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Journal of Cellular Biochemistry 115:1430–1440 (2014)

Journal of Cellular Biochemistry

SUMOylation Attenuates the Transcriptional Activity of the NF-κB Subunit RelB

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ABSTRACT

The NF-κB subunit RelB is known to act either as an activator or repressor of NF-κB-dependent gene expression. The RelB–p52 heterodimer, for instance, is the key element of the alternative NF-κB signaling pathway supporting the expression of a subset of NF-κB target genes. By contrast, RelB is crucial for the repression of important pro-inflammatory cytokines like TNFα or interleukin 1β. Despite accumulating reports describing the functional variability of RelB, the molecular mechanisms underlying these divergent functions are still unknown. One potential explanation could be a functional reprogramming of RelB by different post-translational modifications. Here, we demonstrate that SUMOylation of RelB might be one of these post-translational modifications rendering the function of the NF-κB transcription factor RelB. In vivo SUMOylation analyses using either the UBC9-fusion-directed SUMOylation method or endogenous proteins from Namalwa B cells revealed that RelB is modified by either SUMO1 or SUMO2 attachment at various sites. Functional studies suggest that SUMOylation converts RelB into a transcriptional repressor. For instance, a SUMO1-RelB fusion protein mimicking RelB-SUMOylation displayed a reduced transcriptional activity in comparison to wild type RelB. Consistently, inactivation of specific SUMOylation sites in the central part of RelB augmented the transcription activity of the corresponding RelB mutant. Taken together, our data suggest that SUMOylation might be a potential molecular mechanism involved in reprogramming RelB, thus contributing to its functional diversity. J. Cell. Biochem. 115: 1430–1440, 2014.

KEY WORDS: NF-κB; RelB; SUMOylation; GENE EXPRESSION

The transcription factor NF-κB (nuclear factor κB) plays a crucial role for the regulation of immune and inflammatory responses, development, and cancer (Hayden and Ghosh, 2004; Ghosh and Hayden, 2008; Hayden and Ghosh, 2008). The basis for the pleiotropic effects of this transcription factor is the functional divergence of the different NF-κB dimers, composed of the subunits RelA, RelB, c-Rel, NF-κB1/p50, and NF-κB2/p52. Inactive NF-κB is restrained in the cytoplasm of resting cells by the members of the IκB (inhibitor of NF-κB) family which become site-specifically phosphorylated upon cell stimulation by the multisubunit IKK (IκB kinase) complex, composed of the kinases IKK1 and IKK2, and the adaptor protein NEMO (NF-κB essential modulator), and it is then subsequently degraded by the proteasome. Liberated NF-κB translocates to the nucleus, where it supports the expression of various NF-κB target genes. Besides this canonical NF-κB signalling pathway, an alternative IKK1-dependent pathway has been described which is characterized by the activation of RelB–p52 heterodimers and the expression of a specific subset of NF-κB target genes. In addition to its role in the alternative NF-κB pathway, RelB is also linked to the termination of NF-κB-dependent gene

Abbreviations: EGFP, enhanced green fluorescence protein; IB, immunoblot; IP, immunoprecipitation; NRS, normal rabbit serum; PMA, phorbol-12-myristate-13-acetate; SUMO, small ubiquitin-like modifier; TNFα, Tumor necrosis factor alpha; WCE, whole cell extracts.

JL and CV contributed equally to this work.

The authors have no conflict of interest to declare.

Grant sponsor: German Research Foundation (DFG); Grant number: MA2367/3-2.

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Manuscript Received: 10 October 2013; Manuscript Accepted: 20 February 2014

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 25 February 2014

DOI 10.1002/jcb.24794 • © 2014 Wiley Periodicals, Inc.

expression or to the silencing of genes encoding pro-inflammatory cytokines (McCall and Yoza, 2007). Thus RelB either acts as an activator or a repressor of NF-κB target gene expression by not yet fully understood mechanisms. The recruitment of co-activators or co-repressors like DAXX, EZH2 or G9a by RelB might be an explanation (Croxton et al., 2006; Puto and Reed, 2008; Chen et al., 2009; Lee et al., 2011; Liu et al., 2011). However, it still remains unknown how this recruitment of co-activators or co-repressors by RelB is achieved. One possible mechanism might be specific post-translational modifications of RelB and thereby creating novel interaction platforms. In general, post-translational modifications such as phosphorylation, ubiquitination or acetylation are crucial for the fine tuning of the NF-κB transcription factors. For instance, the recruitment of the co-activator CBP [CREB (cAMP-response-element-binding protein)-binding protein] to the NF-κB subunit RelA depends on either site-specific phosphorylation or acetylation of RelA (Zhong et al., 2002). RelB, on the other hand, has been shown to be phosphorylated or ubiquitinated thereby affecting its stability and transcriptional activity (Marienfeld et al., 2001; Leidner et al., 2008). Another post-translational modification reported to modulate the functionality of transcription factors is the conjugation of SUMO peptides at lysine side chains, a process which is termed SUMOylation. Examples for transcription factors which are converted into transcriptional repressors are NF-ATc1, C-EBP or c-Jun (Muller et al., 2000; Kim et al., 2002; Chang et al., 2005; Nayak et al., 2009; Zimnik et al., 2009). SUMOylation of a target protein involves a set of enzymes quite similar to the ubiquitination machinery with the SUMO activating proteins (E1), the SUMO conjugating protein UBC 9 (E2) and a panel of SUMO ligases (E3). However, in contrast to ubiquitination, SUMOylation does not necessarily require the presence of an E3 protein, while it still requires the E2 protein UBC9 (Jakobs et al., 2007).

In a previous study, we reported that a constitutive RelB polyubiquitination not only modulates the stability of RelB but also augments the activity of this NF-κB subunit (Leidner et al., 2008). Since SUMOylation and ubiquitination frequently alter the function of the modified protein in an antagonistic manner (Anderson et al., 2012), we aimed to determine whether SUMOylation might be a molecular mechanism to transform RelB into a transcriptional repressor. Indeed, by using different in vivo SUMOylation assays we provide evidence that RelB is a SUMOylation target. Furthermore, here we demonstrate that this SUMOylation exerts a negative effect on RelB activity while the DNA-binding capacity of RelB remains unaltered. In line with these findings is our observation that a RelB mutant with inactivated SUMOylation sites displays an augmented activity in comparison to $RelB_{WT}$. Collectively, our results support the idea that SUMOylation might be involved in the negatively reprogramming of RelB.

MATERIALS AND METHODS

CELLS, REAGENTS AND ANTIBODIES

Antibodies for RelB (sc-226), SUMO1 (sc-9060), SUMO2 (sc-32873), RelA (sc-372), NF-κB1/p50 (sc-114X) and UBC9 (sc-10759) were purchased from Santa Cruz Biotechnology. The anti-FLAG antibody (F1804) and anti-FLAG antibody coupled sepharose was from Sigma. The antibody recognizing IκBα (#4812) was from Cell Signaling and the anti-p52 antibody (US 06-413) from Upstate Biotechnology. 293 HEK cells were propagated in DMEM + 10% FCS containing streptomycin and penicillin. For the cultivation of the B cell lines, Namalwa and S107 RPMI-media + 10% FCS including streptomycin and penicillin was used.

EXPRESSION VECTORS AND IN VITRO MUTAGENESIS

The expression vectors encoding p50, p52, full-length FLAG–RelB or FLAG–RelA were described previously (Maier et al., 2003; Marienfeld et al., 2003). The FLAG–SUMO1–RelB plasmid was cloned by inserting a PCR-generated murine SUMO1 cDNA in frame into the HindIII and NotI restriction sites of the pFLAG–RelB vector. The vector encoding for the RelB-UBC9 fusion protein was generated by inserting the PCR amplified murine cDNA of RelB in frame into the pCU vector using the NotI and EcoRI sites. The NF-κB-dependent luciferase reporter construct (3xκB) and the renilla luciferase reporter construct under the control of the ubiquitin-promoter have been described elsewhere (Leidner et al., 2008). Expression vectors for EGFP-SUMO1, EGFP-SUMO2 or UBC9 (pNU, pCU) have been described previously (Jakobs et al., 2007). The luciferase reporter construct driven by the human TNFα promoter was a kind gift of Dr. Stephan Ludwig (University of Münster, Germany). For the construction of the BIRC3 luciferase reporter plasmid, a fragment of the human BIRC3 gene promoter spanning the region −1400 to +1 was inserted into the XhoI and HindIII sites of the pGL4.20 luciferase vector (Promega). For the in vitro mutagenesis, the QuickChange in vitro mutagenesis kit was used following the manufacturers protocol. Sequences of the oligonucleotides used for cloning or in vitro mutagenesis are available upon request.

IN SILICO IDENTIFICATION OF POTENTIAL SUMOylation SITES

For the prediction of potential SUMOylation sites in murine or human RelB cDNA the SUMOsp 2.0, SUMOplotTM, and the PCI-Based Sumo Site Prediction Server software were used.

IN VIVO SUMOylation ANALYSIS

For the in vivo SUMOylation analysis of ectopically expressed proteins, 293 HEK cells were transiently transfected with expression vectors coding for EGFP-SUMO1, UBC9 (pNU) and Flag-tagged RelB. For the UFDS method, 293 HEK cells were transiently transfected with the various pCU-RelB expression vectors alone or in combination with expression vectors encoding either EGFP–SUMO1 or EGFP–SUMO2. After 48 h the cells were treated with 20 mM N-ethylmalemide for 5 min and were afterwards lyzed in TNT-buffer (200 mM NaCl, 20 mM HEPES pH 7.6, 1% Triton X-100, 1 mM DTT, NaF, Na-glycerophosphate) and 90% of the resulting whole cell extracts was subjected to an anti-FLAG or anti-RelB immunoprecipitation, the remaining 10% was used for control immunoblot analyses. For anti-FLAG immunoprecipitations 10 μl anti-FLAG antibody coupled sepharose (Sigma) was used. The precipitates were washed extensively with TNT + 0.5% SDS and PBS prior to the separation of the precipitated proteins by standard SDS–PAGE. Following transfer of the separated proteins on a

nitrocellulose membrane the SUMOylation was monitored by either an anti-SUMO1, anti-SUMO2 or anti-RelB immunoblot analysis.

QUANTIFICATION OF SUMOylated FLAG-TAGGED RelB

To estimate the relative alteration of the SUMOylation level of FLAG-tagged RelA or RelB upon coexpression of EGFP–SUMO1, the intensities of the signals obtained with the anti-SUMO1 and anti-FLAG antibodies as well as the background were measured using the NIH ImageJ software. The background values were subtracted and the values obtained in the anti-FLAG immunoblot were used to normalize the SUMO1 measurements. The overall SUMOylation of FLAG–RelA or FLAG–RelB was calculated by summing all normalized SUMO1 values obtained in an individual lane. For the relative SUMOylation, a ratio of the overall SUMOylation without and with coexpressed EGFP–SUMO1 was calculated.

TRANSFECTION AND LUCIFERASE REPORTER ASSAY

To determine the activity of RelB, cells were transiently transfected with 200 ng per well of a 24 well plate of either a NF-κB-dependent reporter (3xκB-luc), the TNFα promoter controlled firefly luciferase construct (TNFαluc) or the BIRC3 promoter controlled firefly luciferase reporter construct (BIRC3) along with with 15 ng per well of a 24 well plate a plasmid encoding a Renilla luciferase under the control of the human ubiquitin-promoter as internal control. Lipofectamine LTX + PLUS reagent (Invitrogen) was used to transfect S107 cells and the $CaPO_4$ method for the transfection of 293 HEK cells. Briefly, on the day before transfection the cells were plated on an appropriate tissue culture dish. On the day of transfection the DNA was incubated with a 200 mM $CaCl_2$ solution for 5 min prior to the addition of an appropriate volume of 2xHeBS (280 mM NaCl, 1.5 mM Na_2HPO_4, 50 mM HEPES, pH 7.04). After another 5 min of incubation the sample was added to the cells. The cells were subsequently incubated for 24–48 h. For TNFα stimulation, the cells were either left untreated (control, C) or were stimulated with TNFα (10 ng/ml) for 4 h. For determination of the luciferase activity, the cells were lyzed in TNT-buffer and the activity of the renilla as well as the firefly luciferase was measured according to the manufacturer's protocol. Firefly luciferase activity was normalized to the approriate Renilla luciferase values. The experiments were done in duplicates and were repeated at least three times.

RNA EXTRACTION, cDNA SYNTHESIS AND qPCR ANALYSIS

Quantitative RT-PCR was performed by using the iCycler PCR instrument (Bio-Rad). Total RNA was prepared using RNeasy protect kit from Qiagen (Hilden, Germany) according to the manufacturer's protocol. 1 μg of total RNA was used to generate cDNA using First-Strand Synthesis kit (Invitrogen). qPCR was performed using 0.1 μl of cDNA reaction mix in the IQSYBRGreen supermix (Biorad). PCR was carried out as follows: after an initial 3 min preincubation step at 95 °C, 40 amplification cycles were run (95 °C for 30 s, 55 °C for 30 s, and 72 °C for 15 s). All qPCR samples were measured in triplicates. Quantification of gene regulation was performed by the ΔΔCp method. Results are presented relative to the expression of the house-keeping gene b-ACTIN. Sequences of Primers used for qPCR are available upon request. The primers for human TNFα were obtained from RealTimePrimers.

PREPARATION OF NUCLEAR AND CYTOPLASMIC PROTEIN SAMPLES AND WHOLE CELL EXTRACTS

Whole cell extracts were prepared by using TNT buffer (20 mM Tris pH8.0, 200 mM NaCl, 1% TritonX100, 1 mM DTT, 50 mM NaF, 50 mM β-glycerophosphate, 50 μM leupeptin, 1 mM PMSF) and isolation of cytoplasmic and nuclear proteins was achieved by subsequent incubation of the cells in buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) and buffer C (20 mM HEPES pH 7.9, 0.4 M KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, including protease inhibitors (complete protease inhibitor, Roche)). DignamC extracts were prepared by resuspending cells in DignamC buffer (20 mM HEPES pH 7.9, 25% glycerol, 0.42 M NaCl, 1,5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM PMSF, 1 mM DTT, supplemented with Complete mini protease inhibitor cocktail (Roche)) followed by three freeze-and-thaw cycles and a centrifugation step at 13000 rpm for 10 min at 4 °C.

IMMUNOPRECIPITATION AND IMMUNOBLOTTING

Immunoprecipitation and immunoblotting procedures were performed as described previously (Palkowitsch et al., 2011). In brief, 250–500 μg of protein extracts were mixed with 1 μg/sample of the appropriate antibody and samples were incubated for o.n. at 4 °C with agitation. After incubation, 10 μl of a 50% protein G slurry was added and the samples were further incubated for 1 h. Subsequently the precipitates were washed extensively in TNT-buffer (20 mM Tris pH 8.0, 200 mM NaCl, 1% Triton X100, 1 mM DTT, 50 mM NaF, 50 mM β-glycerophosphate, 50 μM leupeptin, 1 mM PMSF). The resulting immunopurified proteins were used for immunoblotting experiments. For the immunoblotting analysis, either the immunopurified protein complexes, or, as indicated, 50–100 μg of a protein extract were loaded on a standard SDS–polyacrylamide gel (PAA). SDS–PAGE and the transfer to nitrocellulose (SchleicherŁSchuell) or nylon membranes (Immobilon PVDF-membrane, Millipore) were performed using standard protocols. The membrane was blocked with 5% milk powder in TBS + Tween 20 prior to the incubation with the primary antibody (1:1000 in TBS + Tween 20), subsequently washed three times for 5 min each and incubated in a TBS-Tween 20 solution containing either horse-radish peroxidase conjugated or IRDye700/800 conjugated secondary antibody (1:5000). The detection was performed using either ECL-substrates from Amersham Biosciences or the Odyssey infrared scanning system (LICOR).

GEL SHIFT ANALYSIS

For the gel shift analysis (EMSA), 5 μg of nuclear proteins or whole cell extracts (DignamC extracts) from untreated or stimulated cells were incubated on ice for 20 min in a reaction containing 0.3 ng ^{32}P-labelled κB-specific or Oct-specific oligonucleotide, 1 μg pdI:dC and 3 μl of a binding buffer. The samples were separated on a native 5% PAA gel, the gel was dried and subjected to autoradiography.

RESULTS

RelB IS A SUMOylation TARGET IN VIVO

RelB is the key transcription factor of the alternative NF-κB pathway but is also known as a repressor of NF-κB target genes. To determine

whether SUMOylation might be one of the mechanisms involved in the reprogramming of RelB, we made use of two different experimental strategies. The first strategy is the UBC9 fusion-directed SUMOylation (UFDS-method) which depends on the expression of a RelB–UBC9 fusion protein (Jakobs et al., 2007). Fusing the SUMO-conjugating enzyme UBC9 enhances the SUMOylation of a given target protein at physiological SUMOylation sites independent of a SUMO-ligase (Fig. 1A). Such SUMO–RelB signals were observed upon transient transfection of HEK293 cells with the RelB–UBC9 encoding plasmid (pCU–$RelB_{FL}$) in combination with EGFP–SUMO1 or EGFP–SUMO2, respectively. Resulting whole cell extracts were either directly analyzed by RelB immunoblot (Fig. 1B, input, lanes 7–12) or used for RelB-specific immunoprecipitation (Fig. 1B, IP, lanes 1–6). As shown in Fig. 1B, expression of RelB–UBC9 alone led to the appearance of a high molecular smear (left part, lane 2) which was enhanced upon co-transfection of either a SUMO1- or a SUMO2-expression vector (left part, line 4 and 6). Moreover, SUMO1 or SUMO2 coexpression caused the appearance of several distinct additional bands visible in the anti-RelB immunoblot (upper panel) as well as by the multiple signals detected with either a SUMO1-specific antibody (middle panel, lane 4) or a SUMO2-specific antibody (lower panel, lane 6) suggesting that SUMOylation of RelB can occur at several sites. Of note, we also observed a SUMO1 signal at a height corresponding to the molecular weight of endogenous RelB (Fig. 1A, middle panel, labeled with an asterix), implying that also endogenous RelB is SUMOylated in HEK293 cells.

An increase of the in vivo SUMOylation of RelB was also observed in a more physiological in vivo SUMOylation assay. FLAG–RelB was transiently expressed in HEK293 cells, either alone or together with a combination of UBC9 and SUMO1. Since a PIAS3 mediated RelA-SUMOylation has recently been reported to modulate the activity of this NF-κB factor (Liu et al., 2012), we also included the analysis of

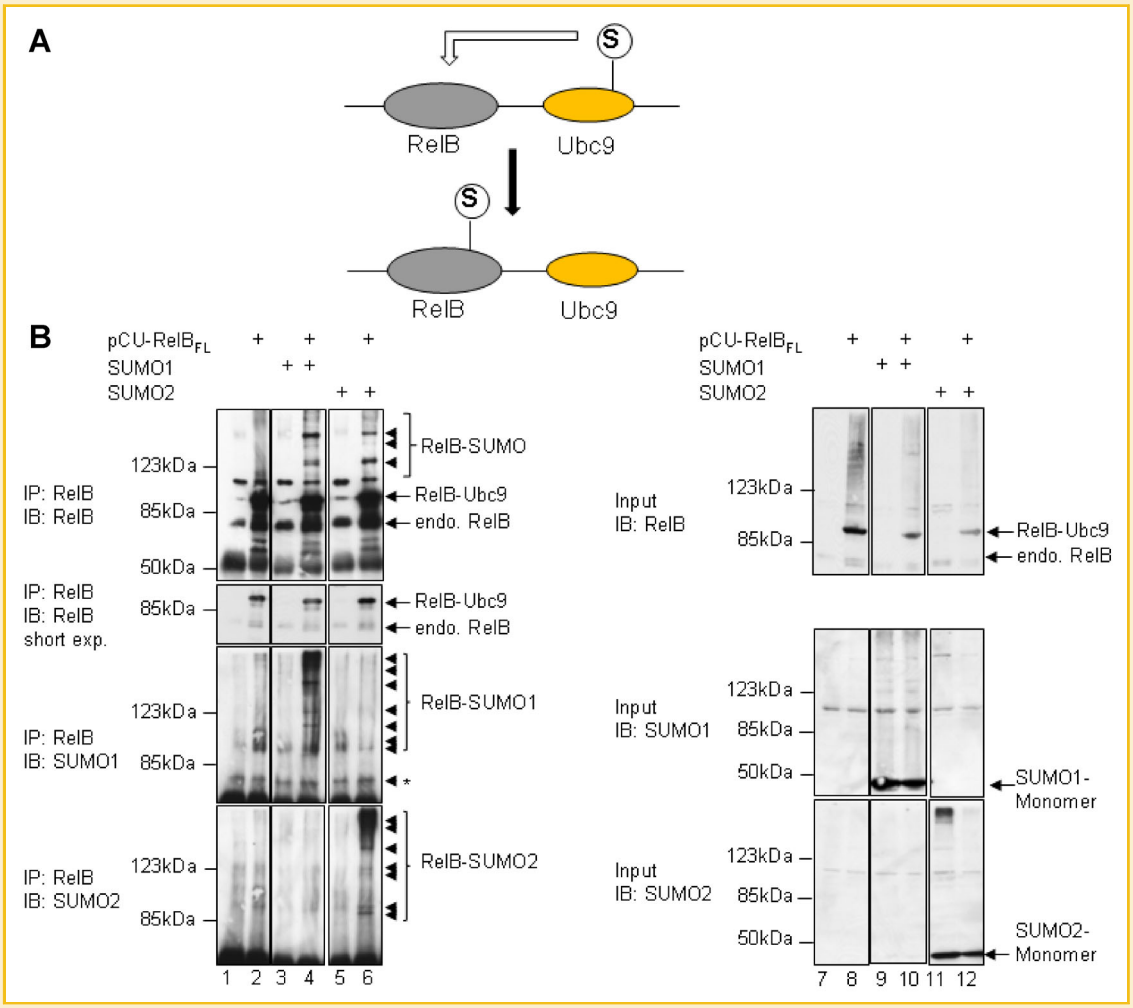

Fig. 1. In vivo SUMOylation of RelB determined by UFDS method. A, Model depicting the UFDS method used to define SUMOylation of RelB. B, In vivo SUMOylation assay of RelB using the UFDS method. HEK293 cells were transiently transfected with expression vectors encoding pCU–RelB, EGFP–SUMO1 and EGFP–SUMO2 as indicated. After 48 h whole cell extracts were prepared and were either subjected to immunoblot analyses (right part) or were used for anti-RelB IPs followed by immunoblot analyses with anti-RelB, anti-SUMO1 or anti-SUMO2 antibodies (left part). Signals corresponding to SUMOylated RelB proteins are marked with arrows. Enodogenous SUMOylated RelB is marked by an asterix.

RelA as a positive control in this experiment. Resulting whole cell extracts were either used for immunoblot analyses (Fig. 2A, input, lower part) or were subjected to anti-FLAG-immunoprecipitation experiments (Fig. 2A, IP, upper part). As shown in Figure 2A, anti-SUMO1 immunoblot analysis revealed a basal SUMOylation of RelB and of RelA (upper part, lane 7 and 8) even in absence of additional UBC9 and SUMO1. Furthermore, SUMOylation of RelB, but not of RelA, was considerably increased after co-expression of UBC9 and SUMO1 (compare lanes 7 + 8 with lanes 10 + 11, see also diagram in the lower part).

To analyze whether SUMOylation also occurs at the level of endogenous RelB, we performed in vivo SUMOylation studies with Namalwa B cells. RelB is highly expressed in B cells and is required for the development and function of B cells as well as for the pathogenesis of different B cell lymphomas (Gasparini et al., 2014). Stimulation of B lymphoma cells, including Namalwa cells, with phorbol myristate acetate and ionomycin (PMA/Iono) induces a degradation of RelB which is accompanied by its increased ubiquitination. To unravel whether PMA/Iono also influences RelB SUMOylation we treated Namalwa B cells with PMA/Iono for different times. The resulting whole cell extracts were used for an anti-RelB immunoprecipitation (IP) experiment including control precipitations with normal rabbit serum (NRS; Fig. 2B, middle part). In contrast to the control IP, anti-RelB IP revealed a basal

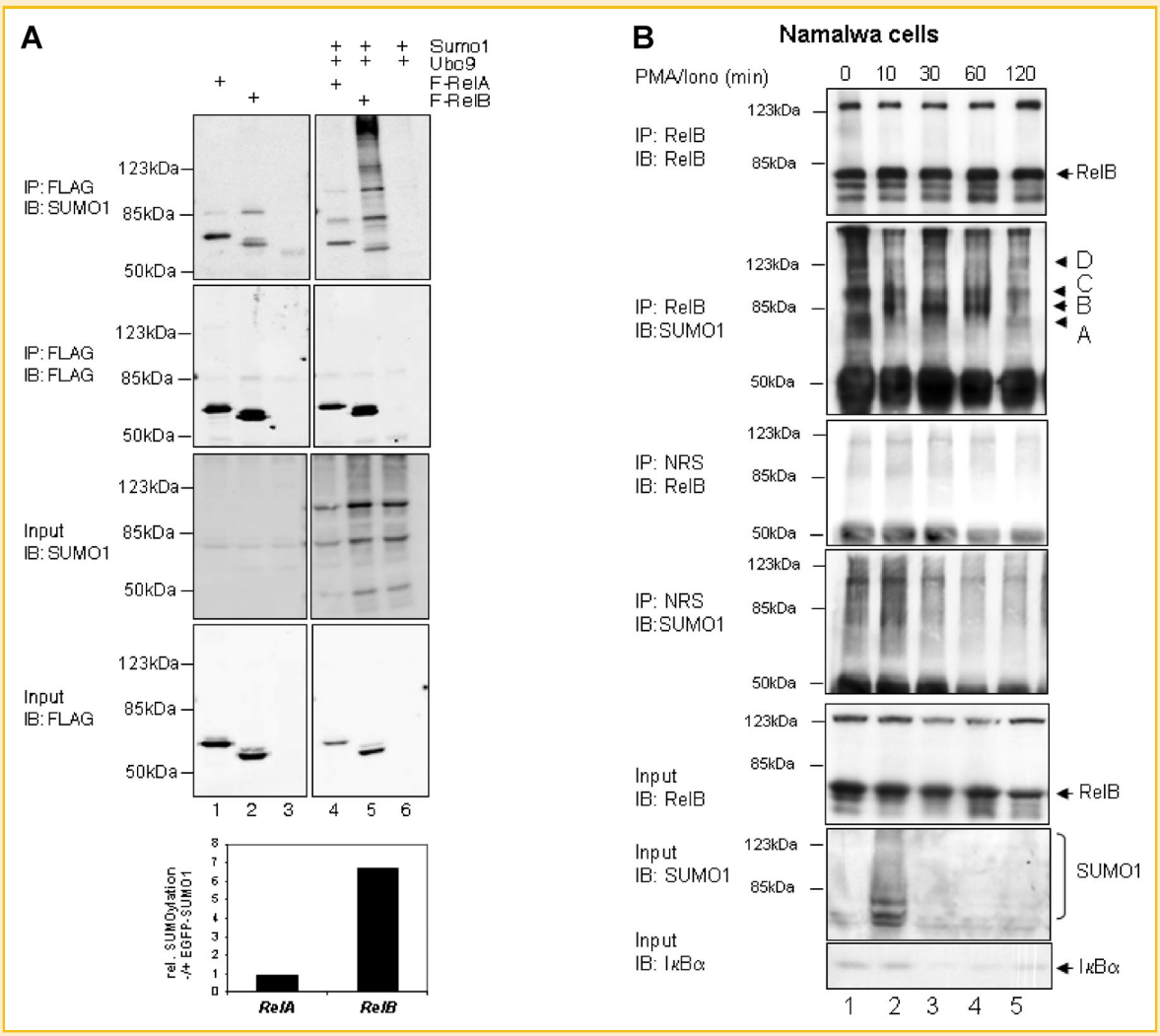

Fig. 2. In vivo SUMOylation analysis of RelB. A, Immunprecipitation was performed using whole cell extracts from HEK293 cells ectopically expressing FLAG–RelB, FLAG–RelA, alone or in combination with UBC9 and EGFP–SUMO1. Resulting precipitates were separated by SDS–PAGE, transferred to nitrocellulose membrane and subjected to immunoblot analyses with indicated antibodies (upper part). As a control for expression levels 10% of the whole cell extracts were subjected to additional immunoblots with the indicated antibodies (middle part). The ratio of SUMOylated FLAG–RelA or FLAG–RelB −/+ addition of UBC9 + EGFP–SUMO1 was calculated (lower part). B, SUMOylation of endogenous RelB in Namalwa B cells. Namalwa B cells were either left untreated (lane 1) or were stimulated with PMA + ionomycin for the indicated times (lanes 2–5). The resulting whole cell extracts were subjected to IP experiments using either RelB specific (upper part) or unspecific (normal rabbit serum, middle part) antibodies. To control the expression levels of RelB and SUMO1 and the successful cell stimulation, a fraction of the lysates (50 μg) was used for additional immunblot analyses using RelB, SUMO1 and IκBα-specific antibodies (lower part). Signal corresponding to SUMO1–RelB as designated as A–D.

endogenous RelB SUMOylation as indicated by several high molecular signals including two prominent SUMO1-specific signals with the size of approximately 85 kDa (signals A + C) and close to 120 kDa (signal D, Fig. 2B, IP, upper part). Interestingly, the SUMOylation pattern of RelB changed in the course of stimulation with a decrease of the basal RelB SUMOylation signals and the appearance of an additional, slightly slower migrating signal (signal B; Fig. 2B, upper part, lanes 2–4). However, this signal disappeared after 120 min (Fig. 2B, upper part, lane 5) while signal A reappeared. Interestingly, the molecular weight of two of the basal SUMOylation signals (signal A + D) corresponded well to major RelB signals of about 70 kDa and 120 kDa (upper panel).

Taken together, the results from the different in vivo SUMOylation analyses demonstrate that RelB can be modified by the conjugation of SUMO proteins.

SUMOylation ATTENUATES THE TRANSCRIPTIONAL ACTIVITY OF RelB

Since only a minority of RelB proteins is SUMOylated in the cell, we used the SUMO1–RelB fusion protein to mimick a constitutive RelB-SUMOylation and thus determine the functional consequences of a RelB SUMOylation (Fig. 3A). A similar experimental design was used to define the role of NF-AT and C/EBP SUMOylation (Berberich-Siebelt et al., 2006; Nayak et al., 2009). For the functional analysis, we performed luciferase reporter assays using three different luciferase reporter constructs. Besides a luciferase reporter construct which is under the control of the κB site from the Ig heavy chain enhancer (3xκBluc), we included also luciferase constructs which are driven by promoters of the described RelB target genes BIRC3 and TNFA. As expected, co-transfection of $RelB_{WT}$ augmented the activity of the 3xκBluc reporter (Fig. 3B, left part). By contrast,

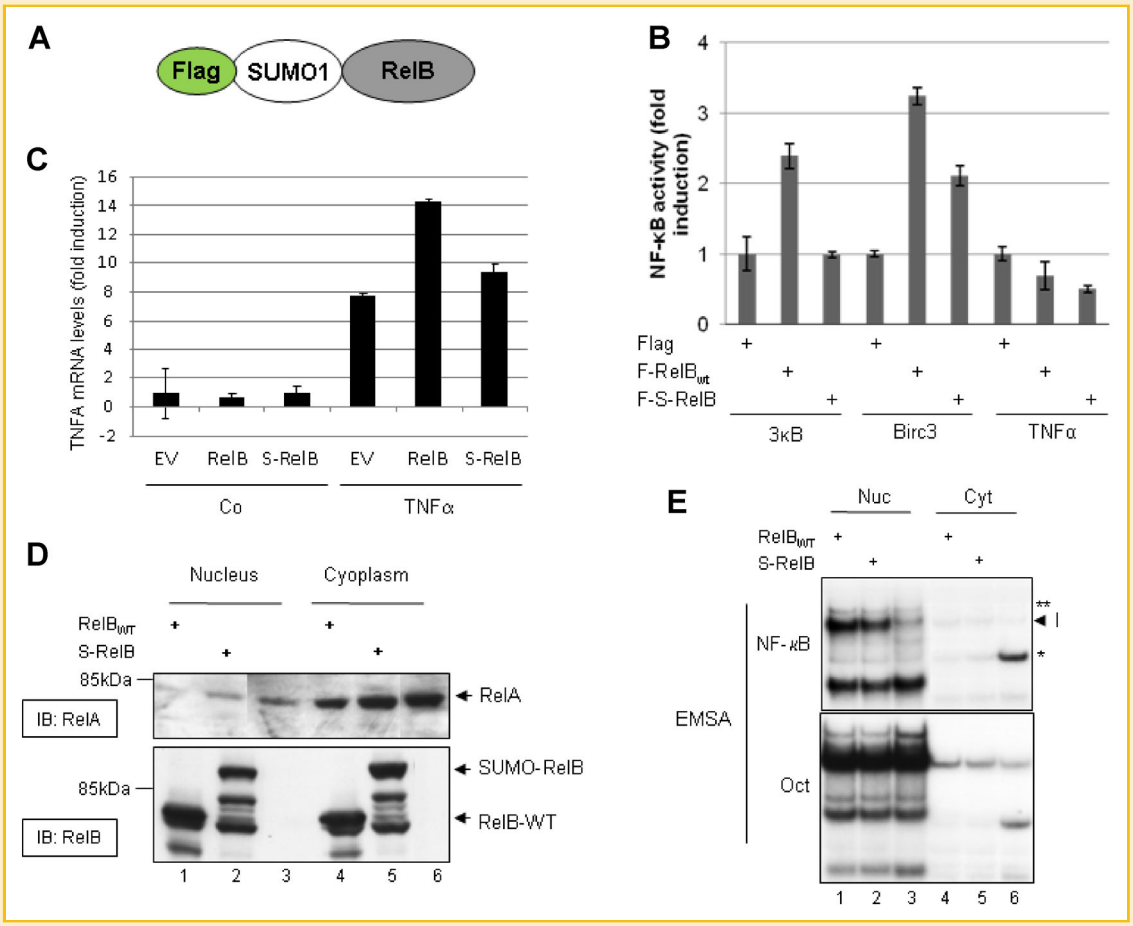

Fig. 3. SUMO1–RelB fusion protein displays a reduced transcriptional activity. A, Schematic of the FLAG-tagged SUMO1–RelB fusion protein. B, Luciferase reporter assays. HEK293 cells were transiently transfected with either 3xκBluc (left), a BIRC3luc (center), or a TNFαluc (right) reporter. Additionally, either the empty vector, the FLAG–RelB or the FLAG–SUMO1–RelB vector was cotransfected. Luciferase activities were measured 24 h post-transfection. All transfections were done in duplicates and the mean value and S.E.M. is depicted. C, Quantitative PCR analysis of TNFα mRNA levels in unstimulated 293 HEK cells (left part) or 293 HEK cells stimulated with TNFα. 48 h before stimulation the cells were transiently transfected with either empty vector, FLAG–RelB, or FLAG–SUMO–RelB. D, Subcellular distribution of FLAG–RelB and FLA–SUMO1–RelB in unstimulated HEK293 cells. Nuclear and cytoplasmic fractions of HEK293 cells transiently transfected with either FLAG–RelB or FLAG–SUMO1–RelB were prepared and subjected to immunoblot analysis with the indicated antibodies. E, DNA binding of the RelB variants. An EMSA experiment was performed using nuclear extracts (lanes 1–3) or cytoplasmic extracts (lanes 4–6) of HEK293 cells ectopically expressing FLAG–RelB or FLAG–SUMO1–RelB.

coexpression of the SUMO1–RelB fusion protein had no effect on basal 3xκB driven luciferase expression. Likewise, coexpression of $RelB_{WT}$ increased the activity of the BIRC3 reporter more efficiently then SUMO1–RelB (Fig. 3B, middle part). TNFA is another NF-κB target gene known to be regulated by RelB in a stimulus- and cell type-specific fashion. Macrophages derived from RelB-deficient mice display an attenuated TNFα expression, while RelB represses TNFα mRNA production in fibroblasts (Weih et al., 1997; El et al., 2007). To analyze the impact of RelB-SUMOylation on TNFα promoter activity, we used an additional luciferase reporter construct under the control of the TNFα promoter (Fig. 3B, right part) as well as a qPCR analysis of the endogenous TNFα mRNA levels (Fig. 3C). Cotransfection of $RelB_{WT}$ and the TNFαluc reporter caused a moderate decrease of the TNFα promoter activity, which was even more pronounced in case of a SUMO1–RelB coexpression (Fig. 3B, right part). We next analyzed the impact of either RelB or SUMO1–RelB on the endogenous TNFα gene expression in unstimulated or TNFα-stimulated HEK293 cells by qPCR. Here, neither RelB nor SUMO1–RelB caused any significant alterations of the basal TNFα expression. However, $RelB_{WT}$ expression augmented the TNFα expression after cell stimulation whereas ectopic expression of SUMO1–RelB had only a slight impact on TNFα expression (Fig. 3B, left part).

To exclude the possibility that the reduced activity of SUMO1–RelB is due to either an attenuated nuclear localization and/or DNA-binding ability of the SUMO1–RelB fusion protein, we determined the subcellular distribution of both RelB proteins and performed an EMSA experiment with nuclear extracts of transiently transfected HEK293 cells. Nuclear and cytoplasmic distribution of ectopic expressed SUMO1–RelB was comparable to the distribution seen with $RelB_{WT}$ (Fig. 3C). Likewise, EMSA analysis did not reveal a reduced DNA-binding activity of SUMO1–RelB compared to $RelB_{WT}$ (Fig. 3E).

Collectively, the functional analysis of SUMO1–RelB suggest that SUMOylation of RelB has a negative effect on the activity of this transcription factor, without affecting its nuclear localization or its DNA-binding capability, suggesting that this effect is mediated by secondary effects probably by the recruitment of co-repressors.

IDENTIFICATION OF RelB SUMOylation SITES

To identify the lysine residues within RelB which serve as SUMOylation acceptor sites, we first performed in silico SUMOylation analysis using three different SUMOylation prediction programs (see table one for detailed results). The results obtained by these in silico analyses varied and most of the potential SUMOylation sites were identified by either one or two of the three prediction programs (see Table I and Fig. 4A for a schematic representation). Only two lysine residues, Lys308 and Lys415, turned up with all SUMOylation prediction programs. However, in subsequent in vivo SUMOylation analyses using a panel of RelB–Ubc9 fusion proteins harbouring lysine-to-arginine substitutions at various positions (RelBM1–M14) no dramatic alteration of the SUMOylation pattern of RelB was observed (Fig. 4A and data not shown). Yet, by using a $RelB_{M15}$-mutant harbouring a combination of arginine substitutions at the positions 386, 387, 410, 413, 414, and 415 a pronounced drop of SUMO–RelB was observed in a further in vivo UFDS analysis [signal I, Fig. 4C, upper part, compare lane 7 (RelBWT) and lane 14 (RelBM15)]. Similarly, a reduction of

TABLE I. Prediction of SUMOylation Sites in RelB

Human RelB	SUMOplot™	SUMOsp2	SUMO site prediction	Murine RelB	SUMOplot™	SUMOsp2	SUMO site prediction
K62	−	−	+	K44	−	−	+
K134	−	−	−	K112	−	−	−
K162	−	−	−	K140	−	−	−
K187	−	−	−	K165	−	−	−
K201	−	−	+	K179	−	−	+
K232	+	+	−	K210	−	+	−
K233	−	−	−	K211	−	−	−
K242	−	−	+	K220	−	−	+
K256	−	+	+	K234	−	+	+
K295	−	−	+	K273	−	−	+
K296	−	−	−	K274	−	−	−
K310	−	−	−	K288	−	−	−
K327	−	−	−	K305	−	+	−
K330	+	+	+	K308	+	+	+
K362	+	−	−	K340	+	−	−
K409	−	−	−	K387	−	−	+
K410	−	−	−	K388	−	−	+
K412	+	+	−	K390	+	+	−
K433	−	+	−	K411	−	+	−
K436	−	+	+	K414	−	+	+
K437	+	+	+	K415	+	+	+
K438	−	−	−	K416	−	−	−

Results of the in silico analysis of the human RelB protein (left part) and the murine RelB protein (right part) using three different freely available SUMOylation prediction programs. Lysine residues identified by the indicated SUMOylation prediction software are labelled as "+", lysine residues not identified are labelled "−". In case of SUMOsp2 a score of >1.5 was considered to be positive for SUMOylation.

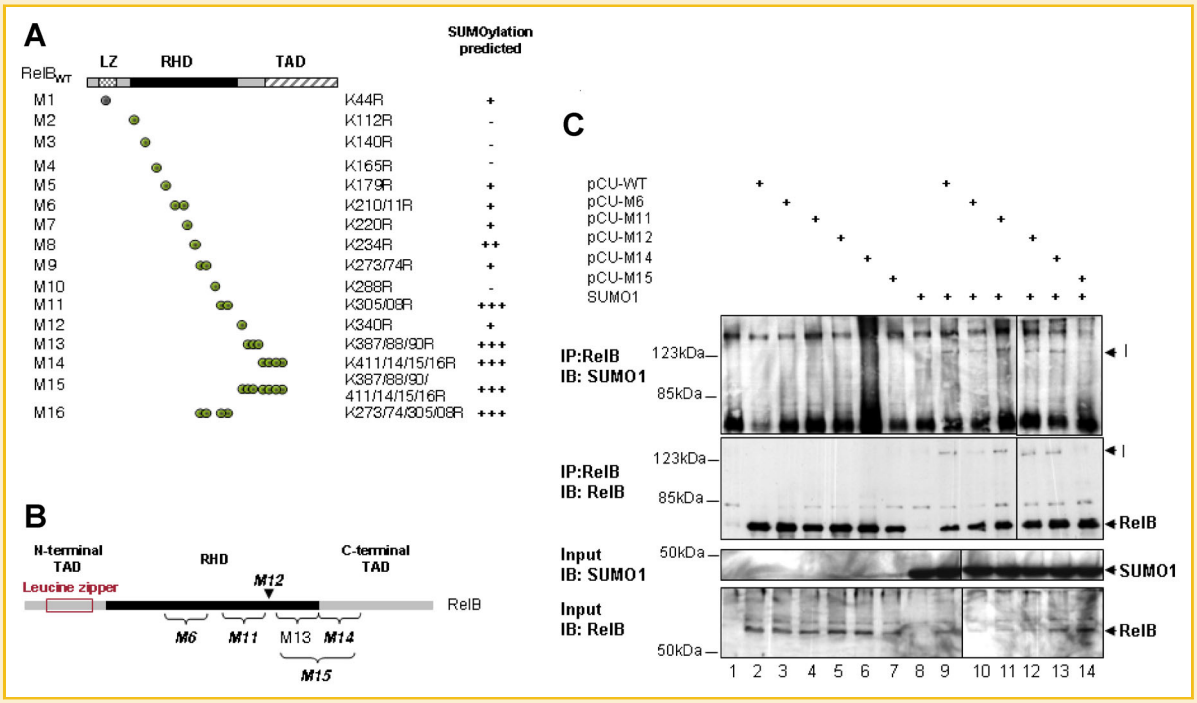

Fig. 4. Identification of SUMOylation sites in RelB. A, Schematic representation of the location of all lysine residues in the RelB protein and the RelB mutants with lysine-to-arginine substitutions. The results of the in silico prediction is given on the right side. Lysine residues predicted by either one, two or all three SUMOylation prediction programs are labeled "+", "++", or "+++", respectively. Lysine residues which are not predicted to be SUMOylation sites are labeled "−". B, Model of RelB depicting the location of the lysine residues analyzed in the in vivo SUMOylation assays. C, In vivo SUMOylation with the UFDS method using a panel of RelB–UBC9 variants as indicated. The indicated RelB–UBC9 fusion variants were expressed in HEK293 cells either alone (lanes 1–7) or in combination with additional EGFR–SUMO1 (lanes 8–14). The resulting whole cell extracts were subjected to anti-RelB IP experiments followed by immunoblot analyses with the indicated antibodies (IP; upper part). Additionally, a fraction of the WCEs was subjected to control immunoblots using the same antibodies (input; lower part). SUMOylated RelB is indicated (signal I).

RelB-SUMOylation was observed in an additional in vivo SUMOylation assay using the ectopic coexpression of distinct FLAG-tagged RelB mutants, UBC9, and SUMO1 (Fig. 5). Again, only the $RelB_{M15}$ mutant showed a loss of one distinct SUMOylation signal [signal I, Fig. 5A, compare lanes 4 ($RelB_{WT}$) and 8 ($RelB_{M15}$)]. In a previous study, we reported that the substitution of the lysines 273 and 274 ($RelB_{M9}$), lysines 305 and 308 ($RelB_{M11}$), and a combination of these lysines ($RelB_{M16}$) caused a reduction in basal and ubiquitination-induced RelB activity. However, as shown in Figure 5 (lanes 6, 7, and 8), SUMOylation of these RelB mutants remained unaltered when compared to $RelB_{WT}$ (lanes 4) or an unaffected RelB mutant ($RelB_{M8}$, lane 5). Thus, ubiquitination and SUMOylation seems to target different lysine residues in RelB.

Given the negative impact of the SUMO1 fusion on the activity of RelB (Figure 3), we hypothesized that an inactivation of a SUMOylation acceptor site would augment the RelB activity. Indeed, in a luciferase reporter assay using the BIRC3 reporter construct, the $RelB_{M15}$ mutant displayed an increased activity in comparison to $RelB_{WT}$ while SUMO1-RelB was less active as expected (Figure 5B).

To exclude the possibility that the higher activity seen with $RelB_{M15}$ is caused by an increased DNA-binding of this mutant, we performed electromobility shift assays (EMSA) with whole cell extracts from transiently transfected HEK293 cells. We compared a panel of RelB variants ($RelB_{WT}$, $RelB_{M13}$ (K386/387/388R), $RelB_{M14}$ (K410/413/414/415R), and $RelB_{M15}$ (K386/387/388/410/413/414/415R)) either alone, or in combination with ectopically expressed NF-κB1/p50 or NF-κB/p52. Of note, heterodimerization with NF-κB1/p50 or NF-κB/p52 is required for the DNA-binding of RelB, since RelB does not form active homodimers. Here, $RelB_{M15}$ showed no increased DNA-binding activity irrespective whether the RelB variants were expressed alone or in combination with either NF-κB1/p50 or with NF-κB2/p52 (Fig. 6).

In conclusion, our data suggest that SUMOylation is a molecular mechanism to negatively regulate the activity of RelB and that this negative influence of RelB-SUMOylation is not based on an altered DNA-binding of this NF-κB transcription factor.

DISCUSSION

NF-κB transcription factors regulate a wide variety of cellular processes, including inflammatory and immune responses. Several of the control mechanisms ensuring the temporal and spatial activity of NF-κB include post-translational modifications of the different NF-κB subunits (Perkins, 2006). For example, a tightly regulated recruitment of co-activators or co-repressors, such as CBP or HDAC1 (histone deacetylase 1) respectively, depends on site-specific phosphorylations or acetylations of RelA (Chen et al., 2002; Zhong et al., 2002). In contrast, the molecular mechanisms which determine whether RelB acts as either a gene-specific transcriptional activator

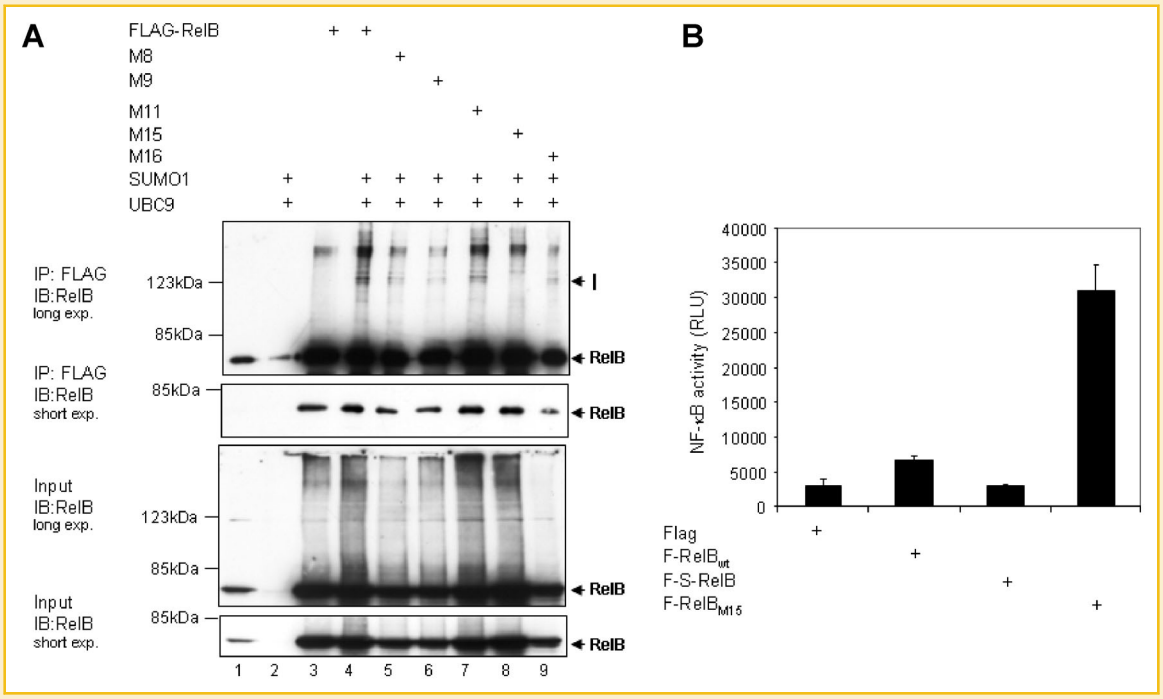

Fig. 5. Characterization of the RelB$_{M15}$ mutant. A, In vivo SUMOylation after coexpression of the indicated FLAG–RelB variants alone or in combination with UBC9 and EGFP–SUMO1. Anti-FLAG IP experiments were performed using the resulting WCEs. Subsequent immunoblot analyses were done using the RelB-specific antibody. B, Luciferase reporter assay. S107 cells were transiently transfected with the indicated RelB variant in conjunction with the BIRCluc reporter plasmid and the Ubi Renilla reporter plasmid. After 24 h the cells were harvested and the luciferase activity was determined as described in the material and methods section. SUMOylated RelB is indicated (signal I).

or repressor are currently largely unknown. In the present study, we set out to clarify whether a specific SUMOylation of RelB might be involved in the regulation of RelB functions. Indeed, we observed a distinct SUMOylation of RelB upon ectopic co-expression of FLAG–RelB, UBC9 and SUMO1 (Fig. 2A) or a UBC9–RelB fusion protein (Fig. 1A) as well as in Namalwa cells (Fig. 2B). SUMOylation of RelB occured at several sites and was achieved by using either SUMO1 or SUMO2 (Figs 1 and 2). Consistently, multiple potential SUMOylation sites in the center and carboxy-terminal part of the Rel homology domain (RHD) of RelB were also identified by three different SUMOylation prediction programs (Fig. 4A and Table I). Mutational analysis of FLAG–RelB as well as the UBC9–RelB fusion protein revealed the necessity of inactivating seven lysine residues at the positions 387, 388, 390, 411, 414, 415, and 416 to impair the formation of the main SUMOylation signal while the substitution of single lysine residues had no effect (Figs 4B and 5A). Taken together, these results clearly show that RelB is a SUMOylation target.

The SUMOylation of endogenous RelB in Namalwa B lymphoma cells changed upon stimulation of the cells with PMA + ionomycin (P + I). While the signals corresponding to the basal SUMOylation (A, C, and D) decreased after P + I stimulation, possibly due to the already described P + I-induced RelB degradation (Marienfeld et al., 2001), another signal (B) newly appeared (Fig. 2B) which might be based on a signal-induced SUMOylation of RelB. Signal induced changes in the SUMOylation pattern have been shown for a variety of transcription factors including heat shock transcription factor 1 (HSF1), myelin expression factor 2 (Mef2) and the erythroid transcription factor GATA-1 (Yang and Chiang, 2013). Additionally, an increased SUMOylation at Lys37 and Lys122 of RelA has been observed in TNFα-stimulated HEK293 cells (Liu et al., 2012). However, the augmented PIAS3-mediated RelA SUMOylation depends on the binding of this transcription factor to its cognate DNA site, whereas the signal-induced SUMOylation is driven by a preceding phosphorylation of the target protein which requires the presence of a specific phosphorylation-dependent SUMOylation motif (PDSM, ψKXEXXSP). Yet, none of the RelB SUMOylation sites identified either by the mutational screening or by the SUMOylation prediction programs is located within the context of such a phosphorylation-dependent SUMOylation motif. Thus, further work is required to clarify the molecular mechanisms underlying the appearance of the novel signal-induced RelB SUMOylation in P + I stimulated Namalwa cells.

Already known post-translational modifications of RelB include the phosphorylation at Thr84 and Ser552 as well as the poly-ubiquitination at various sites (Marienfeld et al., 2001; Leidner et al., 2008). Both post-translational modifications are involved in the regulation of the signal-induced RelB degradation in activated B and T cells. Moreover, poly-ubiquitination also augments the transcriptional activity of RelB, and the lysine residues at position 273, 274, 305 and 308 are required for this ubiquitination-induced activity increase. Interestingly, two of these ubiquitination sites at positions Lys273 and Lys305 were also predicted to be potential SUMOylation acceptors (Fig. 4A). However, no change in the SUMOylation pattern was observed using the appropriate RelB

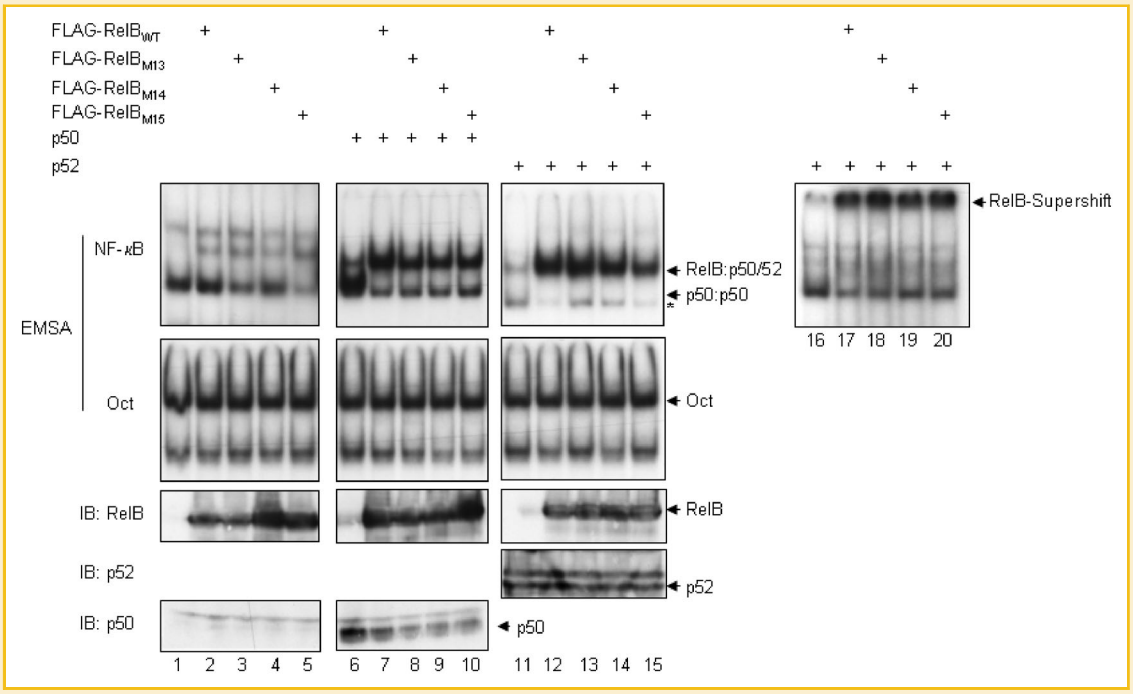

Fig. 6. Unaltered DNA-binding activity of the RelB$_{M15}$ mutant. EMSA experiments using 5 μg of dignamC extracts from HEK293 cells transiently transfected with expression vectors for the indicated FLAG–RelB variants alone or in combination with expression vectors for either NF-κB1/p50 or NF-κB/p52. To ensure similar expression levels of the different RelB-variants, additional immunoblot analyses with the indicated antibodies were performed with 30 μg protein.

mutants (RelBM9, RelBM11 and the combinatorial RelBM16 mutant) in the in vivo SUMOylation assays (Fig. 4B), suggesting that RelB SUMOylation and ubiquitination occurs at different sites.

Functionally, SUMOylation appears to attenuate the transactivation capacity of RelB. The SUMO1–RelB fusion protein, which mimicks its constitutive SUMOylation, for instance, exhibits a distinctly lower activity than wild-type RelB as measured by luciferase reporter assays or by qPCR analysis (Fig. 3B and C). Consistent with the attenuated activity of the SUMO1–RelB fusion protein is our finding that the SUMOylation defective RelBM15 mutant displays a far higher transcriptional activity than its wild-type counter part (Fig. 5B). These results are in line with previously published reports showing the negative impact of SUMOylation on the activity of a wide variety of transcription factors, including p53, c-EBP, SMAD4, the andogen receptor (AR) as well as the NF-κB subunit RelA (Muller et al., 2000; Chang et al., 2005; Cheng et al., 2005; Berberich-Siebelt et al., 2006; Nayak et al., 2009; Rytinki et al., 2011). Thus, although the sites within RelB modified either by ubiquitination or by SUMOylation are not identical, these two post-translational modifications have still antagonistic consequences for the function of RelB. In addition, the fact that SUMOylation and ubiquitination targets different lysine residues in RelB argues against a direct competition of these post-translational modifications which has been reported to control the stability of IκBα by targeting Lys21 (Lens et al., 2011).

Various molecular mechanisms underlying the negative impact of SUMOylation on the activity of transcription factors have been reported. While the SUMOylation of the AR and SMAD4 causes a recruitment of the co-repressor DAXX, it mediates a subnuclear redistribution of C-EBPβ and NF-ATc1 (Lin et al., 2004; Chang et al., 2005; Berberich-Siebelt et al., 2006; Nayak et al., 2009). In case of RelB, SUMOylation seems not to affect its nuclear localization or its DNA binding capacity as neither the SUMO1–RelB fusion protein nor the RelBM15 mutant displayed any changes in nuclear expression and DNA binding in comparison to wild-type RelB (Fig. 3D). Moreover, the efficient DNA binding of the SUMO1–RelB fusion protein or the RelBM15 mutant containing NF-κB dimers seen in Figure 3E and Figure 6 also suggests that the complex formation with either NF-κB/p50 or NF-κB/p52 is not affected by the SUMOylation of RelB. However, it is tempting to speculate that one possibility by which SUMOylation might negatively affect the activity of RelB could be an increased recruitment of co-repressors like DAXX and HDAC1 which are known to bind to SUMO moities by their SUMO interaction motifs (SIM). Importantly, DAXX is a known RelB binding partner, while HDAC1 has been demonstrated to interact with RelA or c-Rel (Zhong et al., 2002; Croxton et al., 2006). Experiments are under way to determine whether an increased binding to DAXX and/or to HDAC1 is molecular mechanism underlying the negative impact of SUMOylation on RelB activity.

ACKNOWLEDGMENTS

This work was supported by a grant from the German Research Foundation (DFG, MA2367/3-2 to R.B.M.). The authors like to thank Bernd Baumann, Thomas Wirth, and Stephan Ludwig for their kind gift of different plasmids.

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