-1, respectively. BL21(DE3) cells were grown at 37°C in LB medium containing ampicillin and were subsequently induced with 1 mM IPTG for 3 h. After cells were centrifuged, the pellet was resuspended in PBST (PBS with 1% Triton X-100) and sonicated for 10 s, four times. After centrifugation, the supernatant was incubated with glutathione-sepharose beads for 1 h at 4°C. The beads were washed three times with PBST. Bound proteins were eluted with 10 mM reduced glutathione in 10 mM Tris-HCl (pH 7.4). The concentration of eluted protein was measured using the Bradford reagent (Bio-Rad).

In vitro SUMO-modification

Reconstituted *in vitro* sumoylation assays were performed as described (Sung et al., 2005). Briefly, *in vitro*-translated Myc tagged Pellino-1 prepared by the TNT-coupled Reticulocyte Lysate System (Promega) was incubated with the SUMO-1 mixture in a total volume of 20 μ l in the presence of 50 mM Tris-HCl (pH 7.5), 2 mM DTT, 0.05% Tween-20, and 3 mM ATP at 37°C for 1 h. The SUMO-1 mixture is composed of 500 ng of purified E1 (GST-SAE1/SAE2) enzyme, 400 ng of GST-Ubc9 (E2 enzyme), and 2 μ g of GST-SUMO-1.

In vitro GST pull-down assays

Myc-tagged Pellino-1 was subjected to *in vitro* translation using a TNT-coupled Reticulocyte Lysate system (Promega). GST pull-down assays were performed by incubating the *in vitro* translated Myc-Pellino-1 with equal amounts of either GST or GST-Ubc9 fusion protein immobilized onto glutathione-sepharose beads.

Yeast two hybrid assay

The AH109 yeast strain was used as the host for a yeast two-hybrid interaction assay. AH109 cells were transformed with the

plasmids encoding GAL4-DBD-fused Pellino-1 and GAL4-AD-fused Ubc9, and the interactions of Pellino-1 with Ubc9 were observed in AH109 cells according to the protocol previously described (Sung et al., 2005). Cells were spread on synthetic drop-out agar plates lacking tryptophan and leucine (TL) or tryptophan, leucine, and histidine (TLH). The specificity of the interaction and binding strengths of the two fusion proteins were verified by streaking several colonies onto agar plates lacking TL and TLH.

Immunoblotting

For immunoblot analysis, cells were lysed in NETN buffer (20 mM Tris-Cl (pH 8.0), 1 mM EDTA, 100 mM NaCl, 0.5% (v/v) Nonidet P-40, protease inhibitor cocktail) with 10 mM N-Ethylmaleimide. Protein extracts were separated by SDS-PAGE, followed by transfer to nitrocellulose membrane and immunoblot analysis.

RESULTS AND DISCUSSION

Since Pellino-1 is known to be ubiquitinated in IL-1R/TLR signaling (Butler et al., 2007), we tested whether other modifications of the Pellino-1 protein exist and found that Pellino-1 is covalently modified by the SUMO-1 protein. Myc-Pellino-1 and SUMO-1 expression plasmids were co-transfected into HeLa cells, and total cell lysates were prepared in the presence of 10 mM N-ethylmaleimide (desumoylation enzyme inhibitor) and subsequently immunoblotted against anti-Myc antibody. SUMO-1-conjugated Pellino-1 was detected as three slower migrating bands compared with unconjugated Pellino-1 (Fig. 1A).

SUMO modification is facilitated by three main enzymatic components. A SUMO-specific E1 activating enzyme, AOS1-UBA2 (also known as SAE1/SAE2 in humans), activates SUMO proteins by conjugation, in an ATP-dependent manner. The E1 activating enzyme subsequently transfers activated SUMO to the E2 conjugating enzyme Ubc9. Ubc9 transfers SUMO to lysine residues in the Ψ KxE/D consensus sequence of the substrate, facilitated by SUMO E3 ligases in a substrate-dependent manner (Hay, 2005; Johnson, 2004; Melchior, 2000; Yeh, 2009). We next examined the SUMO-1 conjugation of Pellino-1 through an *in vitro* sumoylation assay using affinity-purified SAE1/2, Ubc9, and SUMO-1. Three slower migrating bands, representing covalent SUMO-1 modification of Pellino-1, were only detected when all of the components necessary for sumoylation were included (Fig. 1B). In addition, sumoylation of Pellino-1 was inhibited by treatment with SUMO-1 specific protease 1 (SENP1), whereas expression of a dominant negative SENP1 gene, with the catalytic cysteine residue at position 603 changed into serine (Bailey and O'Hare, 2004), further increased the sumoylation of Pellino-1, as expected. These results indicate that Pellino-1 is covalently modified by SUMO-1 at multiple lysine residues.

Since it is known that Ubc9 binding to the target protein is required for SUMO modification (Johnson, 2004), we next investigated whether the Ubc9 protein binds to Pellino-1. A yeast two hybrid assay showed that Pellino-1 fused to the GAL4 DNA binding domain specifically interacts with Ubc9 fused to the GAL4 activation domain in minimal media deficient in leucine, tryptophan, and histidine (Fig. 2A). Also, a GST pull-down assay using *in vitro* translated Myc-Pellino-1 protein revealed that the Pellino-1 protein specifically binds to GST-Ubc9 (Fig. 2B).

To determine the sumoylation sites of Pellino-1, site-directed mutagenesis was performed to change each lysine residue of Pellino-1 to an arginine residue. When an *in vitro* sumoylation assay with full-length Pellino-1 is conducted, three distinct bands

Fig. 1. Pellino-1 is modified by SUMO-1. (A) Immunoblot analysis of Myc-Pellino-1 from the lysates of HeLa cells which were transiently transfected with the Myc-Pellino-1 expression plasmid in the presence or absence of a SUMO-1 expression plasmid. (B) *In vitro* sumoylation assays of Myc-Pellino-1 in the absence of E1 enzyme, E2 enzyme, or SUMO-1, respectively. All components (SUMO-mixture) are needed for the *in vitro* sumoylation to occur (lane 5). (C) The plasmids encoding Myc-Pellino-1 and SUMO-1 were transiently transfected into HeLa cells with or without the plasmid encoding HA tagged SUMO-1-specific protease 1 (SEN1) and dominant negative HA-tagged-SEN1. Pellino-1 sumoylation was detected by immunoblot analysis with anti-HA antibody.

representing the sumoylated Pellino-1 are observed (Fig. 1B). Substitution of Lys-202 (K202R) or Lys-266 (K266R) to arginine abolished the upper or middle band, respectively, in an *in vivo* sumoylation assay (Fig. 3A; lanes 3 and 4). When both Lys-202 and Lys-266 of Pellino-1 were substituted with arginines (2KR), both the upper and middle bands simultaneously disappeared while the lower band was still detected (Fig. 3A; lane 5). To identify the sumoylation sites resulting in the lower band, we constructed an extensive number of Pellino-1 point mutants with various combinations of lysine residues changed into arginines. The simultaneous substitution of Lys-295, Lys-297, and Lys-303 (K295/297/303R) caused the disappearance of the lower sumoylated band, implying that the three lysine residues (Lys-295, Lys-297, and Lys-303) are additional acceptor sites for SUMO-1 during Pellino-1 sumoylation (Fig. 3B; lane 5). Finally, we constructed a point mutant in which all five lysine residues (Lys-202, Lys-266, Lys-295, Lys-297, Lys-303) were substituted with arginines (5KR), and observed that the 5KR Pellino-1 mutant was no longer modified with SUMO-1 (Fig. 3B; lane 9). These results indicate that five lysine residues, Lys-202, Lys-266, Lys-295, Lys-297, and Lys-303, are the acceptor sites for SUMO-1 in the Pellino-1 protein (Fig. 3C).

In this study we demonstrated that Pellino-1 is covalently modified with SUMO-1 protein by the E2 conjugating enzyme Ubc9. We also provided evidence that five lysine residues of Pellino-1 are acceptor sites for SUMO-1 during Pellino-1 sumoylation.

Pellino-1 protein is a scaffolding protein which interacts with proteins such as IRAK1, IRAK4, and Smad6 (Choi et al., 2006; Jiang et al., 2003), and has a distinct structure with a C-terminal RING-like domain (Moynagh, 2009; Schavliege et al., 2006). To date, two post-translational modifications of the Pellino-1 protein have been reported, namely phosphorylation and ubiquitination (Butler et al., 2007; Moynagh, 2009; Smith et al., 2009). IRAK1 phosphorylates seven amino acid residues in Pellino-1 and the phosphorylation of Pellino-1 enhances its E3 ubiquitin ligase activity, which subsequently facilitates K63-linked polyubiquitination of IRAK1 (Ordureau et al., 2008; Smith et al., 2009). Interestingly, polyubiquitinated IRAK1 is reciprocally able to induce the polyubiquitination and degradation of Pellino-1, through an unknown biochemical mechanism (Butler et al., 2007). In addition, three lysine residues (Lys-169, Lys-202, and Lys-266) of Pellino-1 have been identified as the sites for ubiquitina-

Fig. 2. Pellino-1 interacts with Ubc9. (A) Yeast two hybrid assay of GAL4-DBD fused Pellino-1 with GAL4-AD fused Ubc9. AH109 cells transformed with the indicated plasmids were grown on synthetic drop-out agar plates lacking tryptophan and leucine (TL) or tryptophan, leucine, and histidine (TLH). When interacting proteins are co-expressed, cells can survive in the TLH-lacking plate. pGBT7 and pGAD424 plasmids were used as a negative control. p53 and PTD1 expression plasmids were used as a positive control. (B) GST pull-down assay of Myc-Pellino-1 and GST-Ubc9. *In vitro*-translated Myc-Pellino-1 was incubated with GST or GST-Ubc9 and the bound proteins were eluted and resolved in SDS-PAGE, and subsequently immunoblotted with anti-Myc antibody. The amount of GST and GST-Ubc9 proteins loaded in this experiment is shown by Coomassie brilliant blue staining.

Fig. 3. Identification of the five sumoylation sites of Pellino-1. (A) Plasmids expressing Myc-Pellino-1 point mutants were transfected into HeLa cells with or without the SUMO-1 plasmid, and cell lysates were subsequently immunoblotted with anti-Myc antibody. WT: full-length Pellino-1. K202R, K266R, 2KR (K202/266R): Each indicated lysine residue was changed into arginine. (B) Plasmids expressing Myc-Pellino-1 point mutants were transfected into HeLa cells with or without SUMO-1 plasmid, and cell lysates were subsequently immunoblotted with anti-Myc antibody. WT: full-length Pellino-1. K202R, K266R, K295/297R, K303R, K295/297/303R, 2KR (K202/266R), 2KR+K295/297R, 2KR+K303R, 5KR (K202/266/295/297/303R): Each indicated lysine residue, or combination of residues, was mutated into arginine. (C) Schematic representation of Pellino-1 sumoylation sites. The numbers indicate the locations of the amino acids in Pellino-1.

tion *in vitro* (Smith et al., 2009). These findings and our present results indicate that Pellino-1 post-translational modification is likely to be critical for the distinct biochemical functions of Pellino-1.

To our knowledge, this is the first report showing that Pellino-1 is subject to SUMO modification. The acceptor sites for SUMO-1 are five lysine residues of Pellino-1 (Lys 202, Lys 266, Lys 295, Lys 297 and Lys 303), which partially overlap with the three lysine residues (Lys-169, Lys-202, and Lys-266) used for ubiquitination (Smith et al., 2009). The presence of two common lysine residues (Lys-202 and Lys-266) indicates the possibility that Pellino-1 sumoylation may compete with the ubiquitination process.

In general, SUMO proteins have been found to bind to lysine residues in the consensus sequence Ψ Kx Ψ /D (Ψ is hydrophobic amino acid and x is any amino acid residue) of target proteins (Rodríguez et al., 2001). However, similar consensus SUMO binding sequences were not observed surrounding the five lysine residues identified as the sumoylation sites in Pellino-1. These sites may be atypical, or may indicate novel binding sequences.

The sumoylation of a target protein has been found to be re-

lated to a variety of cellular functions, including transcriptional regulation, protein trafficking, DNA repair and mitotic cell division (Hay, 2005; Johnson, 2004; Zhao, 2007). However, it remains unknown how the SUMO modification of Pellino-1 affects the IL-1R/TLR signaling cascades, and how it relates to other modifications of Pellino-1. It is also formally possible that SUMO modification of Pellino-1 may be related to a unique cellular function unrelated to its role in the innate immune response. Thus, functional analysis of sumoylated Pellino-1 will likely yield new insights into the molecular functions of Pellino-1.

ACKNOWLEDGMENTS

We thank Dr. Min Sung Choi for critical reading of the manuscript. This work was supported by a National Research Foundation grant of Korea (2009-008176) and in part by a National Research Foundation grant of Korea (ROA-2007-000-20047-0 and C00650 to S.H.P.) funded by the Korean Government.

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