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The conserved sumoylation consensus site in TRIM5α modulates its immune activation functions

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ABSTRACT

TRIM5α is a type I interferon-stimulated anti-retroviral restriction factor expressed in most primates and homologous proteins are expressed in other mammals. Through its C-terminal PRYSPRY (B30.2) domain, TRIM5α binds to incoming and intact post-fusion retroviral cores in the cytoplasm. Following this direct interaction, the retroviral capsid core is destabilized and progression of the virus life cycle is interrupted. Specific recognition of its viral target by TRIM5α also triggers the induction of an antiviral state involving the activation of transcription factors NF-κB- and AP-1. In addition to PRYSPRY, several other TRIM5α domains are important for anti-retroviral function, including a RING zinc-binding motif. This domain has "E3" ubiquitin ligase activity and is involved in both the direct inhibition of incoming retroviruses and innate immune activation. A highly conserved sumoylation consensus site is present between the RING motif and the N-terminal extremity of TRIM5α. No clear role in restriction has been mapped to this sumoylation site, and no sumoylated forms of TRIM5α have been observed. Here we confirm that mutating the putatively sumoylated lysine (K10) of the Rhesus macaque TRIM5α (TRIM5α_{Rh}) to an arginine has only a small effect on restriction. However, we show that the mutation significantly decreases the TRIM5α-induced generation of free K63-linked ubiquitin chains, an intermediate in the activation of innate immunity pathways. Accordingly, K10R decreases TRIM5α-mediated activation of both NF-κB and AP-1. Concomitantly, we find that K10R causes a large increase in the levels of ubiquitylated TRIM5α. Finally, treatment with the nuclear export inhibitor leptomycin B shows that K10R enhances the nuclear localization of TRIM5α_{Rh}, while at the same time reducing its level of association with nuclear SUMO bodies. In conclusion, the TRIM5α sumoylation site appears to modulate the E3 ubiquitin ligase activities of the adjacent RING domain, promoting K63-linked ubiquitin chains at the expense of auto-ubiquitylation which is probably K48-linked. Consistently, we find this sumoylation site to be important for innate immune activation by TRIM5α. In addition, lysine 10 regulates TRIM5α nuclear shuttling and nuclear localization, which may also be related to its role in innate immunity activation.

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1. Introduction

Proteins belonging to the TRIM5 (tripartite motif 5) family are known for their species-specific anti-retroviral activity (Sayah et al., 2004; Stremlau et al., 2004; Towers, 2007). Restriction mediated by TRIM5 proteins relies mainly on their ability to recognize incoming intact capsids early after entry of the virus (Sebastian and Luban, 2005). TRIM5α orthologs differ from each other mostly in the C-terminal hypervariable PRYSPRY domain that confers specificity to retroviral capsid (CA) (Keckesova et al., 2004; Perron et al., 2004; Song et al., 2005; Yap et al., 2004), e.g. HIV-1 is inhibited by rhesus macaque TRIM5α (TRIM5α_{Rh}), but not by the human version of the protein (Yap et al., 2004). Interactions between TRIM5α proteins and CA lead to the inhibition of early steps of the virus life cycle by different mechanisms: disruption of capsid integrity (Black and Aiken, 2010; Perron et al., 2007; Stremlau et al., 2006), proteasomal degradation (Kutluay et al., 2013; Lukic et al., 2011; Rold and Aiken, 2008) and impairment of nuclear import (Campbell et al., 2007). In addition, recent studies have shown that TRIM5α-CA interactions or the over-expression of TRIM5α lead to the induction of an antiviral state that includes the activation of NF-κB and AP-1

Abbreviations: CA, capsid; HEK, human embryonic kidney; HTLV, human T-cell leukemia virus; IF, immunofluorescence; IP, immunoprecipitation; LMB, leptomycin B; MOI, multiplicity of infection; NB, nuclear body; PBS, phosphate buffer saline; PML, promyelocytic leukemia; SUMO, small ubiquitin-like modifier; TRIM, tripartite motif; VSV G, glycoprotein of the vesicular stomatitis virus; WT, wild type.

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(Pertel et al., 2011; Tareen and Emerman, 2011b; Uchil et al., 2013). This pathway also involves the generation of "free" (unconjugated) K63-linked ubiquitin chains (Pertel et al., 2011).

TRIM5α contains several domains located upstream of the PRYSPRY domain, including RING, B-Box-2 and Coiled-coil (RBCC) domains (Reymond et al., 2001). The RING domain has an intrinsic E3 ubiquitin ligase activity essential for the auto-polyubiquitylation of the protein (Diaz-Griffero et al., 2006; Li et al., 2013; Lienlaf et al., 2011; Maegawa et al., 2010), which is assumed to proceed through K48-linked ubiquitin chains. This process is stimulated by contact with restriction-sensitive viruses and it contributes to the destabilization of retroviral cores (Kutluay et al., 2013; Rold and Aiken, 2008). Deleting the RING domain or disrupting its zinc-binding properties by mutagenesis abrogates the capacity of TRIM5α to activate NF-κB and AP-1 and to stimulate K63-linked poly-ubiquitylation (Pertel et al., 2011; Uchil et al., 2013).

Similar to the ubiquitylation process, sumoylation is a post-translational modification involved in many cellular mechanisms including cell signaling, transcription and regulation of protein stability (Zhao, 2007). Three main isoforms of SUMO are found in mammalian cells, including SUMO-1, which can only achieve mono-sumoylation, and SUMO-2/3 that are able to form SUMO chains (Vertegaal, 2007). SUMO proteins are localized mostly in the nucleus and modify target proteins through covalent attachment to a lysine present in a specific consensus site, Ψ-K-X-D/E (Gocke et al., 2005; Sampson et al., 2001). TRIM5α carries such a sumoylation consensus motif, that is conserved in all known orthologs (not shown), upstream of the RING domain. Despite the fact that TRIM5α was never identified as a sumoylated protein, recent publications proposed a role for SUMO-1 in TRIM5α-mediated restriction (Arriagada et al., 2011; Lukic et al., 2013). However, this conclusion was disputed by another report (Brandariz-Nunez et al., 2013).

The main objective of this study was to determine whether the sumoylation consensus site could have a role in TRIM5α-mediated immune activation. Our results suggest that the putatively sumoylated lysine 10 (K10) of TRIM5α_{Rh} modulates the activity of the RING domain by decreasing auto-ubiquitylation while increasing the generation of K63-linked ubiquitylation. In conclusion, the consensus sumoylation site of TRIM5α_{Rh} is involved in the regulation of ubiquitylation pathways that control the activation of innate immunity.

2. Materials and methods

2.1. Plasmid DNAs and mutagenesis

pMIP-TRIM5α_{Rh} expresses a C-terminal FLAG-tagged version of the Rhesus macaque TRIM5α and has been described before (Bérubé et al., 2007; Sebastian et al., 2006). Directed mutagenesis was performed to introduce K10R, using the following mutagenic primers: 5′-TTCCTCGAGATGGCTTCTGGAATCCTGCTTAATGTAAGG-GAGGAGGTGACCTGT (forward) and 5′-TCCTGAATTCTTACTTA-TCGTCGTCATCCTTGTAATC (reverse). The K10R substitution was confirmed by Sanger sequencing. The vector production plasmids pMD-G, pΔR8.9, pCL-Eco and pTRIP-CMV-GFP have all been extensively described elsewhere (Aiken, 1997; Berthoux et al., 2003, 2004; Naviaux et al., 1996; Zufferey et al., 1997). pRK5-HA-Ubiquitin WT and KO (Lim et al., 2005) were obtained from Ted Dawson (Johns Hopkins University, Baltimore, MD) through Addgene. KO ubiquitin bears the following mutations eliminating all possibilities of ubiquitin chain formation: K6R, K11R, K27R, K29R, K33R, K48R and K63R. pCEP4-NF-κB-Luc expresses firefly luciferase under the control of an NF-κB-dependent promoter, while pCEP4-ΔNF-κB-Luc is transcriptionally deficient due to the deletion of the NF-κB binding site (Tareen and Emerman, 2011a). Both constructs were kind gifts from M. Emerman (University of Washington, Seattle, WA). pHTS-AP1, a kind gift from J. Luban (University of Massachusetts Medical School, Worcester, MA), expresses firefly luciferase under control of an AP-1-dependent promoter (Pertel et al., 2011).

2.2. Cell lines

Human embryonic kidney (HEK) HEK293 T cells, HeLa human adenocarcinoma cervical cells and CRFK feline kidney cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics at 37 °C, 5% CO_2. All cell culture reagents were from Hyclone (Thermo Scientific, Logan, UT, USA).

2.3. Virus production

MLV and HIV-1-based vectors were produced through transient transfection of HEK293 T cells using polyethylenimine (MW 25,000; Polysciences, Warrington, PA) and collected as previously described (Bérubé et al., 2007; Pham et al., 2010). To produce the MLV-based MIP vectors, cells were transfected with the relevant pMIP plasmid and co-transfected with pCL-Eco and pMD-G. All stably transduced cell lines were produced as previously described (Bérubé et al., 2007; Pham et al., 2010). Successfully transduced cells were selected with puromycin, using concentrations of 2 μg/ml (HeLa cells) and 4 μg/ml (CRFK cells). To produce GFP-expressing HIV-1 vectors, cells were co-transfected with pΔR8.9, pMD-G and pTRIP-CMV-GFP as described previously (Bérubé et al., 2007).

2.4. Viral challenges

HeLa and CRFK cells were plated in 24-well plates at 50,000 cells per well and infected the next day with different amounts of HIV-$1_{TRIP-CMV-GFP}$ (nicknamed "HIV-1-GFP") or using a defined multiplicity of infection (MOI). Two days post-infection, cells were trypsinized and fixed in 2% formaldehyde in a PBS solution. The % of GFP-positive cells was then determined by analyzing 10,000 cells using a FC500 MPL cytometer with the CXP software (Beckman Coulter).

2.5. Western blotting

Cells were lysed in RIPA buffer (NaCl 150 mM, Triton 1%, SDS 0.1%, TRIS 50 mM (pH 8.0), sodium deoxycholate 0.5% and Complete protease inhibitor cocktail (Roche, Bale, Switzerland)). Whole cell lysates were then boiled in protein sample buffer (60 mM Tris–Cl pH6.8, 10% glycerol, 0.002% bromophenol blue, 2% SDS, 2% beta-mercaptoethanol) and resolved by SDS-PAGE. After transfer to nitrocellulose membranes, blots were probed with various antibodies as specified throughout the text and visualized using secondary antibodies coupled to horseradish peroxydase (Santa Cruz, Dallas, TX) and a chemiluminescence detection system (SuperSignal West Femto, Thermo scientific, Waltham, MA). The horse radish peroxidase-conjugated anti-actin antibody (Santa Cruz) was used to control for equal loading across lanes. Images were recorded on a UVP (Upland, California) EC3 imaging system, and densitometry analyses were performed using the area density tool of the VisionWorks LS software (UVP).

2.6. Immunoprecipitation and auto-ubiquitylation

HEK293 T cells were co-transfected with either WT or mutant FLAG-tagged TRIM5α, and a plasmid expressing HA-tagged WT

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