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SUMOylation Regulates the Transcriptional Activity of JunB in T Lymphocytes

Johan Garaude,1,2 Rosa Farrás,4 Guillaume Bossis, Seyma Charni, Marc Piechaczyk, Robert A. Hipskind, and Martin Villalba2

The AP-1 family member JunB is a critical regulator of T cell function. JunB is a transcriptional activator of various cytokine genes, such as IL-2, IL-4, and IL-10; however, the post-translational modifications that regulate JunB activity in T cells are poorly characterized. We show here that JunB is conjugated with small ubiquitin-like modifier (SUMO) on lysine 237 in resting and activated primary T cells and T cell lines. Sumoylated JunB associated with the chromatin-containing insoluble fraction of cells, whereas nonsumoylated JunB was also in the soluble fraction. Blocking JunB sumoylation by mutation or use of a dominant-negative form of the SUMO-E2 Ubc-9 diminished its ability to transactivate IL-2 and IL-4 reporter genes. In contrast, nonsumoylatable JunB mutants showed unimpaired activity with reporter genes controlled by either synthetic 12-O-tetradecanoylphorbol-13-acetate response elements or NF-AT/AP-1 and CD28RE sites derived from the IL-2 promoter. Ectopic expression of JunB in activated human primary CD4+ T cells induced activation of the endogenous IL-2 promoter, whereas the nonsumoylatable JunB mutant did not. Thus, our work demonstrates that sumoylation of JunB regulates its ability to induce cytokine gene transcription and likely plays a critical role in T cell activation. The Journal of Immunology, 2008, 180: 5983–5990.

Activator protein-1 collectively describes a class of structurally and functionally related proteins that are characterized by a basic DNA-binding domain and a leucine-zipper dimerization motif. It principally comprises members of the Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra-1, and Fra-2) protein families. These proteins bind as dimers to specific sequences in the promoter and enhancer regions of target genes; the canonical site mediates activation by 12-O-tetradecanoylphorbol-13-acetate (TPA) and is termed a TPA response element (TRE). Additionally, some members of the activating transcription factor and Jun dimerization protein families can form AP-1 complexes, primarily with Jun proteins, and bind to TRE-like sequences (1, 2). Unlike the Jun proteins, Fos family members cannot form homodimers, but heterodimerize with Jun partners, giving rise to transactivating or transrepressing complexes with different biochemical properties.

Jun proteins are implicated in numerous cellular processes, such as transcriptional control, proliferation, differentiation, and tumorigenesis (3–6). In particular, JunB has a unique, nonredundant function in vivo, because its inactivation in mice causes vascular defects in extra-embryonic tissues that lead to embryonic lethality (7). In adult mice, JunB plays an important regulatory role in the hematopoietic system and tumorigenesis. The embryonic lethality of JunB−/− mice is reversed by expression of a junB transgene under the control of the Ubiquitin promoter (Ubi-JunB). However, these animals develop a myeloid hyperproliferation that progresses to a disease resembling chronic myeloid leukemia; remarkably, this corresponds to silencing of the Ubi-JunB transgene (8, 9). In agreement with this, junB expression is diminished in some human chronic myeloid leukemia (10) and B cell leukemias (11, 12). Similarly, JunB expression blocks leukemia induced by knockdown of PU.1 in mouse hematopoietic stem cells (13). Although this suggests that JunB is a tumor suppressor, it appears to be oncogenic in T cells, because some hyperproliferative T cell lymphomas show JunB overexpression (14–18). Accordingly, JunB is a critical regulator of the expression of cytokines important for T lymphocyte proliferation and differentiation, namely IL-4 (5, 19, 20) and IL-2 (21–23). Moreover, JunB expression and binding capacity are decreased in anergic T cells, which do not produce IL-2 (24–27).

The activity of AP-1 is critically modulated by post-translational modifications, in particular, phosphorylation mediated by MAPK cascades. The JNK pathway is an important regulator of c-Jun function; however, its role in JunB phosphorylation remains unclear, even though JunB contains a docking site for JNKs (5, 28, 29). Instead, JNKs regulate the E3 ubiquitin ligase Itch that targets JunB for ubiquitylation in T cells (30). Thus, mice lacking Itch show an accumulation of JunB in helper T cells, which leads to an increase in TH2 differentiation (31).

Sumoylation is a reversible modification that conjugates an ubiquitin-like peptide, SUMO (for small ubiquitin-like modifier),
onto specific lysine residues of substrate proteins (32–34). Three SUMO isotypes are expressed in mammalian cells. SUMO-1 shows 47% homology at the protein level with SUMO-2 and -3, whereas SUMO-2 and -3 are 95% homologous (33). Sumoylation of target proteins is a multistep process that is mechanistically analogous to ubiquitination. An E1-activating enzyme, namely Aos1/Uba2, and an E2-conjugating enzyme, Ubc9, are sufficient for SUMO conjugation in vitro. In vivo, this sometimes requires E3 factors, such as PIAS proteins, PC2 and RanBP2 (32). Sumoylation affects target protein function in a variety of ways and has now been implicated in many cellular processes, notably regulation of gene expression (33, 35). Numerous proteins involved in transcriptional control are sumoylated, including transcription factors, coactivators, corepressors, and histones, to name a few. In most cases, sumoylation represses transcription; the mechanisms include recruiting repressor complexes directly on promoters (35) or by sequestering proteins in nuclear subcompartments. Although sumoylation represses the activity of the AP-1 components c-Fos and c-Jun (36), it enhances that of other transcription factors, such as NF-AT1 and Oct4 (37, 38).

In the present study, we show that endogenous and ectopically expressed JunB is sumoylated in both resting and activated T lymphocytes. Of the three SUMO consensus sites, the site containing lysine 237 is the primary site of conjugation of either SUMO-1 or -2 in T cells. Remarkably, blocking JunB sumoylation, either by mutation or by coexpression with inactive Ubc9, strongly diminishes JunB-mediated transactivation of reporter genes controlled by the IL-2 and IL-4 promoters. In contrast, JunB sumoylation plays no role in its activity on synthetic TRE or AP-1 binding sites derived from the IL-2 promoter. These data suggest that sumoylation of JunB plays an important role in the transcriptional activation of certain cytokine genes in T cells.

Materials and Methods

Plasmids

Human hemagglutinin (HA)-tagged JunB wild type (wt), the single mutants (K237R, K267R, and K301R) and the JunB triple mutant (K237R/K267R/K301R, termed JunB-3R) were cloned in the pcDNA3 vector (Invitrogen). Point mutations were made using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s protocol. The expression vectors pcDNA3-His6-SUMO-2 and pcDNA3-His6-SUMO-1 (39), the TRE-luciferase reporter (40), and IL-2 Luc reporter containing the 300bp upstream of the initiation site (41) have been described. The IL-4 Luc reporter plasmid was provided by Dr. Glimcher (42). wt Ubc9 in (39), the TRE-luciferase reporter (40), and IL-2 Luc reporter containing the empty vector. Cells were incubated for 10 min at room temperature with the same total amount of DNA by adding the required quantities of plasmid by electroporation (40). In each experiment, cells were transfected logarithmic growth phase were transfected with the indicated amounts of plasmid. All cells were grown in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FBS, 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES (pH 7.4), 1× MEM nonessential amino acid (Invitrogen), 50 μM 2-ME, and 100 U/ml each of penicillin G and streptomycin. Jurkat cells in logarithmic growth phase were transfected with the indicated amounts of plasmid by electroporation (40). In each experiment, cells were transfected with the same total amount of DNA by adding the required quantities of empty vector. Cells were incubated for 10 min at room temperature with the DNA mix and electroporated at 260 mV. 960 mF in 400 μl of RPMI 1640. Murine primary T cells were isolated from spleens using a murine T cell negative isolation kit (Dynal Biotech) according to the manufacturer’s protocol. Human PBMCs were isolated as previously described (45). Human CD4+ T cells were isolated from fresh whole blood using RosetteSep human CD4+ T cell enrichment (Stem Cell Technologies) according to the supplier’s protocol. Five to ten million CD4+ T cells were transfected using a Human T cell Nucleofector kit (Amazza) following the manufacturer’s protocol. When required, CD4+ T cells were activated 6 h post-transfection as described below.

T cell activation

Plates were coated with 2 μg/ml each anti-human or -mouse CD3 and CD28 Abs in PBS for 4 h at 4°C. T cells were incubated on these plates for the indicated times. Plates were placed on ice, and cells were lysed with SDS-PAGE sample buffer (2% SDS, 0.1 M DTT, 50 mM Tris [pH 6.8], and 10% glycerol). PMA (100 ng/ml) and ionomycin (1 μg/ml) were added to the cell suspension for the indicated times. Cells were washed once with PBS and processed.

Reporter gene assays

In all experiments, cells were transfected with a β-galactosidase reporter plasmid as control of transfection (40). Transfected cells (1 x 10^6) were harvested after 2 days and washed twice with PBS. Cells were lysed in 100 μl luciferase lysis buffer (PROMega) and luciferase assays (40 μl) performed according to the manufacturer’s instructions (PROMega) using a Berthold luminometer. For β-galactosidase assays, 40 μl of lysates were added to 200 μl of β-galactosidase assay buffer (50 mM phosphate buffer [pH 7.4], ortho-nitro-phenyl-galactopyranoside 200 μM, 1 mg MgcCl2, and 50 mM 2-ME) and the absorbance measured at 400 nm. The results were expressed as luciferase units normalized to the corresponding β-galactosidase activity. The expression level of the transfected proteins was routinely controlled by immunoblotting analysis.

Small-scale fractionation

Small-scale biochemical fractionation was performed as described previously (46). Briefly, 2 x 10^6 cells were collected, washed with precooled PBS, and resuspended in 0.5 ml of buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM MgCl2, 0.34 M sucrose, 10% glycerol, 1 mM DTT, “complete” protease inhibitor mixture, and 20 mM NEM). After addition of Triton X-100 (0.1% final concentration), cells were incubated on ice for 8 min, and nuclei (fraction P1) were collected by centrifugation (5 min, 13000 g, 4°C). The supernatant (fraction S1) was clarified by high-speed centrifugation (5 min, 20000 g, 4°C), and the supernatant (fraction S2) was collected. The P1 nuclei were washed once in buffer A and lysed on ice for 30 min in buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, protease inhibitor mixture, 20 mM NEM, and 20 mM iodoacetamide). The insoluble chromatin (fraction P3) and soluble (fraction S3) fractions were separated by centrifugation (5 min, 17000 g, 4°C). The P3 fraction was washed once with buffer B and resuspended in SDS-PAGE sample buffer.

Purification of SUMO conjugates

Forty million cells were lysed in 2 ml of Solution A (6 M guanidium-HCl, 0.1 M Na2HPO4/NaH2PO4, 0.1 M Tris-HCl [pH 8.0], 20 mM NEM, 20 mM iodoacetamide, and 10 mM imidazole). Samples were sonicated (30 s) and centrifuged for 15 min at 14000 rpm at 4°C. Supernatants were transferred to a new tube containing 30 μl of prewashed Ni2+ -NTA magnetic beads (Qiagen) and incubated for 4 h at room temperature (on a rotating platform). Twenty five μl of the supernatants were used as inputs. Beads were sequentially washed with 2 ml of the following solutions: A, B (8 M urea, 0.1 M Na2HPO4/NaH2PO4, 0.1 M Tris-HCl [pH 8.0], 20 mM NEM, 20 mM iodoacetamide, and 10 mM imidazole), and C (8 M urea, 0.1 M Na2HPO4/NaH2PO4, 0.1 M Tris-HCl [pH 6.3], 20 mM NEM, 20 mM iodoacetamide, 10 mM imidazole, and 0.2% Triton X-100). After two additional washes in buffer C with 1% Triton X-100, proteins were eluted by adding 50 μl of SDS-PAGE sample buffer and analyzed by SDS-PAGE.

RNA isolation and quantitative-PCR (Q-PCR)

Total RNA was isolated from 3 x 10^6 human CD4+ T cells using a GenElute Mammalian total RNA Miniprep kit (Sigma-Aldrich) using the supplier’s instructions. One to 2 μg of RNA were treated with DNaseI (Promega), and 1 μg RNA was used to synthesize cDNA using pdn9 primer and MuLV reverse transcriptase (New England Biolabs) using the supplier’s protocol. Q-PCR for IL-2 and S26 were performed using Taq polymerase and SYBR green reagent (Invitrogen) according to the manufacturer’s instructions. The Q-PCR was performed in a 96-well plate using
the Stratagene MX3000P, and analysis was performed with MXPro soft-
ware. All samples were analyzed in duplicates, and the results were ex-
pressed as fold induction compared with the empty pCDNA3 vector. Primers
used in this study were hIL-2, sense: TCA CCA GGA TGC TCA CAT
TTA AGT; antisense: GA GTT TGT AGT TCT TCT AGA CAC
TTG A; and for hRPS26, sense: GAA CGC ATT TCC ACC CTA GA; an-
tisense: GCA CGA CCA TTG TTC CT.

ELISA

Human CD4+ T cells were seeded at 2.10^6 cells/ml in 24-well plates
(Pharmingen) precoated with 2 μg/ml anti-CD3 and anti-CD28 Abs and
left at 37°C for 12 h. IL-2 secretion was measured using Human IL-2
quantikine ELISA kit (R&D systems) following the manufacturer’s in-
structions. All samples were analyzed in duplicates, and the results were ex-
pressed as fold induction compared with cells transfected with empty
pcDNA3 vector.

Statistical analysis

The statistical analysis of the differences between means of paired samples
was performed using the paired t test. The results are given as the confi-
dence interval (p). All the experiments described in the figures were per-
formed at least three times with similar results.

Results

JunB is sumoylated in primary and leukemic T lymphocytes

JunB migrates in SDS-PAGE as a doublet with an apparent mo-
lecular mass of 40 kDa in the murine leukemic T cell line EL-4, as
well as in mouse and human primary T cells (Fig. 1). This com-
plements the list of resting T cells of different origins that express
this protein constitutively (5, 25, 27). In EL-4 cells, activation with
PMA/ionomycin treatment increases JunB expression, as previ-
ously described in other cells (47, 48). Moreover, we observed a
species of JunB migrating at 60 kDa that is more easily visualized
after PMA/ionomycin treatment (Fig. 1). This band was also ob-
erved when we used a mouse monoclonal JunB Ab against the
JunB N-terminal. The 20 kDa increase in size indicated a post-
translational modification, such as sumoylation, which has already
been described for ectopically expressed JunB in Hela cells (49).
Importantly, the 60 kDa JunB band was also observed in mouse and
human primary T cells activated either with PMA/ionomycin
or anti-CD3/anti-CD28. Maximal activation with the latter was
observed 2 h after stimulation; at 1 h, we found a small increase in
nonmodified and modified proteins (Fig. 1B).

To confirm that this band comigrates with sumoylated JunB, we
cotransfected Jurkat T cells with expression vectors for His6-
SUMO-2 and the SUMO E2 enzyme Ubc9. After activation with
PMA/Ionomycin, cells were lysed under denaturing conditions to
prevent desumoylation, and SUMO-2 conjugates were purified by
metal affinity chromatography. A fraction of endogenous JunB
was detected in the latter by immunoblot analysis, where it migrated at
60 kDa (Fig. 1C). This strongly suggests that the 60 kDa band
observed in Fig. 1, A and B, represents monosumoylated JunB.

Most studies have needed to use protein enrichment or overex-
pression of the protein or SUMO isoforms to detect the sumoylated
forms. These include the transcription factors NF-AT, Oct-4, and
ELK-1 (38, 50, 51). In contrast, we readily detected the upper JunB
band in total cell extracts without enrichment of sumoylated pro-
teins or ectopic expression of JunB or SUMO isoforms. Hence, the
proportion of endogenous JunB appearing to be sumoylated is
higher than many other proteins.

To facilitate further molecular analysis, we checked whether
ectopically expressed JunB is also sumoylated in Jurkat cells.
Transfections were performed as described above with the addition of
an expression vector for HA-tagged JunB wt. Affinity-purified
His-tagged proteins were separated by SDS-PAGE, and ectopic
JunB was visualized in Western blots by immunodetection of the
HA epitope. Like the endogenous protein, a proportion of exoge-
nous JunB was conjugated to SUMO-2 in both resting and acti-
vated T cells (Fig. 2A). Similar results were obtained using an ex-
pression vector for His6-SUMO-2 and 0.5 μg of Ubc-9. Two days later, cells were treated with
100 ng/ml PMA and 1 μg/ml ionomycin or plated on anti-(CD3+CD28)-coated
plates for the indicated periods. Cells were processed as described in A.
The arrow indicates sumoylated JunB. C. Endogenous JunB is sumoy-
lated in Jurkat cells. Forty million Jurkat cells were transfected with 2 μg
of His6-SUMO-2 and 0.5 μg of Ubc-9. Two days later, cells were treated with
100 ng/ml PMA and 1 μg/ml ionomycin for 4 h and lysed. Sumoylated
proteins were purified by immobilized metal affinity chromatography
(IMAC) and JunB visualized by immunoblotting (top panel). Immunoblot
analysis of the inputs, representing 10% of the total protein, is shown in the
bottom panel.

FIGURE 1. JunB is sumoylated in T cells. A. JunB expression in stimu-
lated EL-4 cells. One million EL-4 cells were treated with 100 ng/ml
PMA and 1 μg/ml ionomycin for the indicated times and then lysed. Equiv-
alent amounts of protein were separated by SDS-PAGE, transferred to
membranes and incubated with Abs against JunB and β-Actin. B. JunB
expression in stimulated human PBMCs and murine T lymphocytes. Hu-
man PBMCs or murine T lymphocytes were purified and treated with 100
ng/ml PMA and 1 μg/ml ionomycin or plated on anti-(CD3+CD28)-coated
plates for the indicated periods. Cells were processed as described in A.
The arrow indicates sumoylated JunB. C. Endogenous JunB is sumoy-
lated in Jurkat cells. Forty million Jurkat cells were transfected with 2 μg
of His6-SUMO-2 and 0.5 μg of Ubc-9. Two days later, cells were treated with
100 ng/ml PMA and 1 μg/ml ionomycin for 4 h and lysed. Sumoylated
proteins were purified by immobilized metal affinity chromatography
(IMAC) and JunB visualized by immunoblotting (top panel). Immunoblot
analysis of the inputs, representing 10% of the total protein, is shown in the
bottom panel.

Lyseine 237 is the primary sumylation site on JunB in vivo

Sumoylation generally occurs on the lysine residue within a KXE
consensus motif, where ψ is an aliphatic amino acid and X any
amino acid (33). Three motifs are present in JunB, surrounding
lysines 237, 267, and 301 (Fig. 2B). To determine which sites are
used in vivo, these lysines were mutated to arginines individually
(JunB-K237R, -K267R, and -K301R, Fig. 2B) or in combination
(JunB-K237R/K267R/K301R, termed JunB-3R). The expression
vectors were transfected into Jurkat cells, which were then acti-
vated by PMA/Ionomycin and lysed. Overexpressed wt or mutant
JunB in the whole cell extracts was detected in Western blots using
an anti-HA Ab. Mutation of lysine 237, but not that of lysines 267
or 301, strongly diminished JunB modification by endogenous
Nevertheless, these two lysines were used, albeit weakly, because the residual level of the 60 kDa band seen with JunB-K237R completely disappeared in JunB-3R (Fig. 3A). To confirm this, we overexpressed either JunB wt, K237R, or 3R in Jurkat cells together with His6-SUMO-1 or His6-SUMO-2 and purified SUMO conjugates from cell lysates by nickel affinity chromatography under denaturing conditions (Figs. 2A and 3B). Indeed, mutation of lysine 237 strongly impaired JunB conjugation with SUMO (36, 49), and sumoylated c-Fos preferentially localizes in an insoluble nuclear fraction in HeLa cells (36). To investigate whether this was also the case for sumoylated JunB, we performed nuclear fractionation experiments (Fig. 4). Because we wished to localize sumoylated vs nonsumoylated endogenous showed endogenous JunB to be primarily nuclear in primary T cells and Jurkat cells, without revealing any preferential subnuclear association (data not shown). This confirms fractionation experiments in Jurkat cells, where JunB was exclusively in the nucleus (40).

Like JunB, the AP-1 family members c-Jun and c-Fos are conjugated to SUMO (36, 49), and sumoylated c-Fos preferentially localizes in an insoluble nuclear fraction in HeLa cells (36). To investigate whether this was also the case for sumoylated JunB, we performed nuclear fractionation experiments (Fig. 4). Because we wished to localize sumoylated vs nonsumoylated endogenous

**FIGURE 2.** Mutation of the three consensus sumoylation sites of JunB prevents sumoylation. A, Forty million Jurkat cells were transiently transfected with 2 μg of a His6-SUMO-2 expression vector, together with 5 μg of vectors encoding either wt HA-Tagged JunB (JunB wt) or the mutant HA-JunB-3R, where lysines 237, 267, and 301 are changed to arginine. Half of the cells were stimulated with 100 ng/ml PMA and 1 μg/ml ionomycin for 4 h. After lysis, sumoylated proteins were purified by IMAC, separated by SDS-PAGE, and visualized by immunoblotting with an anti-HA Ab (top panel). Immunoblot analysis of the inputs, representing 10% of the total protein (WCE), is shown in the bottom panel. The arrow indicates sumoylated JunB. B, Diagram depicting the major structural features of JunB. The consensus site for Ubc9 binding and sumoylation is shown, where ψ is an aliphatic amino acid, X is any amino acid, and K the lysine conjugated to SUMO. Also indicated are the sequences harboring the three major consensus sites in JunB, surrounding lysines 237, 267, and 301 (bold letters). DBD: DNA-binding domain; NLS: Nuclear Localization Signal; and bZip: basic domain-leucine zipper.

**FIGURE 3.** Lysine 237 is the SUMO acceptor in Jurkat cells. A, Ten million Jurkat cells were transiently transfected with 5 μg of expression vectors encoding the indicated HA-Tagged JunB proteins. Cells were activated for 4 h with 100 ng/ml PMA and 1 μg/ml ionomycin, then processed as described in the legend to Fig. 2 to visualize HA-tagged JunB. The panel contains a long exposure to reveal the band of sumoylated JunB, indicated by the arrow. B, Forty million Jurkat cells were transiently transfected with 2 μg of an expression vector for His6-SUMO-1, together with 5 μg of vector encoding HA-Tagged JunB wt or -K237R. Two days later, cells were treated with 100 ng/ml PMA and 1 μg/ml ionomycin for 4 h and lysed. Sumoylated proteins were purified by IMAC, separated on SDS-PAGE, and JunB visualized by immunoblotting. The asterisk indicates nonspecific binding of JunB.

**FIGURE 4.** Subcellular distribution of sumoylated JunB in human PBMCs. Human PBMCs were either untreated or stimulated for 1 h with PMA (100 ng/ml) and ionomycin (1 μg/ml), followed by a small-scale cell fractionation. The S2 fraction contains soluble proteins, P3 is the Triton-insoluble nuclear fraction, and W is the wash of P3. Cell equivalent amounts of each fraction were separated by SDS-PAGE and proteins visualized by immunoblotting with the indicated Abs. For the input, 2% of total protein was analyzed the same way. The arrow indicates sumoylated JunB.

**SUMOYLATION OF JunB IN T CELLS**

**Sumoylated JunB is enriched in a nonsoluble nuclear fraction**

SUMO conjugation has been linked to nuclear localization of numerous transcription factors (33), such as the T cell regulatory protein NF-AT1 (38). Moreover, certain sumoylated transcriptional regulators are found associated with a nuclear subdomain that is implicated in leukemia, namely promyelocytic leukemia nuclear bodies (Ref. 33). Indirect immunofluorescence microscopy
JunB in activated vs nonactivated cells, we performed a 1-h stimulation of PBMCs with anti-CD3/CD28 Abs (Fig. 1). Although unconjugated JunB was equally distributed in the soluble and insoluble nuclear fractions (S2 and P3, respectively, left panels), sumoylated JunB was detectable uniquely in the insoluble fraction. The purity of the two fractions was confirmed by the selective presence of the chromatin- and matrix-associated protein Topoisomerase I in P3 and the nucleoplasmic protein PHAX in S2. This indicates that SUMO-conjugated JunB associates with the insoluble fraction of the nucleus, possibly a subdomain tightly associated with chromatin. In addition, we detected similar levels of the 60 kDa band representing endogenous JunB conjugated to SUMO (Fig. 4, right panel) in resting and stimulated cells. These data show that T cell activation did not increase the percentage of sumoylated JunB or change JunB intracellular localization. The sumoylated form of JunB was easily observed after activation because activated cells expressed more JunB (Fig. 1). Consistent with this, ectopically expressed JunB was sumoylated to a similar extent in resting Jurkat cells as endogenous JunB in activated cells (Fig. 3). Therefore, sumoylation of JunB was constitutive and did not require T cell activation; still, as the total amount of JunB increased in activated T cells via the de novo expression of the protein (Fig. 1), the amount of sumoylated JunB increased comparably.

Sumoylation controls JunB transcriptional activity on the minimal IL-2 and IL-4 promoters

Sumoylation regulates transactivation by numerous transcription factors and, in many cases, has a repressive effect (35). To evaluate the effect of SUMO conjugation on trans-activation by JunB, we performed cotransfection experiments with reporter genes controlled by the IL-2 and IL-4 gene promoters, two well-known JunB targets (5, 22, 52). In Jurkat cells, wt JunB activated the IL-2 promoter by 70-fold (Fig. 5A). Interestingly, induction was half as strong with JunB-K237R, and mutation of all three consensus lysines almost abolished activation of the IL-2 promoter. All three proteins were expressed at similar levels (Fig. 5A). Our results show that K237 was the primary site for sumoylation; when this site was mutated to alanine, we detected a minor level of sumoylated JunB (Fig. 3). This indicates that other lysines, especially K267 and K301 in the other sumoylation consensus sites, were also conjugated with SUMO. Therefore, we used the triple mutant that was barely sumoylated to investigate the role of sumoylation on the transcriptional activity of JunB. This mutant activated the TRE, NF-AT/AP-1, and CD28RE reporters to the same extent as wt JunB, indicating that the mutation did not affect basal function (Fig. 6). In agreement with Li et al. (5), wt JunB activated
the IL-4 reporter gene 4-fold (Fig. 5C); this was reduced to control levels with JunB-3R (Fig. 5C). These data suggested that sumoylation of JunB potentiates its ability to transactivate the IL-4 and IL-2 promoters.

Ubc9 is the only SUMO-E2 identified in human cells. As expected, cotransfection of Ubc-9DN led to inhibition of sumoylation of ectopically expressed JunB (Fig. 5B). Consistent with the results using nonsumoylatable mutants, cotransfection of Ubc-9DN led to a 2-fold reduction in JunB-driven activation of the IL-2 reporter gene (Fig. 5B). Although this strongly suggests that Ubc9-DN inhibits JunB activity in this context, we cannot exclude that part of the Ubc9-DN effect was mediated by an effect on another protein.

Surprisingly, wt JunB and JunB-3R showed indistinguishable activity on the reference AP-1 reporter gene, namely the TRE reporter plasmid that is driven by four canonical binding sites upstream of the TATA box (Fig. 6A). Thus, sumoylation regulates JunB activity on specific cytokine promoters. The proximal region of the IL-2 promoter contains several regulatory elements targeted by different transcription factors (23). The composite elements ARRE-2 and CD28RE are recognized by NF-AT and NF-kB, respectively, in combination with AP-1, in particular JunB (22, 25, 26). Surprisingly, JunB-K237R and JunB-3R transactivated reporter genes driven by these composite sites at the same level as wt JunB (Fig. 6B and C). Taken together, these results suggest that sumoylation controls JunB transcriptional activity on certain cytokine promoters without intrinsically affecting JunB transactivation.

**Sumoylation is essential for endogenous IL-2 promoter activation by JunB**

To unambiguously investigate the role of JunB sumoylation in primary T cells, we transfected human CD4+ T cells with JunB and JunB-3R. Resting CD4+ T cells did not express measurable levels of IL-2, and ectopically expressed JunB did not induce its expression (data not shown). Therefore, in the next set of experiments, we used activated CD4+ T cells. Like in Jurkat T cells, ectopically expressed JunB wt, but not JunB-3R, was sumoylated in CD4+ T cells (Fig. 7A). wt JunB, but not JunB-3R, significantly increased expression of endogenous IL-2 mRNA in CD4+ T cells (Fig. 7B). We confirmed this differential effect of JunB wt and -3R by measuring IL-2 levels in supernatants of transfected cells (Fig. 7C). Thus, in primary CD4+ T cells, sumoylation of JunB cooperates with other endogenous signals to fully activate the IL-2 promoter.

**Discussion**

We report here that JunB is covalently modified by either SUMO-1 or SUMO-2 in both human and mouse T cells. The major acceptor site is lysine 237, although weak levels of sumoylation are detectable on other sites. Like the majority of sumoylated proteins (32, 33, 35), a minor proportion of JunB, both endogenous and exogenous, was conjugated with SUMO under steady-state conditions. However, we could detect sumoylated JunB in whole cell extracts, which was enhanced by enrichment of SUMO-conjugated proteins. Activation of T cells also allowed us to more easily visualize sumoylated JunB. This reflected the increase in JunB expression, because the ratio between sumoylated and nonsumoylated JunB remained the same as found in resting cells. The latter observation suggests that this modification of JunB is constitutive and does not require T cell activation. However, this does not exclude that other signals or stresses could regulate JunB sumoylation, as observed for c-Fos and c-Jun in Hela cells (36, 39).

Sumoylation is often linked to a specific intranuclear distribution of the target proteins. Many sumoylated nuclear proteins localize in nuclear subdomains, such as promyelocytic leukemia nuclear bodies, which may serve to sequester them and thereby suppress their activity. Endogenous JunB is nuclear in primary T cells and Jurkat cells but showed no concentration in a subdomain. Nevertheless, the modified protein does show a striking localization, as it was found exclusively in the insoluble nuclear fraction of human primary T cells, i.e., associated with a detergent-resistant nuclear matrix and/or chromatin structure. In contrast, nonmodified JunB was equally distributed between soluble and nonsoluble nuclear chromatin fractions. Thus, JunB resembles c-Fos and several other nuclear factors in the preferential binding of the sumoylated protein to this insoluble nuclear structure (36, 53). Further work will determine whether sumoylation is a cause or a consequence of this localization.

In certain transcriptional regulators, such as Elk-1, the lysines in the sumoylation consensus motif were initially identified as being essential for repressing their activity (54). Similarly, this motif is found in synergy control domains present in c-Myb, C/EBP, SP3,
and nuclear receptors (55). Synergy controls down-regulate synergistic activation of reporter genes driven by compound transcriptional regulatory elements but do not affect the activity of single response elements. Subsequently, this negative regulation was shown to be dependent upon sumoylation, and, in fact, SUMO conjugation to most transcription factors, including c-Fos and c-Jun, represses their activity (36, 49). It is striking that we observe the opposite result with JunB, whose ability to transactivate reporter genes driven by cytokine promoters was strongly compromised by blocking sumoylation, either by mutation or using DN-Ubc9. Thus, JunB joins the small subset of transcription factors, such as Oct4 (37), NF-AT (38), and NF-E2 (56), whose activity is potentiated by SUMO conjugation. JunB appears unique in that sumoylation is not a prerequisite for its transcriptional activity per se, because the mutants JunB-K237R and JunB-3R resemble JunB wt in the activation of reporter genes driven by isolated AP-1 binding sites of the IL-2 promoter.

How might sumoylation affect JunB transcriptional activity on cytokine promoters but not isolated elements? It could be that sumoylated JunB is targeting a different regulatory element than AP-1, NF-AT, or NF-xB, or that sumoylated JunB interacts with different proteins than nonsumoylated JunB. This will be difficult to establish, because JunB might interact with several AP-1 components, e.g., c-Jun and c-Fos. Moreover, the resulting AP-1 dimer might interact with other transcription factors, e.g., NF-AT or NF-xB, as well as coactivators. Given the multiple AP-1 sites in the IL-2 and IL-4 promoters, we cannot rule out that sumoylation of JunB subtly regulates its binding to different sites. Nevertheless, there are other potential explanations. For example, JunB sumoylation might facilitate recruitment and assembly of an enhancerome on the promoter that is also dependent upon other transcriptional regulators, clearly present under our experimental conditions. There is some precedent for protein-protein interactions dependent upon SUMO conjugation (55). It is also possible that sumoylated JunB directs its target gene to a nuclear subcompartment of highly active transcription, or transcription factory (57); this might be reflected by the presence of SUMO-conjugated JunB in the detergent-insoluble fraction of the nucleus. Once again, strong transcriptional activation would then require promoter assembly with other transcriptional regulators induced by T cell activation. Any of these could explain the strong activation of the IL-2 promoter by sumoylated JunB.

In the immune system, this would be particularly important in T cell anergy, where a defect in JunB-dependent transcription leads to impaired IL-2 production (24). We would argue that a failure to sumoylate JunB would have the same consequence, namely a weak activation of IL-2 resulting in anergy. This is supported by our findings that nonsumoylatable JunB mutants fail to increase IL-2 production in activated CD4+ T cells. This means that JunB needs to be sumoylated to fully activate the IL-2 promoter. We note that ectopically expressed JunB does not activate the IL-2 promoter in resting cells; this indicates that other factors or signals are required. Further work will be required to prove this hypothesis and characterize the mechanism by which SUMO enhances JunB activity on cytokine promoters.

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References


