SUMOylation is a regulator of the translocation of Jak2 between nucleus and cytosol

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Jak2 (Janus kinase 2) initiates the signal transduction of many cytokine receptors. We discovered that Jak2 is SUMOylated on multiple lysine residues by SUMO2/3 (small ubiquitin-related modifier 2/3) chains. Analysis of Jak2 mutants revealed that Jak2 SUMOylation depends on the presence of an active catalytic site. We used the GH (growth hormone) receptor to study the physiological relevance of Jak2 SUMOylation. Both GH stimulation and several other environmental stressors increased Jak2 SUMOylation. Cell fractionation showed that SUMOylated Jak2 is mainly present in the nucleus. The constitutively active V617F Jak2 mutant, implicated in myeloproliferative diseases, was highly SUMOylated in the absence of stimuli. These data provide evidence that Jak2 SUMOylation controls Jak2 shuttling between cytoplasm and nucleus.

Key words: growth hormone, Janus kinase 2 (Jak2), nuclear translocation, stress, SUMOylation.

INTRODUCTION

Jak2 (Janus kinase 2) belongs to the family of Janus kinases that are necessary for cytokine signalling. They associate with oligomeric cytokine receptors and, upon cytokine addition, transphosphorylate themselves and the accompanying receptor, allowing the signalling cascade to proceed. This leads to a variety of biological responses in haemopoiesis and immunity. Cytokine receptors associated with Jak2 include the prolactin, erythropoietin and GHR (growth hormone receptor). In the present study, we used GHR as a model to study Jak2 function.

Upon GH (growth hormone) stimulation, Jak2 triggers signalling cascades via the STAT5 (signal transducer and activator of transcription 5)/MAPK (mitogen-activated protein kinase) pathways. This signalling is implicated in various biological processes, including cell-cycle progression, apoptosis, mitotic recombination and alteration of heterochromatin. The best known somatic alteration of Jak2 is a gain-of-function mutation, V617F, associated with human myeloproliferative diseases [1]. The diverse roles of Jak2 in normal and leukaemic haemopoiesis are believed to be restricted to cytoplasmic events. However, Dawson et al. [2] reported that Jak2 can translocate to the nucleus, where it phosphorylates histone H3, which leads to activation of gene transcription. Additionally, it was reported that the Jak2 V617F mutation occurs more often in the nucleus of haemopoietic cells compared with wild-type Jak2 [3]. A selective Jak2 inhibitor was used to chase the kinase back to the cytoplasm, suggesting that blocking the nuclear translocation of Jak2 could be a new treatment strategy for patients bearing the V617F mutation. The mechanism of this translocation is still unknown, as Jak2 lacks either typical nuclear localization or nuclear export signals.

SUMOylation of proteins is an essential process within the cells that alters protein–protein interactions. This can lead to changes in protein localization, stability and activity (reviewed in [4]). The human genome encodes four SUMO (small ubiquitin-related modifier) proteins: SUMO1–SUMO4. SUMO2 and SUMO3 are 97 % identical and can contribute to poly-SUMO chains. SUMO1 occurs only as a mono-substituent and is less ubiquitously expressed than SUMO2 and SUMO3. The role of SUMO4 is unknown. SUMOylation begins with the activation of a SUMO molecule by the SUMO-activating enzymes SAE1 or SAE2 (SUMO E1). Next, activated SUMO molecules are transferred to the specific SUMO-conjugating enzyme Ubc9 (SUMO E2) and are finally attached to lysine residues in targeted proteins.

Many proteins that are modified by SUMO contain the consensus motif ΨKX(D/E), where Ψ is a large hydrophobic residue. This motif is recognized by Ubc9 [5]. Additionally, SUMO modification can also occur on residues located outside this motif, and an increasing number of studies have found other possible SUMO consensus motifs [6]. Other modifications such as phosphorylation can enhance SUMOylation, especially when they include phosphorylation sites downstream of SUMO acceptor sites within the phosphorylation-dependent SUMO motif [7,8]. In other instances, phosphorylation prevents SUMOylation [9,10].

Depending on the target protein, SUMOylation can occur in both the cytoplasm and the nucleus. For several proteins such as CtBP1 (C-terminal binding protein 1) [11] or the IκB (inhibitor of nuclear factor κB) kinase regulator NEMO (nuclear factor κB essential modulator) [12], SUMOylation is required for nuclear translocation. For the IGF (insulin-like growth factor)-1 receptor, it was shown that stimulation with IGF causes receptor modification with SUMO1 and its subsequent translocation to the nucleus [13]. However, lack of SUMOylation did not affect the kinase-dependent signalling. Additionally, there is evidence that SUMO modification could regulate nuclear export of some substrates as has been reported for the Dictostelium factor Mek1 where nuclear SUMOylation is responsible for its translocation to the cytoplasm [14], whereas mutation of SUMO-acceptor lysine

Abbreviations used: CtBP1, C-terminal binding protein 1; EEA1, early endosome antigen 1; FERM, 4.1/ezrin/radixin/moesin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GH, growth hormone; GHR, growth hormone receptor; HA, haemagglutinin; HEK, human embryonic kidney; IGF, insulin-like growth factor; LMO2, LIM domain only 2; NEMO, nuclear factor κB essential modulator; Ni-NTA, Ni2+ -nitrilotriacetate; siRNA, small interfering RNA; SUMO, small ubiquitin-related modifier; TEL, translocated Ets leukaemia.

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residue of TEL (translocated Ets leukaemia) protein increases its level in the nucleus [15].

Since post-translational modifications can alter protein activity, stability, cellular distribution and interactions with other proteins, we decided to study SUMOylation of Jak2. We found that Jak2 is modified by SUMO2/3 up to a high molecular mass. Both GH and extracellular stresses, like elevated temperature or serum starvation, stimulate Jak2 SUMOylation. SUMOylation and phosphorylation of Jak2 appear to be connected. The kinase-inactive mutant K882E shows substantially less SUMOylation signal, whereas the constitutively active Jak2 V617F mutant has increased SUMOylation signal. Furthermore, we provide evidence that SUMOylation of Jak2 facilitates its translocation to the nucleus. We propose that SUMOylation provides an important regulatory mechanism controlling Jak2 shuttling between nucleus and cytoplasm.

EXPERIMENTAL

Reagents

Mouse monoclonal antibody against Jak2 was purchased from Invitrogen (AHO1352), anti-SUMO2/3 antibody was from Abcam (ab81371), anti-[HA (haemagglutinin) tag] 12CA5 antibody was from Babco, antibody against phosphoarginine residues was from Upstate (Millipore) (clone 4G10), anti-KDEL antibody was from Merck (10C3), anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody was from Millipore (MAB374) and anti-actin antibody was from MP Biomedicals (clone C4). Rabbit polyclonal antibody against FLAG tag was purchased from Sigma, anti-EA1 (early endosome antigen 1) antibody was from BD Biosciences and anti-Ubc9 antibody was from Santa Cruz Biotechnology (sc-10759). Rabbit polyclonal anti-(GHR B) antibody used for Western blot detection and anti-(GHR T) antibody used for immunoprecipitation were as described previously [16,17]. Rabbit polyclonal anti-Jak2 antibody raised against a synthetic peptide corresponding to the hinge region (amino acids 758–777) between domains 1 and 2 of murine Jak2 was used for immunoprecipitation [18]. Rabbit monoclonal anti-(histone H3) antibody was from Cell Signaling Technology (D1H2). The Alexa Fluor® 568, 680 and IR 800-conjugated goat anti-mouse and anti-rabbit IgG secondary antibodies were obtained from Molecular Probes/Invitrogen. Beads coupled to antibody against FLAG tag M2 were obtained from Sigma. Protein A beads were purchased from RepliGen Corporation. Ni-NTA (Ni2+-nitrilotriacetate) beads were from Qiagen. Human GH was kindly provided by Eli Lilly Research Labs. Culture media, fetal bovine serum, L-glutamine and antibiotics for tissue culture were purchased from Invitrogen.

Plasmids

The FLAG–Jak2 mouse construct was a gift from Professor Christin Carter-Su (University of Michigan, Ann Arbor, MI, U.S.A.). The pcDNA3 HA-SUMO1, SUMO2 and SUMO3 and pcDNA3 His-SUMO1 and SUMO2 plasmids were gifts from Professor Frauke Melchior (Zentrum für Molekulare Biologie der Universität Heidelberg, Heidelberg, Germany). FLAG–Jak2 truncations (1–842, 1–525 and 1–280) were constructed by the introduction of a stop codon using the QuikChange® site-directed mutagenesis kit from Stratagene. To generate plasmids encoding FLAG–Jak2 K882E, Y1007F, V617F, K630R, K912R, K914R and K912R/K914R mutants, a pair of mutagenic primers with a point mismatch at the desired site were designed for each mutation (see Supplementary Table S1 at http://www.biochemj.org/bj/453/bj4530231add.htm) and used to generate mutated sequences on the FLAG–Jak2 plasmid as a template with the QuikChange® site-directed mutagenesis kit. The mutations were confirmed by DNA sequencing (Macrogen).

Cell culture, transient transfections and gene silencing

The HEK (human embryonic kidney)-293 cell line was cultured under standard conditions in DMEM (Dulbecco’s modified Eagle’s medium) with high glucose containing 10% fetal bovine serum. The HEK-293 cells stably expressing the wild-type GHR were grown in the same medium supplemented with 0.6 mg/ml Geneticin (G418; Gibco). Transfections were performed with FuGENE™ (Roche). Cells were plated to 60% confluence (10° cells in a 6-cm-diameter Petri dish) 24 h before the transfections, and then transfected with 2 μg of DNA and 6 μl of FuGENE™. Cells were silenced for 48 h with Lipofectamine™ 2000 under standard conditions. siRNA (small interfering RNA) used for Ubc9 silencing was purchased from Ambion (s14591).

SDS/PAGE and Western blotting

Proteins were solubilized in sample buffer and electrophoresed on denaturing SDS/polyacrylamide gels. The proteins were transferred on to Immobilon-FL PVDF membranes (Millipore) and analysed by Western blotting with the indicated antibodies. Blots were washed and incubated with fluorescent secondary antibodies. Reactive bands were detected with an Odyssey IR imaging system (Li-Cor Biosciences).

Cell lysis and immunoprecipitation

Cells were washed once with PBS and harvested in denaturing lysis buffer (1 mM EDTA, 1% SDS, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 15 mM N-ethylmaleimide, 15 mM NaF and 1 mM Na3VO4 in PBS, pH 7.4). The lysates were boiled for 6 min, sheared five times with a 25 gauge needle, boiled again for 3 min, vortex-mixed and clarified for 5 min at 16 100 g. Supernatants were diluted to a final concentration of 0.5% SDS with Immunnix special (2% Triton X-100, 1 mM EDTA, 1% BSA, 0.5% sodium deoxycholate, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 15 mM N-ethylmaleimide, 15 mM NaF and 1 mM Na3VO4 in PBS, pH 7.4). Each sample was incubated with polyclonal anti-Jak2 antibody for 2 h before adding Protein A beads for 45 min in an end-over-end rotator at 4°C. Beads were washed twice with Immunonix (1% Triton X-100, 0.1% SDS, 1 mM EDTA, 1% BSA, 0.5% sodium deoxycholate, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 15 mM N-ethylmaleimide, 15 mM NaF and 1 mM Na3VO4 in PBS, pH 7.4) and twice with a 0.1× concentration of PBS and boiled with sample loading buffer for 5 min. To assess the phosphorylation state of Jak2, cells were grown under serum conditions or serum-starved for 1–2 h before stimulation with 180 ng/ml human GH. For co-immunoprecipitation experiments, cells were lysed in non-denaturing lysis buffer (1% Triton X-100, 1 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 15 mM N-ethylmaleimide, 15 mM NaF and 1 mM Na3VO4 in PBS, pH 7.4). Subsequently, the same amount of Immunonix was added (1% Triton X-100, 0.5% sodium deoxycholate, 1% BSA, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 15 mM N-ethylmaleimide, 15 mM NaF and 1 mM Na3VO4 in PBS, pH 7.4) and GHR was immunoprecipitated with anti-(GHR T) antibody as described above.
Subcellular fractionation

The high-salt nuclear fraction protocol was performed as follows. HEK-293 cells in a 10-cm-diameter dish were scraped into 1 ml of ice-cold PBS and centrifuged for 5 min at 16 000 g. Supernatant was discarded and cells were resuspended in 200 μl of buffer 1 (25 mM Hepes, pH 7.9, 5 mM KCl, 0.5 mM MgCl2, 1 mM dithiothreitol, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 15 mM N-ethylmaleimide, 15 mM NaF and 1 mM Na3VO4, pH 7.4) and lysed by adding 200 μl of buffer 2 (buffer 1 supplemented with 1 % Nonidet P40) and incubating for 15 min end-over-end at 4 °C. Nuclei were pelleted at 500 g for 5 min and washed once with buffer 3 (1:1 mixture of buffer 1 and 2). The cytoplasmic fraction was collected and immunoprecipitation was performed by adding 500 μl of buffer (2 % Triton X-100, 1 % SDS, 1 mM EDTA, 1 % BSA, 0.5 % sodium deoxycholate, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 15 mM N-ethylmaleimide, 15 mM NaF and 1 mM Na3VO4 in PBS, pH 7.4) followed by incubation with appropriate antibody as described above. To the nuclear fraction, 500 μl of denaturing lysis buffer was added and the procedure was performed as described in the ‘Cell lysis and immunoprecipitation’ section.

Immune fluorescence microscopy

HEK-293 cells grown on coverslips were incubated with human GH (180 ng/ml). Cells were washed with PBS to remove unbound GH and fixed for 1 h in 4 % (w/v) paraformaldehyde. After fixation, the cells were permeabilized in 0.02 % saponin for 30 min and the coverslips were incubated with anti-Jak2 antibody and subsequently with Alexa Fluor® 568-labelled anti-mouse secondary antibody. For nucleus visualization, ToPro-3 Iodide was used (Molecular Probes/Invitrogen). The coverslips were embedded in ProLong Gold Antifade Reagent (Invitrogen) and images were captured with the Zeiss LSM 510 meta system.

Statistical analysis

For data analysis, SPSS 15.0 software was used. All data are presented as means ± S.E.M. The results were analysed by ANOVA with $P \leq 0.05$ considered significant.

RESULTS

Jak2 is modified by SUMO2/3

In order to determine whether Jak2 is SUMOylated, HEK-293 cells were transiently transfected with FLAG–Jak2 and HA-tagged SUMO1, SUMO2 or SUMO3, and analysed by Western blotting (Figure 1A). Cells transfected with either SUMO2 or SUMO3 showed a clear high-molecular-mass signal, indicating SUMO modification. SUMO1 expression levels were too low to determine a potential modification. In Figure 1(B), we used pre-immune serum to demonstrate that the SUMO signal on Jak2 is specific.

Next, we asked whether endogenous Jak2 is also modified by SUMO2/3. HEK-293 cells were transiently transfected with HA–SUMO3 and endogenous Jak2 was immunoprecipitated. The most abundant SUMOylated Jak2 migrated as high-molecular-mass species, whereas clear bands were visible, probably representing Jak2 molecules with five to eight SUMO moieties (Figure 1C). In Figure 1(D), we show that SUMOylated endogenous Jak2 was also detectable with antibody recognizing endogenous SUMO2/3. Furthermore, we detected high-molecular-mass Jak2 signal in an isolate of total SUMOylated proteins. In an alternative approach, HEK-293 cells were transiently transfected with FLAG–Jak2 and His6–SUMO1 or –SUMO2 and anti-His6 pull-down was performed under denaturing conditions using Ni-NTA beads. As seen in Figure 1(E), clear bands corresponding to SUMOylated Jak2 were visible. An asterisk marks Jak2 bound non-specifically to the beads. ev, empty vector; IP, immunoprecipitation; PD, pull-down; WB, Western blot. Molecular masses are indicated in kDa.

Jak2 SUMOylation requires an intact kinase domain

To determine the mechanism of SUMOylation, we first analysed truncated Jak2 molecules, as depicted in Figure 2(A). As shown in Figure 2(B), only wild-type Jak2 was modified. This indicated that SUMOylation requires the presence of the kinase domain.

Figure 1  Jak2 is SUMOylated by SUMO2/3

(A) HEK-293 cells were transfected with FLAG–Jak2 and with HA–SUMO1 (S1), HA–SUMO2 (S2), HA–SUMO3 (S3) or no SUMO (—). Cells were lysed under denaturing conditions and anti-FLAG immunoprecipitation was performed. The blot was detected with antibodies against HA and FLAG. (B) HEK-293 cells were transfected with FLAG–Jak2 and HA–SUMO3. Cells were lysed under denaturing conditions and immunoprecipitation was performed with pre-immune serum or anti-Jak2 antibodies. (C) HEK-293 cells were transfected with HA–SUMO3 and endogenous Jak2 was immunoprecipitated under denaturing conditions. Western blots were analysed with anti-HA and anti-Jak2 antibodies. (D) Endogenous Jak2 immunoprecipitation was performed under denaturing conditions and the blot was developed with anti-Jak2 antibody and antibody recognizing endogenous SUMO2/3 moieties. The blot represents three independent experiments. An asterisk indicates background band. (E) HEK-293 cells were transfected with FLAG–Jak2, His6–SUMO1 and His6–SUMO2. Subsequently, an anti-His pull-down on Ni-NTA beads was performed under denaturing conditions. The blot was analysed with anti-Jak2 antibodies. An asterisk indicates Jak2 bound nonspecifically to the beads. ev, empty vector; IP, immunoprecipitation; PD, pull-down; WB, Western blot. Molecular masses are indicated in kDa.
Figure 2  Jak2 SUMOylation and phosphorylation signals are connected

(A) Structure of Jak2. Wild-type Jak2 consists of several different domains. The FERM domain is responsible for receptor binding, the SH2 (Src homology 2) domain is responsible for protein–protein interactions and the pseudokinase domain is an inhibitory domain for the kinase domain. Three Jak2 truncations were used: truncated after amino acid 842 lacking the kinase domain, after amino acid 525 lacking both the pseudokinase and kinase domains, and after amino acid 280 consisting only of part of the FERM domain. (B) HEK-293 cells were transfected with HA–SUMO3 and different FLAG–Jak2 truncations (wild-type, 842, 525 and 280), lysed under denaturing conditions and immunoprecipitated with anti-FLAG antibodies. The blot was analysed with antibodies against HA and FLAG. (C) HEK-293 cells were transiently transfected with different FLAG–Jak2 plasmids (wild-type, 1–842, Y1007F, K882E and V617F) and anti-FLAG immunoprecipitation was performed under denaturing conditions. Phosphorylation of Jak2 mutants was assessed with anti-phosphotyrosine antibody (pY) and of GHR with anti-GHR serum. ev, empty vector. (D) HEK-293 cells were transiently transfected with different FLAG–Jak2 constructs as indicated. Anti-FLAG immunoprecipitation was performed under denaturing conditions. Results are from 15 different experiments and are means ± S.E.M. expressed as the ratio of SUMOylation and Jak2 signals. *P < 0.05. IP, immunoprecipitation; WB, Western blot; wt, wild-type. Molecular masses are indicated in kDa.

To test this further, we analysed the SUMOylation state of three phosphorylation mutants: K882E, preventing ATP binding; Y1007F, inhibiting full kinase activation; and V617F inducing constitutive activity (Figures 2A and 2C) [19]. Figure 2(D) shows a summary of 15 independent experiments. The two mutants lacking kinase activity (one truncated at amino acid 842, called 842stop, and K882E) showed significantly less SUMOylation, whereas the constitutively active V617F mutant had a 50% increased SUMOylation signal compared with wild-type. These results alone indicate that Jak2 SUMOylation depends on the phosphorylation status of the kinase. However, the kinase-inactive Y1007F mutant contained an almost normal SUMOylation signal, indicating that the presence of an active catalytic centre is important for SUMOylation. This is probably due to the kinase conformation facilitating SUMOylation machinery for binding. Results presented in Figure 2(C) support the notion that K882E and Y1007F have different conformations, since only the Y1007F mutant caused accumulation of GHR. Together, these results indicate that SUMOylation of Jak2 depends on the presence of an active catalytic centre.

In an effort to identify functional SUMOylation sites, we mutated several lysine residues within or near SUMOylation consensus sites within the kinase domain (Figures 3A and 3B). In several cases, the mutants lost kinase activity, probably due to structural changes caused by the mutations (results not shown). All kinase-inactive mutants showed a decreased SUMOylation signal, supporting our hypothesis that Jak2 SUMOylation depends on an active catalytic centre. On the other hand, mutations K991R and K1011R (within SUMO consensus sites assessed by the SUMOplot™ prediction program at http://www.abgent.com/tools; Figure 3B, all consensus sites are conserved among mammals), together with K912R and K914R, had a decreased SUMOylation signal (Figure 3C), but normal kinase activity as inferred from the degree of autophosphorylation measured with anti-phosphotyrosine antibodies and ability to phosphorylate GHR (Figure 3E). Additionally, mutations within SUMO consensus motifs at K167R, localized in the FERM (4.1/ezrin/radixin/moesin) domain, and K630R, localized in the pseudokinase domain also showed a lower SUMO signal (Figure 3D). All single or double mutants had decreased
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SUMOylation signal (Figure 3C). As the decrease amounted to not more than 50%, this finding suggests that Jak2 is SUMOylated on multiple lysine residues. However, we cannot exclude the possibility that mutations decrease SUMOylation by affecting Jak2 conformation making it less susceptible for the SUMO machinery to bind. Jak2 contains ~80 lysine residues and SUMO2/3, in contrast with SUMO1, is able to make chains. Since attachment of each SUMO moiety increases the apparent molecular mass by 20 kDa, modification of many lysine residues with the SUMO chains would result in a very high-molecular-mass signal. Our data therefore indicate that Jak2 is modified by SUMO chains on multiple lysine residues.

Both GH-induced activation and stressors stimulate Jak2 SUMOylation

To analyse further the relationship between Jak2 phosphorylation and SUMOylation, we used GHR as the signalling receptor system. GHR-expressing HEK-293 cells were stimulated with GH and endogenous Jak2 was analysed under denaturing conditions. Figure 4(A) shows that short GH stimulus caused a 40% increase in endogenous SUMOylation of Jak2 (P = 0.05).

Figure 3 Jak2 SUMOylation sites

(A) Cartoon of the distribution of mutations in putative SUMOylation sites. (B) The most probable locations of SUMO consensus sites assessed using the SUMOplotTM prediction program. The score indicates the probability. All sites are conserved among mammals. (C) Relative SUMOylation signal of several Jak2 lysine to arginine mutants. HEK-293 cells were transiently transfected with FLAG–Jak2 constructs and anti-FLAG immunoprecipitation under denaturing conditions was performed. SUMOylation signals were normalized and expressed relative to wild-type (wt) FLAG–Jak2. Results are means ± S.E.M. and represent three experiments. (D) HEK-293 cells were transiently transfected with FLAG–Jak2 constructs, anti-FLAG immunoprecipitation was performed and the amount of SUMOylation signal was assessed with anti-SUMO2/3 antibodies. The blot represents two independent experiments. (E) GHR-expressing HEK-293 cells were transiently transfected with different FLAG–Jak2 mutants. Cells were stimulated with GH, immunoprecipitation was performed and phosphoryrosine (pY) signal and GHR shift were assessed with appropriate antibodies. IP, immunoprecipitation; WB, Western blot; wt, wild-type.

Figure 4 GH effects on Jak2 SUMOylation

(A) HEK-293 cells were stimulated with 180 ng/ml GH as indicated. Endogenous Jak2 immunoprecipitation was performed under denaturing conditions. The blot was analysed with antibodies against endogenous SUMO2/3 and Jak2. Phosphorylation was assessed with anti-phosphotyrosine antibody. The blot is representative of three independent experiments. *P < 0.05. (B) GHR expressing HEK-293 were transiently transfected with FLAG–Jak2 and HA–SUMO3. Anti-GHR immunoprecipitation was performed. The blot was analysed with antibodies against HA, FLAG and GHR. (C) GHR-expressing HEK-293 cells were transfected with HA–SUMO3 and FLAG–Jak2. Ubc9 was silenced for 48 h. Anti-FIAG immunoprecipitation was performed under denaturing conditions. The blot was detected with anti-HA, anti-GHR and anti-FIAG antibodies. Phosphorylation of Jak2 was detected with anti-phosphotyrosine. Actin staining was used as loading control. (D) Cells were silenced with either control (ctrl) siRNA or Ubc9-specific siRNA. The amount of Ubc9 was assessed with anti-Ubc9 antibody. IP, immunoprecipitation; WB, Western blot. Molecular masses are indicated in kDa.
was performed. The blot was analysed with antibodies against SUMO2/3 and Jak2. The blot for 0, 30 or 60 min and lysed under denaturing conditions, and anti-Jak2 immunoprecipitation was immunoprecipitated. The blot was analysed with antibodies against SUMO2/3 and Jak2.

**Figure 2C.** HEK-293 cells were subjected to 4 h of serum starvation and lysed under denaturing conditions, and Jak2 was immunoprecipitated. The blot was analysed with antibodies against SUMO2/3 and Jak2. The blot represents three independent experiments. In contrast, the kinase is already phosphorylated (compare with Figure 2C), and a strong signal of SUMOylated Jak2, it suggests that high-molecular-mass SUMOylated Jak2 accumulates in the cytosol. As can be seen in Figure 6(D), endogenous SUMOylated Jak2 is clearly present in the nuclear fraction with only a relatively small amount in the cytosolic fraction. Non-modified Jak2 in the nucleus runs slightly higher than the cytoplasmic one. Whether this is due to additional modifications remains to be investigated. The difference in ratios of SUMOylated Jak2 and unmodified Jak2 between the two cell fractions is striking, considering the limited transfer efficiency of high-molecular-mass protein complexes during the electrophoretic transfer. This shows that high-molecular-mass SUMOylated Jak2 accumulates in the nucleus.

Since Jak2 SUMOylation depends on its activity (Figure 2D), we asked whether there is a relation between Jak2’s enzyme activity and its cellular distribution. Cells were transfected with different FLAG-tagged Jak2 mutants, cytosolic and nuclear fractions were isolated and analysed with anti-FLAG antibodies (Figure 6E). In Figure 6(F), Jak2 mutants were expressed relative to wild-type Jak2. Kinase-inactive species showed a 50% decreased nuclear localization, whereas both the Y1007F and the V617F mutants showed a 20% increase in nuclear localization compared with wild-type Jak2. Thus there is a positive correlation between SUMOylation and nuclear translocation of Jak2.

To prove that GH stimulation indeed drives nuclear translocation of Jak2, we used fluorescent microscopy and visualized endogenous Jak2. As can be seen in Figure 7, upon GH stimulation, there is a clear increase in the amount of nuclear Jak2 which supports our hypothesis that SUMOylation drives nuclear translocation of Jak2. To support further our hypothesis that SUMOylation of Jak2 drives its nuclear translocation, we silenced Ubc9 for 48 h and analysed Jak2 in cytosolic and nuclear fractions under denaturing conditions. Ubc9 silencing substantially reduced Jak2 SUMOylation. However, treatment with staurosporin alone caused a substantial increase in Jak2 SUMOylation, independently of GH. We therefore reasoned that general stressors might induce Jak2 SUMOylation. As seen in Figure 5, general stressors could induce Jak2 SUMOylation. As seen in Figure 5(B), starvation caused a 40% increase in Jak2 SUMOylation. To test further the hypothesis that certain stressors might induce Jak2 SUMOylation, we exposed cells to elevated temperature. As seen in Figure 5(C), incubation at 39°C increased Jak2 SUMOylation more than 100% (P = 0.03).

Thus, although there is a basic level of SUMOylated Jak2 in the cells, both cytokine receptor activation and several stressors increase Jak2 SUMOylation.
an increase in high-molecular-mass Jak2 in the nuclear fraction indicating that the cellular concentration of either factor drives both SUMOylation and nuclear translocation (Supplementary Figure S1C).

In conclusion, we have shown that Jak2 is present in the nucleus, almost exclusively in its SUMOylated state. Our data provide the first evidence that SUMOylation might be responsible for Jak2 translocation to the nucleus.

**DISCUSSION**

In the present study, we have demonstrated that Jak2 is modified by SUMO2/3 chains up to a high molecular mass. This modification is induced both by cytokine stimulation and by stressors and depends on the presence of an active catalytic centre. Importantly, we provide evidence that (poly)SUMOylation of Jak2 induces nuclear translocation of the kinase. This mechanism might control the concentration of Jak2 in the cytosol. Although we cannot exclude that SUMOylation prevents Jak2 from being exported from the nucleus, a nuclear targeting function for the poly-SUMO chains on Jak2 is most obvious.

The majority of SUMOylated Jak2 migrates as high-molecular-mass species in the gel. Usually, SUMOylation of proteins generates smaller conjugates. In addition, we observe a ladder characteristic for five to eight SUMO moieties (Figure 1C). Analysis of Jak2 lysine mutants (Figure 3C and 3D) indicates that Jak2 is modified on several lysine residues, probably by SUMO2/3 chains. However, multiple mono-SUMOylation remains possible. As Jak2 contains ~80 lysine residues, SUMOylation of only a few would already result in the observed high-molecular-mass signal. Whether other modifications, such as ubiquitylation, contribute to the high-molecular-mass appearance remains to be determined.

Our results show that cytokine stimulation increases SUMOylation of Jak2 and this modification accompanies its translocation into the nucleus (Figures 4A and 7). Jak2 is an important player in activating signalling cascades leading to activation of gene transcription and cell proliferation. Also, it is well established that Jak2 stabilizes cytokine receptors on the plasma membrane, preventing their endocytosis and degradation in the lysosomess [21]. Thus the more Jak2 is available in the cytosol for cytokine receptor binding, the more sensitive cells are for cytokine-induced signalling. Since the kinase is a stable protein with a half-life of more than 12 h, a tight regulation of its activity is crucial for cells. Our results suggest that SUMOylated Jak2 is not binding the GHR (Figure 4B). It is reasonable to assume that a rapid deSUMOylation process in the cytosol would make the kinase once again available for receptor binding. SUMOylation-induced nuclear translocation of the kinase provides an elegant regulatory mechanism decreasing/preventing Jak2-related cytokine receptor signalling by cytosolar depletion. As Jak2 is a stable protein, it is not likely that stress-induced SUMOylation leads to proteosomal degradation. However, it remains to be investigated how the cells regulate the balance between nuclear translocation and degradation of Jak2.

Serum deprivation, heat shock and staurosporin treatment all increase the SUMOylation of Jak2. The increase in SUMO2/3...
modification of proteins in response to various stressors has been reported previously [22–27]. This common cellular response of enhanced SUMOylation suggests that Jak2 SUMOylation constitutes a protective response [23]. In such a scenario, SUMOylation induced by specific stressors would serve as a regulatory mechanism to control Jak2-induced activity in order for the cells to cope with the stressor first. Keeping Jak2 in such a state would be beneficial for the cell under stress especially given that unregulated active Jak2 leads to cancer. Whether a comparable strategy also applies for the other three Janus kinase family members remains to be investigated. Although we did not probe the reversibility of the system, nuclear segregation via poly-SUMOylation offers a reversible mechanism for the cell to respond to specific stresses. Our results indicate that Jak2 SUMOylation depends on the presence of an active catalytic site. Mutants lacking the kinase domain or harbouring an inactive catalytic site (R42Stop and K882E respectively) show a strongly decreased SUMOylation signal. However, mutation of the activating Tyr797 did not prevent SUMOylation. This indicates that the conformation of Jak2 might be a critical factor for substrate recognition by the SUMOylation machinery. As both cytokine stimulation and various stressors increase Jak2 SUMOylation, the mechanism is probably similar. Since stressors do not induce Jak2 tyrosine phosphorylation, kinase activity is probably not necessary for Jak2 SUMOylation. This supports further the hypothesis that specific Jak2 conformation is a prerequisite for SUMO E3 ligase recognition and attachment.

Aberrant Jak2 signalling has been reported in many malignancies [2,3,28–32]. Dawson et al. [2] found that Jak2 translocates to the nucleus where it phosphorylates histone H3 on Tyr41 (H3Y41), which leads to increased expression of haemopoietic oncogene LMO2 (LIM domain only 2) and can be circumvented by Jak2 inhibitors. Additionally, it was reported that constitutively active Jak2 V617F is found more often in the nucleus of haemopoietic cells [3]. The selective Jak2 inhibitor AG490 was able to chase the kinase back to cytoplasm and normalized LMO2 levels in vitro. The authors suggest that blocking Jak2 nuclear translocation might be a new treatment strategy for patients bearing the V617F mutation. This is in line with our results showing increased nuclear localization of the V617F mutant. However, our results indicate that nuclear translocation is connected with SUMOylation rather than with phosphorylation. Since the authors were not focused on Jak2 SUMOylation, their conclusion was possibly based on the phosphorylation status of the kinase. Analysis of the kinase-inactive Y1007F mutant that is SUMOylated and translocated to the nucleus like the wild-type kinase suggests that SUMOylation is a prerequisite for nuclear translocation. It is also possible that Jak2 activation increases SUMOylation and nuclear translocation of mutant Jak2. Indeed, we show an increased SUMOylation of V617F, which is likely to be due to constitutive activation. Thus a drug that interferes with Jak2-poly-SUMOylation would inhibit nuclear translocation and might be equally efficient in treatment of myeloproliferative diseases dependent on the V617F mutation as drugs that inhibit the kinase activity.

The results of the present study demonstrate a new post-translational modification of Jak2 that regulates its localization. Additionally, we show that Jak2 SUMOylation depends on the presence of an active catalytic centre and increases upon cytokine stimulation and exposure to various stresses. We conclude that such a mechanism provides a novel tool to regulate the concentration of Jak2 in the cytosol. Since Jak2 contributes to several human diseases including cancer development, and myeloproliferative and autoimmune diseases, our discovery may be valuable for the development of new therapeutic strategies.

**AUTHOR CONTRIBUTION**

Ger Strous and Magdalena Sedek designed the experiments, Magdalena Sedek performed the experiments, and Ger Strous and Magdalena Sedek wrote the paper.

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**REFERENCES**

16. van Kerkhof, P., Govers, R., Alves dos Santos, C. M. and Strous, G. J. (2000) The cytokine stimulation and exposure to various stresses. We conclude that such a mechanism provides a novel tool to regulate the concentration of Jak2 in the cytosol. Since Jak2 contributes to several human diseases including cancer development, and myeloproliferative and autoimmune diseases, our discovery may be valuable for the development of new therapeutic strategies.


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SUPPLEMENTARY ONLINE DATA
SUMOylation is a regulator of the translocation of Jak2 between nucleus and cytosol

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Figure S1 Effect of Ubc9 silencing and overexpression on Jak2 SUMOylation

(A) GHR-expressing HEK-293 cells were treated with anti-Ubc9 or control (ctrl) siRNA for 48 h. Anti-Jak2 immunoprecipitation was performed under denaturing conditions. The blot was detected with anti-Jak2 and anti-SUMO2/3 antibodies. Phosphorylation of Jak2 was detected with anti-phosphotyrosine antibodies (pY). Actin staining was used as a loading control. (B) HEK-293 cells were treated with control siRNA and Ubc9-specific siRNA for 48 h. Subsequently, cells were fractionated, Jak2 was immunoprecipitated under denaturing conditions, and analysed by Western blotting with antibodies against SUMO2/3 and Jak2. An asterisk indicates a background band. (C) HEK-293 cells were transiently transfected with HA–Ubc9 or HA–SUMO3 and cytosolic/nuclear fractionation was performed. Subsequently, anti-Jak2 immunoprecipitation was performed on both fractions under denaturing conditions. The blot was analysed with antibodies against SUMO2/3 and Jak2. The asterisk indicates a background band. cyt, cytosol; ev, empty vector; IP, immunoprecipitation; nuc, nuclear; WB, Western blot.

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## Table 1  Primers used for mutagenesis of FLAG–Jak2 plasmid

F, forward; R, reverse.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>K882E F:</td>
<td>5′-GGGAGTTGCTGTAGAAAAGCTCAGCATAG-3′</td>
</tr>
<tr>
<td>R:</td>
<td>5′-CTATGAGCCTTTTCTACGGGACACCTCCC-3′</td>
</tr>
<tr>
<td>Y1007F F:</td>
<td>5′-CCGCAGGACAAAGTCTCAGATTCAGAAGGC-3′</td>
</tr>
<tr>
<td>R:</td>
<td>5′-GCGTTGGTCTGTCGAGGAGGAGA-3′</td>
</tr>
<tr>
<td>V617F F:</td>
<td>5′-GGGAGTTGCTGTAGAAAAGCTCAGCATAG-3′</td>
</tr>
<tr>
<td>R:</td>
<td>5′-CTATGAGCCTTTTCTACGGGACACCTCCC-3′</td>
</tr>
<tr>
<td>K167R F:</td>
<td>5′-GACGGATGAGAAAGGGACACCTCCC-3′</td>
</tr>
<tr>
<td>R:</td>
<td>5′-GCCAGGAGGACCAAGGACACCTCCC-3′</td>
</tr>
<tr>
<td>K530R F:</td>
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<tr>
<td>R:</td>
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<tr>
<td>K912R F:</td>
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<td>K914R F:</td>
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<td>K991R F:</td>
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<td>R:</td>
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<tr>
<td>K1011R F:</td>
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<tr>
<td>R:</td>
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</tr>
</tbody>
</table>

K991R/K1011R Mutagenesis of K991R was performed on plasmid K1011R