TRIM5α DETERMINANTS FOR THE ACTIVATION OF INNATE IMMUNITY

201LB

Marie-Édith Nepveu-Traversy and **Lionel Berthoux**

Dept of Medical Biology and BioMed research group, Université du Québec à Trois-Rivières, Canada.

ABSTRACT

Background: Specific recognition of incoming viral capsids by the anti-retroviral factor TRIM5α disrupts progression of the virus life cycle and also triggers the induction of an antiviral state involving the activation of transcription factors NF-κB and AP-1. Additionally, TRIM5α promotes the formation of K63-linked ubiquitin chains which are thought to be important for innate immunity activation. These functions can also be revealed by over-expressing TRIM5α in the absence of virus, providing a convenient experimental model. A RING zinc-binding motif with E3 ubiquitin ligase activity is found at the N-terminus of TRIM5α and is involved in both the direct inhibition of incoming retroviruses and in innate immune activation. Other TRIM5α motifs were found to be related to the sumoylation pathway, specifically a putatively sumoylated lysine (lysine 10) just upstream of the RING domain, and putative SUMO interaction motifs (SIMs) in the C-terminal PRYSPRY domain which also determines interaction with capsid. Here, we analyzed the role of lysine 10 and the SIMs in the activation of innate immunity by TRIM5α.

Methodology: We searched for novel SIMs in Rhesus macaqueTRIM5α by computational analysis. Lysine 10, the RING domain and the various SIMs of the macaque TRIM5α PRYSPRY domain were mutated and the impact on HIV-1 restriction and on markers of innate immunity activation was measured using specific antibodies for K63-linked ubiquitin and reporter plasmids for NF-κB and AP-1 activation. Co-localization of TRIM5α and nuclear SUMO-1 was observed by IF microscopy.

Results: As expected, mutating the RING domain completely abrogated activation of NF-κB, AP-1 or the generation of K63-linked ubiquitin. Mutating Lysine 10 significantly decreased the activation of NF-κB and AP-1, and reduced the generation of K63-linked ubiquitin. The K10R mutation also caused an increase in TRIM5α self-ubiquitination, unlike mutations in the RING domain which also abrogate this activity. We identified a novel putative SIM motif in PRYSPRY, which we named SIM4. Mutating SIM4 strongly decreased activation of NF-κB while having no effect on the capacity of TRIM5α to trigger the formation of K63-linked ubiquitin chains. Mutations in both lysine 10 and SIM4 decreased the transient nuclear association of TRIM5α with SUMO-1.

Conclusions: Motifs in TRIM5α that are linked to the sumoylation pathway are important for the efficient activation of innate immunity. Lysine 10 modulates the ubiquitin ligase activity of the nearby RING domain to promote K63-linked ubiquitination, while SIM4 has no effect on K63-linked ubiquitin but is nonetheless important for the activation of NF-κB. Thus, we have identified motifs of TRIM5α that differentially modulate activation of innate immunity.

Acknowledgements: Michael Emerman, Ted Dawson and Jeremy Luban (reagents); Canadian Institutes of Health Research (funding).

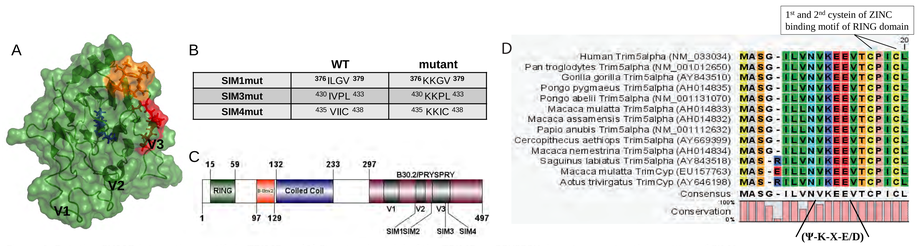


Figure 1. Putative SUMO interacting motifs in TRIM5α$_{Rh}$ (A) Structural model of the TRIM5α$_{Rh}$ PRYSPRY domain mapping the position of SIM1 (blue), SIM3 (red) and SIM4 (orange). The localization of variable regions V1, V2 and V3 is shown. **(B)** Table showing the mutations introduced in TRIM5α$_{Rh}$ with their respective amino acid position. **(C)** Schematic of the full-length TRIM5α$_{Rh}$ protein depicting its main domains and the positions of the various SIMs. **(D)** A conserved putative sumoylation site upstream of the RING domain

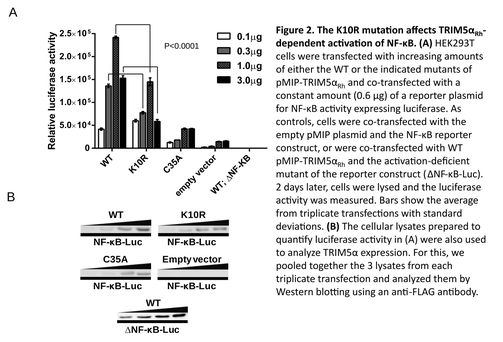


Figure 2. The K10R mutation affects TRIM5α$_{Rh}$-dependent activation of NF-κB. (A) HEK293T cells were transfected with increasing amounts of either the WT or the indicated mutants of pMIP-TRIM5α$_{Rh}$ and co-transfected with a constant amount (0.6 μg) of a reporter plasmid for NF-κB activity expressing luciferase. As controls, cells were co-transfected with the empty pMIP plasmid and the NF-κB reporter construct, or were co-transfected with WT pMIP-TRIM5α$_{Rh}$ and the activation-deficient mutant of the reporter construct (ΔNF-κB-Luc). 2 days later, cells were lysed and the luciferase activity was measured. Bars show the average from triplicate transfections with standard deviations. **(B)** The cellular lysates prepared to quantify luciferase activity in (A) were also used to analyze TRIM5α expression. For this, we pooled together the 3 lysates from each triplicate transfection and analyzed them by Western blotting using an anti-FLAG antibody.

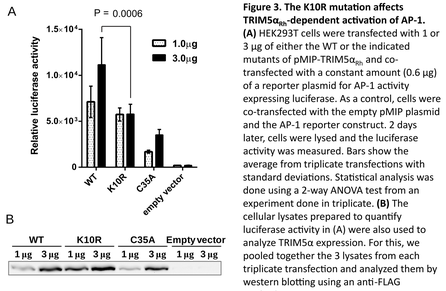


Figure 3. The K10R mutation affects TRIM5α$_{Rh}$-dependent activation of AP-1. (A) HEK293T cells were transfected with 1 or 3 μg of either the WT or the indicated mutants of pMIP-TRIM5α$_{Rh}$ and co-transfected with a constant amount (0.6 μg) of a reporter plasmid for AP-1 activity expressing luciferase. As a control, cells were co-transfected with the empty pMIP plasmid and the AP-1 reporter construct. 2 days later, cells were lysed and the luciferase activity was measured. Bars show the average from triplicate transfections with standard deviations. Statistical analysis was done using a 2-way ANOVA test from an experiment done in triplicate. **(B)** The cellular lysates prepared to quantify luciferase activity in (A) were also used to analyze TRIM5α expression. For this, we pooled together the 3 lysates from each triplicate transfection and analyzed them by Western blotting using an anti-FLAG antibody.

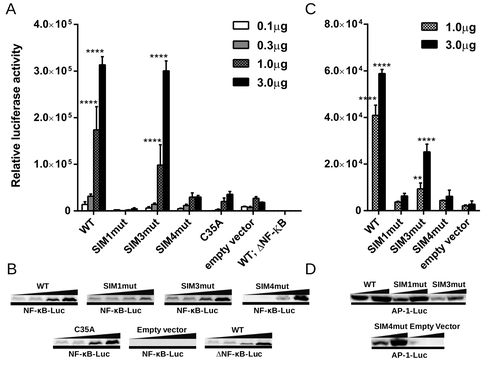


Figure 4. SIM4mut affects TRIM5α$_{Rh}$-mediated activation of NF-κB and AP-1. (A) HEK293T cells were transfected with increasing amounts of either the WT or the indicated mutants of pMIP-TRIM5α$_{Rh}$ and co-transfected with a constant amount (0.6 μg) of a reporter plasmid expressing luciferase from a NF-κB-dependant promoter. As controls, cells were co-transfected with the empty pMIP plasmid and the NF-κB reporter construct, or were co-transfected with WT TRIM5α$_{Rh}$ and the activation-deficient mutant of the reporter construct (ΔNF-κB-Luc). Two days later, cells were lysed and luciferase activity was measured. Bars show averages from triplicate transfections with standard deviations. *P*-values were calculated using a two-way ANOVA test. **(B)** The cellular lysates prepared to quantify luciferase activity in (A) were also used to analyze TRIM5α expression. Specifically, the three lysates from each triplicate transfection were pooled and analyzed by western blotting using an anti-FLAG antibody. **(C)** Same as (A), using a reporter plasmid expressing luciferase from an AP-1-dependant promoter. Two different DNA concentrations (1.0 and 3.0 μg) of the different pMIP plasmids expressing WT, SIM1mut, SIM3mut or SIM4mut TRIM5α$_{Rh}$ were co-transfected with a constant concentration of AP-1-Luc (0.6 μg). The empty vector was used as a negative control. The graph shows relative luciferase activity 48 h post transfection. The experiment was performed in triplicate and standard deviations are shown. *P*-values were calculated with a two-way ANOVA test: * < 0.05, **<0.01, ***<0.001, ****<0.0001. **(D)** TRIM5α protein expression was analyzed as described in (B)

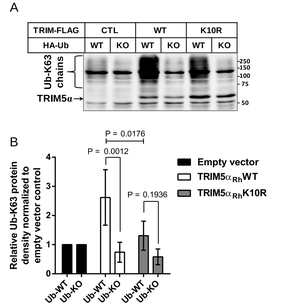


Figure 5. The K10R mutation decreases the ability of TRIM5α$_{Rh}$ to form K63-linked ubiquitin chains. (A) HEK293T cells were transfected with either the "empty" pMIP or with pMIP expressing FLAG-tagged WT or K10R pMIP-TRIM5α$_{Rh}$, and co-transfected with the plasmid expressing WT or KO HA-Ubiquitin. KO Ubiquitin has all its lysines mutated to arginines. Two days later, whole protein lysates were analyzed by western blotting using rabbit antibodies directed at K63-linked ubiquitin chain or at FLAG. **(B)** Density of bands detected using the K63 chains-specific antibody (ranging from 75 to >250 kDa) normalized to the control consisting of cells co-transfected with WT or KO HA-Ub and the "empty" pMIP. Graph shows the average data from 4 independent experiments with standard deviations, and *P*-values were calculated using a two-way ANOVA test.

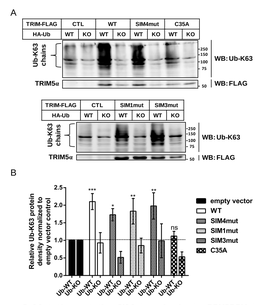


Figure 6. Mutating putative SIM domains in the PRYSPRY domain does not affect TRIM5α$_{Rh}$ ability to trigger K63-linked ubiquitin chains formation. (A) HEK293T cells were transfected with either the empty pMIP or with pMIP expressing FLAG-tagged WT, SIM1mut, SIM3mut or SIM4mut TRIM5α$_{Rh}$, and co-transfected with WT or KO HA-Ubiquitin. KO Ubiquitin has all its lysines mutated to arginines to prevent poly-ubiquitylation. C35A TRIM5α$_{Rh}$ was used as a negative control for its incapacity to significantly trigger Ub-K63 chains formation. Two days later, whole protein lysates were analyzed by Western blotting using rabbit antibodies directed at K63-linked ubiquitin chain or at FLAG. A single experiment representative of three independent experiments is presented. **(B)** The graph represents the mean densities of bands detected using the K63 chains-specific antibody (ranging from 75 to >250 kDa) normalized to the control (CTL) consisting of cells co-transfected with WT or KO HA-Ub and the empty pMIP. Data were obtained in three independent experiments and standard deviations are shown on the graph. *P*-values were calculated using a two-way ANOVA test: *< 0.05, **<0.01, ***<0.001.

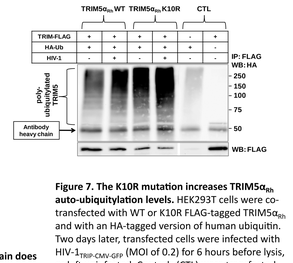


Figure 7. The K10R mutation increases TRIM5α$_{Rh}$ auto-ubiquitylation levels. HEK293T cells were co-transfected with WT or K10R FLAG-tagged TRIM5α$_{Rh}$ and with an HA-tagged version of human ubiquitin. Two days later, transfected cells were infected with HIV-1$_{TRIM-CAN-GFP}$ (MOI of 0.2) for 6 hours before lysis, or left uninfected. Controls (CTL) were transfected with WT FLAG-TRIM5α$_{Rh}$ alone or with HA-ubiquitin alone and were not infected. FLAG-tagged proteins were immunoprecipitated as described in Materials and Methods. The IP products were analyzed by western blotting first using a mouse anti-FLAG antibody and subsequently with a rabbit anti-HA antibody. All lanes shown are from a single western blot experiment and were exposed the same amount of time but some irrelevant lanes were cut out.

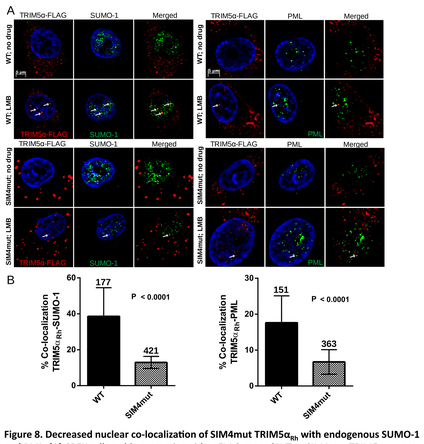


Figure 8. Decreased nuclear co-localization of SIM4mut TRIM5α$_{Rh}$ with endogenous SUMO-1 and PML. (A) CRFK cells stably expressing either FLAG-tagged WT or SIM4mut TRIM5α$_{Rh}$ were treated or not with LMB for 12 h and then processed for IF microscopy. A rabbit anti-FLAG antibody was used for the detection of TRIM5α$_{Rh}$ (red) and mouse antibodies were used for the detection of endogenous SUMO-1 and PML (green). DNA was stained with Hoechst33342 (blue). White arrows point to examples of co-localizations of nuclear TRIM5α$_{Rh}$ with SUMO-1 (left panels) or PML (right panels). **(B)** Quantitative analysis of the frequency of WT or SIM4mut TRIM5α$_{Rh}$ nuclear bodies (NBs) co-localization with either SUMO-1 (left panel) or PML (right panel). The percentage of WT and SIM4mut TRIM5α$_{Rh}$ NBs co-localizing with SUMO-1 or PML is shown. Each bar represents the mean % of co-localization observed in 15 randomly chosen fields considered as replicates. *P*-values were calculated using Student T-test and standard deviations are shown. The total number of TRIM5α$_{Rh}$ NBs in the 15 fields is shown on top of each bar.

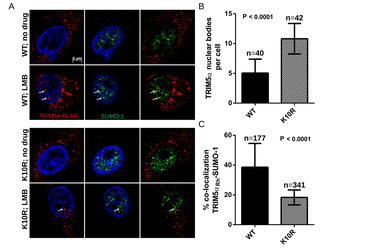


Figure 9. Decreased nuclear co-localization of K10R TRIM5α$_{Rh}$ and SUMO-1. (A) CRFK cells stably expressing either WT or K10R FLAG-tagged TRIM5α$_{Rh}$ and treated or not with LMB were processed for IF microscopy. A rabbit polyclonal anti-FLAG antibody was used for the detection of TRIM5α$_{Rh}$ (red) and a mouse monoclonal antibody was used for the detection of endogenous SUMO-1 (green). White arrows point to examples of co-localizations of nuclear TRIM5α$_{Rh}$ and SUMO-1. **(B)** Average number of TRIM5α$_{Rh}$ NBs per cell with standard deviation. The total number of nuclei analyzed is shown on top of each bar, and statistical analysis was done using a Student's t-test. **(C)** Quantitative analysis of the frequency of TRIM5α$_{Rh}$-SUMO-1 co-localization in nuclei. The percentage of WT and K10R TRIM5α$_{Rh}$ NBs co-localizing with SUMO-1 is shown. Each bar represents the % of co-localization observed in 25 randomly chosen nuclei (excluding nuclei that had no TRIM5α NBs) with standard deviation. Statistical analysis was done using a Student's t-test. The total number of NBs analyzed is shown on top of each bar.

CIHR IRSC
Canadian Institutes of Health Research
Instituts de recherche en santé du Canada