Janus kinases promote cell-surface expression and provoke autonomous signalling from routing-defective G-CSF receptors

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INTRODUCTION

The G-CSFR [G-CSF (granulocyte colony-stimulating factor) receptor; now referred to as CSF3R] is the major haematopoietic growth-factor receptor involved in neutrophil development [1]. The CSF3R contains four conserved tyrosine residues in the cytoplasmic domain, which upon phosphorylation by JAKs (Janus kinases) become docking sites for SH2 (Src homology 2)-containing signalling proteins such as STAT (signal transducer and activator of transcription 3) [2]. The cytoplasmic domain of CSF3R further comprises a dileucine-based motif crucial for internalization [3], and five conserved lysine residues. We have recently shown that one of these lysine residues, the juxtamembrane Lys632, is the major determinant for ligand-induced lysosomal routing of the activated CSF3R, a process which involves SOCS3-mediated ubiquitination of Lys632 ([4], but see [4a]).

Ligand-induced CSF3R internalization and lysosomal sorting are both crucial for a balanced signalling output, and disruption of either of these processes results in increased proliferation signalling at the expense of G-CSF-induced neutrophil differentiation ([3,4], but see [4a]). Perturbed signalling from CSF3R due to mutations in the CSF3R gene or expression of signalling-defective splice variants, has been implicated in the development of AML (acute myeloid leukaemia) [5]. Importantly, C-terminal truncation mutants of CSF3R found in patients with severe congenital neutropenia who are at high risk of developing AML lack the dileucine-based internalization motif as well as the recruitment site Tyr729, essential for SOCS3-induced ubiquitination of Lys632 ([4], but see [4a], [6]). Although signal attenuation involving ligand-induced endocytosis and lysosomal degradation has been studied in considerable detail for different receptor systems, less attention has been paid to mechanisms regulating ligand-independent distribution and stability of cytokine receptors and their consequences for signal intensity and duration.

Recently, JAKs have been implicated in the regulation of cell-surface expression of various cytokine receptors. For instance, JAK1 binding to the OSMR (oncostatin M receptor) has been suggested to mask a signal within the juxtamembrane part of the receptor that inhibits expression of the mature form of the OSMR at the plasma membrane [7]. Similarly, binding of JAK2 or TYK2 (tyrosine kinase 2) to the TpoR (thrombopoietin receptor) augmented membrane expression and stability of its mature [EndoH (endoglycosidase H)-resistant] form [8,9]. Studies on the IFNAR1 (interferon receptor-α1) revealed that binding of TYK2 attenuates degradation and increases cell-surface expression by masking dileucine- and tyrosine-based internalization motifs present in the JAK-binding domain of the receptor [10,11]. Taken together, these findings fit into a model in which JAKs interfere with the function of motifs involved in receptor endocytosis and lysosomal degradation, thereby increasing receptor expression on the cell surface. On the other hand, an alternative mechanism was demonstrated for EpoR (erythropoietin receptor), where JAK2 binding does not affect receptor endocytosis, but enhances the appropriate folding of the EpoR protein in the ER (endoplasmic reticulum) instead of its lysosomal degradation, thereby increasing receptor expression on the cell surface. On the other hand, an alternative mechanism was demonstrated for EpoR (erythropoietin receptor), where JAK2 binding does not affect receptor endocytosis, but enhances the appropriate folding of the EpoR protein in the ER (endoplasmic reticulum) instead of its lysosomal degradation, thereby increasing receptor expression on the cell surface.

Key words: endocytosis, granulocyte colony-stimulating factor (G-CSF) receptor, Janus kinase (JAK), signal transducer and activator of transcription 3 (STAT3), signal transduction.

CSF3R [G-CSF (granulocyte colony-stimulating factor) receptor] controls survival, proliferation and differentiation of myeloid progenitor cells via activation of multiple JAKs (Janus kinases). In addition to their role in phosphorylation of receptor tyrosine residues and downstream signalling substrates, JAKs have recently been implicated in controlling expression of cytokine receptors, predominantly by masking critical motifs involved in endocytosis and lysosomal targeting. In the present study, we show that increasing the levels of JAK1, JAK2 and TYK2 (tyrosine kinase 2) elevate steady-state CSF3R cell-surface expression and enhanced CSF3R protein stability in haematopoietic cells. This effect was not due to inhibition of endocytotic routing, since JAKs did not functionally interfere with the dileucine-based internalization motif or lysine-mediated lysosomal degradation of CSF3R. Rather, JAKs appeared to act on CSF3R in the biosynthetic pathway at the level of the ER (endoplasmic reticulum). Strikingly, increased JAK levels synergized with internalization- or lysosomal-routing-defective CSF3R mutants to confer growth-factor independent STAT3 (signal transducer and activator of transcription 3) activation and cell survival, providing a model for how increased JAK expression and disturbed intracellular routing of CSF3R synergize in the transformation of haematopoietic cells.

INTRODUCTION

The G-CSFR [G-CSF (granulocyte colony-stimulating factor) receptor; now referred to as CSF3R] is the major haematopoietic growth-factor receptor involved in neutrophil development [1]. The CSF3R contains four conserved tyrosine residues in the cytoplasmic domain, which upon phosphorylation by JAKs (Janus kinases) become docking sites for SH2 (Src homology 2)-containing signalling proteins such as STAT (signal transducer and activator of transcription 3) [2]. The cytoplasmic domain of CSF3R further comprises a dileucine-based motif crucial for internalization [3], and five conserved lysine residues. We have recently shown that one of these lysine residues, the juxtamembrane Lys632, is the major determinant for ligand-induced lysosomal routing of the activated CSF3R, a process which involves SOCS3-mediated ubiquitination of Lys632 ([4], but see [4a]).

Ligand-induced CSF3R internalization and lysosomal sorting are both crucial for a balanced signalling output, and disruption of either of these processes results in increased proliferation signalling at the expense of G-CSF-induced neutrophil differentiation ([3,4], but see [4a]). Perturbed signalling from CSF3R due to mutations in the CSF3R gene or expression of signalling-defective splice variants, has been implicated in the development of AML (acute myeloid leukaemia) [5]. Importantly, C-terminal truncation mutants of CSF3R found in patients with severe congenital neutropenia who are at high risk of develop-

Abbreviations used: 7-AAD, 7-amino-actinomycin D; AML, acute myeloid leukaemia; BioG, biotinylated granulocyte colony-stimulating factor; CHX, cycloheximide; CSF3R, granulocyte colony-stimulating factor receptor; DMEM, Dulbecco’s modified Eagle’s medium; EndoH, endoglycosidase H; EpoR, erythropoietin receptor; ER, endoplasmic reticulum; ESCRT, endosomal sorting complex required for transport; FCS, foetal calf serum; G-CSF, granulocyte colony-stimulating factor; GGA, Golgi-associated α-adaptin ear homology domain Arf (ADP-ribosylation factor)-interacting protein; IFNAR1, interferon receptor-α1; IL, interleukin; IRES, internal ribosome entry site; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; MPI, mean fluorescence intensity; OSMR, oncostatin M receptor; PE, phycoerythrin; SOCS3, suppressor of cytokine signalling 3; STAT, signal transducer and activator of transcription; TpoR, thrombopoietin receptor; TYK2, tyrosine kinase 2; wt, wild-type.

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reticulum), leading to more efficient Golgi processing and enhanced forward routing of mature protein to the cell surface [12].

Increased JAK expression has been implicated in several haematopoietic malignancies, including AML, and amplification of the JAK2 gene has been reported in Hodgkin’s lymphoma [13,14]. In the present study, we examined whether JAKs control the expression of CSF3R and how this might affect signalling in the absence of ligand-induced receptor activation. We show that JAKs elevate the steady-state CSF3R cell-surface expression, independently of the major determinants of receptor internalization (dileucine motif) or lysosomal routing (conserved lysine residues). Strikingly, increased JAK levels resulted in growth-factor-independent cell survival involving activation of multiple kinases and spontaneous STAT activation in cells expressing internalization- or lysosomal-routing-defective CSF3R mutants.

**EXPERIMENTAL**

**Antibodies**

Mouse anti-human CSF3R antibodies were purchased from Becton Dickinson/PharMingen. Monoclonal rabbit anti-JAK2 antibody, polyclonal rabbit anti-JAK1, rabbit anti-TYK2 and rabbit anti-phosphoSTAT3 antibodies, and the secondary goat anti-rabbit antibody coupled to horseradish peroxidase (termed GAR–HRP) were obtained from Cell Signaling Technology. Mouse anti-phosphoSTAT5a/b antibody was obtained from Upstate Biotechnology. Goat anti-actin and mouse anti-ubiquitin (P4D1) antibodies were purchased from Santa Cruz Biotechnology. Anti-mouse CD2 antibody and anti-human CD4 antibody, both coupled to FITC, were purchased from BD Biosciences. Secondary goat anti-mouse, goat anti-rabbit and donkey anti-goat antibodies coupled to either IRDyeTM680 or IR-DyeTM800CW were purchased from LI-COR Biosciences. Goat anti-mouse immunoglobulin coupled to PE (phycoerythrin) (termed GAM-PE) was obtained from Dako. Donkey anti-mouse Alexa Fluor® 488 and 7-AAD (7-amino-actinomycin D) were purchased from Molecular Probes (Invitrogen).

**Expression constructs**

The pLNCX and pBabe retroviral plasmids containing wt (wild-type) CSF3R, the single lysine mutants K632R, K672R, K681R, K682R, K762R, the lysine null-mutant K5R, and the single lysine add-back mutants mKA, mKB, mKC, mKD and mKE have been described previously [4], but see [4a], as have the W650R and add-back mutants mKA, mKB, mKC, mKD and mKE have been previously reported [8,17].

**Cell culture, transfection and retroviral transduction**

Murine myeloid 32D.c8.6, a subline of the IL (interleukin)-dependent murine myeloid 32Dc3 cell line that lacks endogenous CSF3R expression [18] and Ba/F3 cells, mouse pro-B cells that lack endogenous CSF3R [18,19], were maintained in RPMI-1640 medium supplemented with 10% (v/v) FCS (foetal calf serum), 10 ng/ml murine IL-3, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C and 5% CO2. Ba/F3 cells expressing murine JAK1, murine JAK2 or human TYK2 have been described previously [8]. Phoenix E, HeLa and the parental human fibrosarcoma 2C4 cells [20] were cultured in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% (v/v) FCS, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C and 5% CO2. JAK1-deficient U4C [21] and JAK2-deficient γ2A [20] human fibrosarcoma cell lines were cultured in DMEM containing 400 μg/ml Geneticin (G418). The TYK2-deficient human fibrosarcoma cell line 11.1 [22] was cultured in DMEM in the presence of 400 μg/ml hygromycin. Ba/F3 cells and 32D cell lines were retrovirally transduced with virus from the Phoenix E packaging cell line (a gift from Dr G. Nolan, Department of Microbiology and Immunology, Stanford University, Stanford, CA, U.S.A.) as previously described [3].

**Constitutive and ligand-induced internalization determined by flow cytometry**

To determine constitutive internalization, 32D cells expressing wt or K5R CSF3R were incubated with a mouse anti-human CSF3R antibody in PBS [PBS supplemented with 1% (v/v) FCS and 0.05% (w/v) sodium azide] for 1 h at 4°C. After washing, cells were incubated at 37°C for 0, 30, 60, 120 and 240 min. Next, cells were stained with a goat anti-mouse–PE secondary antibody in PSA for 1 h at 4°C and, after a final wash, analysed by flow cytometry using FACS Calibur (BD Biosciences). The MFI (mean fluorescence intensity) of histograms was taken as a measure of the average CSF3R expression.

To study ligand-induced internalization of the CSF3R along with JAK overexpression, Ba/F3 cells were incubated with 10 ng/ml human G-CSF for 30 min at 4°C. Thereafter the cells were incubated at 37°C for 0, 15, 30 and 60 min. Cell-surface expression of the G-CSF receptor was determined by labelling the cells with PE-conjugated mouse anti-human CSF3R antibody for 1 h at 4°C followed by flow cytometric analysis.

**Receptor protein stability assay**

Phoenix E cells were transiently transfected with CSF3R constructs in pBabe with or without co-transfecting murine JAK2 using calcium phosphate precipitation. At 2 days after transfection, cells were incubated for 3 h with 50 μg/ml CHX (cycloheximide) to block protein synthesis or were left untreated. Cells were washed with ice-cold PBS and resuspended in lysis buffer [20 mM Tris/HCl (pH 8.0), 137 mM NaCl, 10 mM EDTA, 100 mM NaF, 1% (v/v) Nonidet P40, 10% (v/v) glyc erol, 50 μg/ml aprotinin, 1 mM pefabloc and 2 mM sodium orthovanadate]. Lysates were put on ice for 30 min and then centrifuged for 15 min at 21 000 g to remove insoluble proteins. CSF3R was pulled down from lysates by adding magnetic Protein-G beads (Dynabeads, Invitrogen) pre-incubated with CSF3R overnight at 4°C. Beads were incubated in Laemmli buffer for 5 min at 95°C and then were subjected to SDS/PAGE and Western blot analysis as described previously [23]. Proteins were detected by fluoroescence labelled secondary antibodies followed by detection using an Odyssey IR Imaging System (LI-COR Biosciences).

**Ubiquitination of CSF3R**

Ba/F3 cells were incubated with 500 nM bafilomycin A1 (Calbiochem) for 30 min. Cells were stimulated with BioG (bionylinated G-CSF) prepared using a biotin-labelling kit according to the manufacturer’s protocol (Roche Molecular Biochemicals) for 1 h at 37°C. Cells were washed twice with ice-cold PBS and lysed. CSF3R was pulled down using streptavidin-coated magnetic beads (Dynabeads, Invitrogen). Beads were washed and resuspended in Laemmli buffer, incubated for 5 min at 95°C and subjected to Western blot analysis as described previously [23].

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JAKs enhance CSF3R cell-surface expression

To determine the effects of elevated JAK1, JAK2 or TYK2 expression on CSF3R cell-membrane levels, we used the previously described Ba/F3 cell system co-expressing CD2 or CD4 from an IRES-containing expression vector [8]. First, we confirmed that

Proliferation and survival assay

JAK2-overexpressing Ba/F3 cells stably expressing wt, K5R or d715 were washed and transferred to RPMI medium without growth factors. Cells were counted and the cell viability was assessed on a daily basis by flow cytometry using 7-AAD. For the inhibitor study, 10 μM LY294002, 10 μM JAK inhibitor I, 10 μM SB203580, 10 μM U0126, 0.2 μM Akt inhibitor IV or 10 μM PP2 (Calbiochem; all dissolved in DMSO), or DMSO as solvent control was added to the medium. Half of the medium was replaced daily by fresh medium with fresh inhibitors.

Statistical analysis

For comparing two groups, Student’s t tests were performed. When comparing multiple groups, one-way ANOVA was performed followed by a post hoc test.

RESULTS

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EndoH treatment

Phoenix E cells were transiently transfected with wt CSF3R. For membrane receptor pulldown, cells were incubated with 1:100 BioG for 30 min at 37°C. Cells were then washed twice with ice-cold PBS and resuspended in lysis buffer (as above). For total CSF3R levels, cells were first lysed and subsequently 1:50 BioG was added after overnight incubation at 4°C. CSF3R was then pulled down using streptavidin-coated magnetic beads. For EndoH treatment (New England Biolabs), beads were washed and resuspended in 50 μl 1 × GD denaturation buffer (supplied by New England Biolabs) and boiled for 10 min at 100°C. After adding 10 × G5 reaction buffer (supplied by New England Biolabs), the supernatant was divided into two portions. EndoH was added to one portion and water, as a control, was added to the other. The samples were incubated for 3 h at 37°C and thereafter subjected to Western blot analysis.

Confocal microscopy

HeLa cells were seeded on to glass coverslips and transfected with wt or K5R CSF3R in pLNCX using LipofectamineTM (Invitrogen). 32D.c12.6 clones stably expressing wt or K5R CSF3R were spun down on glass slides. Cells were prepared and analysed by confocal microscopy using a LSM510 microscope equipped with argon and helium/neon lasers as described previously ([4], but see [4a]).

Figure 1  JAK1, JAK2 and TYK2 augment cell-surface expression of CSF3R

(A) JAK overexpression in Ba/F3 cells assessed by flow cytometry using CD2– or CD4–FITC antibodies. The control is Ba/F3 cells without antibody staining. (B) Western blot analysis showing JAK levels in parental Ba/F3 cells and JAK transfectants. To control for loading, the blot was restained for actin. (C) Expression of wt CSF3R in stably transduced Ba/F3 cell lines determined by flow cytometry using the CSF3R–PE antibody. The control is Ba/F3 parental cells without antibody staining. (D) Immunoblots of total cell lysates from human parental fibrosarcoma cells (2C4) and derivatives deficient for either JAK1, JAK2 or TYK2 (U4C, γ2A and 11.1 respectively). Blots were stained for anti-JAK1, anti-JAK2 and anti-TYK2 to confirm JAK-deficiencies. Stainings with anti-STAT5 and anti-STAT3 were included for loading controls. (E) Flow cytometric analysis of CSF3R cell-surface levels in the human fibrosarcoma cell lines stably transduced with wt CSF3R. The control is 2C4 cells without antibody staining.

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Figure 2  JAK-induced surface expression of CSF3R depends on a tryptophan residue (Trp<sup>650</sup>) that is critical for JAK binding

(A) Left-hand panel: JAK2-overexpressing Ba/F3 cells with the indicated CSF3R variants were stimulated with biotinylated G-CSF (BioG) for 30 min. Receptor complexes were isolated using streptavidin pulldown and analysed by Western blotting for the presence of CSF3R, JAK2 or phospho-JAK2. For loading control, blots were stained for actin. A representative blot of three independent experiments is shown. Right-hand panel: lack of EndoH sensitivity of BioG-precipitated plasma-membrane bound CSF3R (left two lanes). CSF3R immunoprecipitates of total cell lysates (TCL, right two lanes) serve as positive controls for EndoH sensitivity of immature CSF3R (lower arrows). (B) Quantification of total JAK2 (left-hand panel) and phospho-JAK2 (right-hand panel) binding to wt CSF3R, mutant K5R and W650R. Values are expressed as means ± S.D. from three experiments. (C) Expression of mutant W650R relative to wt CSF3R in Ba/F3 parental and JAK-overexpressing cells. Cell-surface expression was determined by flow cytometry. Values are expressed as MFI ± S.E.M. *P < 0.05 compared with parental Ba/F3 cells expressing wt CSF3R.

the amount of CD2 or CD4 on the cell surface, determined by flow cytometry, correlated with the expression of the different JAKs, as determined by Western blot analysis (Figures 1A and 1B). Parental Ba/F3 cells and the JAK-overexpressing cells were then retrovirally transduced to express wt CSF3R and cell-membrane expression was determined by flow cytometry. JAK1, JAK2 or TYK2 transduced cells showed a 2- to 4-fold higher level of CSF3R expression on the cell surface compared with parental control cells (Figure 1C). Complementary to the experiments in the Ba/F3 transfectants, we studied the consequences of the absence of each of these JAK proteins on CSF3R expression using JAK-deficient fibrosarcoma cell lines (Figure 1D). All cell lines showed comparable cell-surface expression of CSF3R, suggesting that JAK1, JAK2 and TYK2 are redundant in controlling steady-state CSF3R cell expression (Figure 1E). These results raised the additional question as to whether CSF3R cell-surface expression would be significantly hampered in the absence of any JAK binding. To address this, we introduced the CSF3R mutant W650R.
which fails to activate JAKs [15], into Ba/F3 cells. W650R CSF3R was severely hampered in both JAK2 binding and activation (Figures 2A and 2B). In agreement with its inability to bind JAKs, no significant increase in cell-surface expression of W650R CSF3R was seen in the JAK-overexpressing Ba/F3 cells relative to parental controls (Figure 2C). Notably, membrane expression of W650R CSF3R in parental Ba/F3 cells was equal to wt CSF3R, suggesting that JAK binding as such is not required for CSF3R expression (Figure 2C).

Role of receptor lysine residues in steady-state cell-surface expression and stability of CSF3R

One of the mechanisms implicated in the JAK-mediated effects on cell-surface expression of cytokine receptors is that motifs involved in receptor endocytosis and lysosomal degradation are masked by stearic interference [7,10,11]. We have previously demonstrated that ubiquitination of lysine residues in the intracellular domain of CSF3R is important for ligand-induced lysosomal targeting, but not for receptor internalization ([4], but see [4a]). To determine whether receptor lysine residues are involved in ligand-independent (constitutive) endocytosis, we studied membrane expression of a receptor mutant in which all five cytoplasmic lysine residues were replaced with arginine residues (mutant K5R). Myeloid 32D cells stably expressing K5R showed higher membrane expression levels compared with wt CSF3R (Figure 3A), a result that was confirmed by confocal microscopy in 32D cells (Figure 3B, top panels) and in HeLa cells (Figure 3B, bottom panels). However, kinetic analysis of ligand-independent internalization of wt and K5R CSF3R were similar, indicating that lysine residues are not critical in this process (Figure 4A). Taken together, these results suggest that lysine residues within the CSF3R cytoplasmic region are involved in the control of membrane expression through the biosynthetic pathway. To confirm this, we studied the role of the receptor cytoplasmic lysine residues in CSF3R stability. To this end, Ba/F3 cells stably expressing wt or K5R CSF3R were incubated for 3–6 h with CHX to inhibit de novo protein synthesis. While degradation of wt CSF3R was observed after inhibition of protein synthesis, K5R CSF3R was found to be stable (Figure 4B). The lysosome inhibitor bafilomycin prevented degradation of wt CSF3R, suggesting that steady-state receptor levels are regulated via lysosomal degradation and that lysine residues are important for this mechanism.

Because the lysine residues in the cytoplasmic domain of CSF3R are fully conserved between multiple species, we investigated which of these are specifically involved in the negative control of steady-state CSF3R membrane expression. Using a panel of lysine substitution (K → R) and single lysine add-back (mKA–mKE) mutants, we found that Lys632 is the major inhibitory determinant for constitutive CSF3R cell-surface expression (Figure 4C).

**JAKs do not mask motifs involved in CSF3R endocytosis or lysosomal routing**

Next, we examined whether increased JAK levels alter the kinetics of CSF3R internalization. As shown in Figure 5(A), none of the JAKs affected the kinetics and magnitude of CSF3R endocytosis. On the other hand, JAK2 and TYK2, and to a lesser extent JAK1, significantly enhanced surface expression of d715 CSF3R and K5R CSF3R (Figure 5B), showing that JAKs do not elevate CSF3R expression by blocking internalization or lysine-based lysosomal-sorting motifs, which are lacking in these mutants. Also, in the presence of excess JAKs, Lys632 remains the most prominent lysine residue involved in the down-regulation of CSF3R surface expression (Figure 5C, compare with Figure 4C).

**JAKs do not inhibit ubiquitination of Lys632 in ligand-activated CSF3R**

From the studies described above, Lys632 emerges as a major negative-determinant in constitutive CSF3R expression. Upon ligand activation of the CSF3R–JAK complex, Lys632 is ubiquitinated, which is essential for G-CSF-induced lysosomal routing of CSF3R ([4], but see [4a]). Because JAKs are activated in this setting, we wondered whether increased JAK levels may...
Figure 4  Lysine residues are important for steady-state CSF3R cell-surface expression, but are not involved in constitutive internalization

(A) 32D cells stably expressing wt or K5R CSF3R were labelled with CSF3R antibody and were allowed to internalize for 4 h in the absence of ligand. CSF3Rs remaining at the cell surface were labelled with goat anti-mouse-PE and analysed by flow cytometry. Values from three independent experiments are expressed as MFI ± S.E.M. relative to t = 0, which was set at 100 %. (B) Protein synthesis in Ba/F3 cells stably expressing wt or K5R was blocked by CHX added to the medium for 0, 3 and 6 h. The amount of CSF3R in total cells lysates was visualized by Western blot analysis using a CSF3R-specific antibody (lanes 1–3). The addition of the lysosomal inhibitor bafilomycin shows that wt CSF3R is constitutively degraded via the lysosomal pathway (lanes 4–6). (C) CSF3R cell-surface expression on Ba/F3 parental cells transduced with wt CSF3R, lysine substitution (K632R, K672R, K681R, K682R and K762R) or add-back mutants (mKA, mKB, mKC, mKD and mKE). Results are shown as MFI ± S.E.M. from four to seven clones relative to the wt receptor. *P < 0.05 compared with wt receptor. ***P < 0.001 mKA compared with K5R CSF3R.

Figure 5  Enhanced CSF3R cell-surface expression by JAKs is not due to masking of internalization or lysosomal sorting motifs

(A) Ba/F3 parental and JAK-overexpressing cells stably expressing wt CSF3R were incubated with G-CSF (100 ng/ml) and at the time points indicated were stained with anti-CSF3R–PE followed by flow cytometric analysis. Values from three independent experiments are expressed as MFI ± S.E.M. relative to t = 0, which was set at 100 %. (B) Cell-surface expression of internalization-defective d715 CSF3R and lysosomal-routing defective K5R is strongly increased by JAK2 or TYK2, and to a lesser extent JAK1 overexpression. Receptor membrane expression was determined as in (A) and values are expressed as MFI ± S.E.M. from four to seven clones relative to parental Ba/F3 cells expressing wt CSF3R (set at 100 %). (C) CSF3R cell-surface expression on JAK2-overexpressing Ba/F3 cells transduced with wt CSF3R, lysine substitution (K632R, K672R, K681R, K682R and K762R) or add-back mutants (mKA, mKB, mKC, mKD and mKE) and parental Ba/F3 cells transduced with wt receptor (set at 100 %). Values are expressed as in Figure 4(C). *P < 0.05 K632R compared with wt CSF3R. ***P < 0.001 mKA compared with K5R CSF3R.
Figure 6  JAKs control CSF3R expression in the biosynthetic pathway irrespective of receptor ubiquitination

(A) Ubiquitination (Ub) status of the lysine residue at position 632 of membrane CSF3R determined in Ba/F3 parental and JAK-overexpressing cells. Cells were stimulated with BioG for 30 min in the presence of bafilomycin, whereafter CSF3R was pulled down with streptavidin-coated magnetic beads, eluted and subjected to Western blot analysis using an anti-ubiquitin antibody P4D1. Vertical line (left-hand side) indicates the size variation of receptor–ubiquitin complexes. Blots were restained with anti-CSF3R to determine CSF3R loading. The black line between lanes 4 and 5 indicates that two parts of the blot have been juxtaposed. (B) Stability of wt and K5R CSF3R transiently expressed with or without JAK2 in Phoenix E cells. Cells were left untreated or treated with CHX for 3 h to block protein synthesis. CSF3R was immunoprecipitated using CSF3R antibodies and subjected to Western blotting using the same antibodies. The top panel shows the relative intensities between CSF3R mutants with and without overexpression of JAK2. In the second panel the intensities are adjusted to show the difference between the mature and immature CSF3R band. The quantification of mature and immature forms of CSF3R is shown in the right-hand panel; protein levels are expressed in arbitrary units (AU). Total cell lysates were also stained for JAK2 content and for actin (loading control). Endogenous JAK2 is below the detection level on this blot. The results shown are representative of three independent experiments.

JAKs enhance protein stability of CSF3R

To determine whether JAKs influence CSF3R protein stability, e.g. as shown for the TpoR [8], Phoenix E cells transiently expressing JAK and wt CSF3R were cultured in the presence or absence of CHX for 5 h to block protein synthesis. Lysates were then subjected to immunoprecipitation with anti-CSF3R antibodies. Immunoblots revealed two CSF3R species, the higher band representing the mature form. The lower band (not to be confused with the glycosylation variant shown in Figure 2A), was sensitive to EndoH treatment, thus representing immature CSF3R protein. Overexpression of JAK1, JAK2 and TYK2-overexpressing cells relative to parental cell controls. Taken together, these results indicate that JAKs do not interfere with the function of Lys632 in either forward or retrograde routing of CSF3R.

Increased JAK levels confer ligand-independent signalling to internalization- and lysosomal-routing-defective CSF3R mutants

Having established that JAKs increase the stability and membrane expression of CSF3R, we wondered whether increased JAK levels can co-operate with mutant CSF3R defective in lysosomal degradation to enhance downstream signalling. As reported previously [24], both parental and JAK2-overexpressing Ba/F3 cells failed to proliferate or survive in the absence of growth factor (IL-3) (Figure 7A). Expression of wt CSF3R in these cells did not alter this growth behaviour. In contrast, JAK2-overexpressing Ba/F3 cells transduced with either the lysosomal-routing-defective mutant K5R or the internalization-defective mutant d715 showed a moderately enhanced proliferation and a pronounced increase in cell survival (Figure 7B). To determine which pathways are responsible for the growth-factor-independent cell survival of the JAK2-overexpressing Ba/F3 cells stably expressing K5R CSF3R, cells were cultured in the presence of a variety of inhibitors (Figure 7C). JAK inhibitor I, a potent inhibitor of JAK2 (IC50 = 1 nM), MEK [MAPK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase) kinase] inhibitor U0126 and Akt (also known as protein kinase B) inhibitor IV completely abolished cell survival.
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Figure 7 JAK2 overexpression provokes ligand-independent pro-survival signalling by internalization-defective and lysosomal-routing-deficient CSF3R mutants

Parental and JAK2-overexpressing Ba/F3 transfectants were cultured in medium with IL-3 and transferred to medium without growth factors. (A) Total cell numbers and (B) the percentage of living cells based on 7-AAD staining were assessed at the time points indicated. Representative graphs of three independent experiments are shown. (C) Effects of various kinase inhibitors on cell survival. See the main text for details on the specificity of different compounds. DMSO, solvent control. Representative graphs of three independent experiments are shown. (D) Cell lysates were made after removal of growth factor (time w/o GF) at the indicated time points (h) and subjected to Western blot analysis with antibodies against phosphoSTAT3, phosphoSTAT5 and actin as a loading control.

Blocking of PI3K (phosphoinositide 3-kinase), acting upstream of Akt, by LY294002 also severely diminished survival, as did inhibition of Src activity by the tyrosine kinase inhibitor PP2. This result indicates that multiple pathways contribute to the growth-factor-independent cell survival of the routing-defective K5R CSF3R mutant. In contrast, inhibition of the stress-associated p38 MAPK [25] by SB203580, resulted in significantly increased survival. Because both STAT3 and STAT5 have been implicated as important signalling molecules for G-CSF-induced proliferation and survival [26,27], we investigated whether these STATs are constitutively activated in JAK2-overexpressing Ba/F3 cells harbouring K5R CSF3R or d715 CSF3R. A prominent ligand-independent activation of STAT3, but not STAT5, was observed in JAK2-overexpressing Ba/F3 cells transduced with the mutants K5R or d715, but not with wt CSF3R (Figure 7D).

DISCUSSION

In the present study, we investigated the role of JAK proteins in controlling CSF3R expression and intracellular routing. Specifically, we addressed to what extent JAKs interfere with the internalization and lysosomal-routing machinery, linked to the dileucine-based internalization and conserved cytoplasmic lysine residues respectively. Major observations were: (i) increased JAKs significantly elevated CSF3R membrane expression and protein levels, (ii) JAKs did not exert this effect by masking motifs essential for internalization, as previously demonstrated for IFNAR1, or by reducing the ubiquitination of the lysosomal-routing determinant Lys632 of CSF3R, and (iii) simultaneous perturbation of internalization or lysosomal routing and a JAK-mediated increase in forward routing resulted in ligand-independent activation of CSF3R complexes.

Enhanced cell-surface expression after JAK overexpression has been reported for a number of cytokine receptors [7,8,10,12,28], but whether endogenous JAKs control receptor routing has been a matter of controversy. For example, a variety of EpoR mutants that failed to bind JAK2 showed only moderately reduced cell-surface expression compared with wt EpoR and EpoR was still expressed on the cell surface of JAK2-deficient embryonic fibroblasts [29]. Because EpoR exclusively binds JAK2, these results implied that cell-surface expression of EpoR does not require JAKs. On the other hand, a clear relationship between loss of TYK2 and reduced membrane expression was reported for IFNAR1, owing
to TYK2-mediated masking of endocytosis motifs [10,11] and, similarly JAK1 was found to inhibit internalization of OSMR [7]. In this respect, CSF3R resembles the EpoR, because increased JAK levels did not affect endocytosis and because cell-surface expression of the CSF3R mutant W650R, which fails to activate JAKs and is hampered in JAK binding, is approximately similar to that of wt CSF3R. However, it remains possible that the residual 10–20% JAK binding observed with W650R CSF3R might still suffice to promote cell-surface routing.

Among the five conserved lysine residues in the cytoplasmic tail of CSF3R, the juxtamembrane residue Lys632, in addition to being a major determinant for ligand-induced lysosomal routing ([4], but see [4a]), also appeared to be uniquely involved in the control of constitutive CSF3R cell-surface expression. Although lysine residues have been reported to regulate ligand-independent internalization of the short isoform of the leptin receptor [30], we could not detect any role for lysine residues in internalization of the CSF3R. Hence loss of Lys632 rather leads to enhanced cell-surface expression by redirection of receptors in the biosynthetic pathway from the ER–lysosomal degradative route towards the plasma membrane. Major players involved in intracellular trafficking of surface membrane proteins are the ESCRT (endosomal sorting complex required for transport) and GGA [Golgi-associated γ-adaptin ear homology domain Arf (ADP-ribosylation factor)-interacting protein] protein complexes respectively [31]. Because key proteins within these complexes, such as Hrs and GGA, interact with ubiquitinated lysine residues in cargo proteins, but simultaneously require interaction with phosphoinositides to increase affinity [32,33] this might explain why membrane proximal positioning of Lys632 is imperative for lysosomal routing of CSF3R. This is supported by recent experiments showing that relocation of Lys632 to a more membrane-distant position disrupted its role in routing despite the fact that ubiquitination was unaffected (A. Wöllfer, M. Irandoust, A. Meenhuis, J. Gits, O. Roovers and I. P. Touw, unpublished work).

How JAKs control basal cell-surface expression of CSF3R remains to be resolved. Because JAKs bind with their N-terminal FERM (4.1/ezrin/radixin/moesin) domain to the juxtamembrane domain of CSF3R that encompasses Lys632, a feasible explanation was that JAK overexpression would mask the function of this lysine residue, e.g. by preventing its ubiquitination or by hindering the binding of effector proteins. This hypothesis can now be discarded because increased JAK levels did not prevent ubiquitination of Lys632 and elevated cell-surface levels of mutants K632R and K5R as efficiently as the wt CSF3R. Most probably, JAKs associate with the CSF3R early in the biosynthetic pathway and help to protect CSF3R from misfolding and degradation, similar to what has been proposed for the EpoR [12].

Abnormal responses to haematopoietic growth factors, including G-CSF, have long been suspected to be involved in leukemic cell growth in AML [34]. The discovery of acquired mutations in CSF3R in patients with severe congenital neutropenia which are strongly associated with progression to AML have supported this idea [35]. Although CSF3R mutations are rarely detected in de novo AML, a scenario can be envisaged in which increased levels of JAK confer spontaneous pro-survival signalling from lysosomal-routing defective CSF3R via multiple downstream pathways. Such a combinatorial mechanism might, e.g. involve deubiquitinating enzymes that attenuate lysosomal routing by deubiquitination of critical lysine residues in CSF3R or proteins of the ESCRT machinery. Notably, this is not without precedent, since it was demonstrated previously that expression of the deubiquitinating enzyme DUB-2 increases survival of haematopoietic cells following cytokine withdrawal [36].

ACKNOWLEDGEMENTS
We thank Dr Stefan Constantinescu (Ludwig Institute for Cancer Research, Brussels, Belgium) for providing Ba/F3 parental and JAK-overexpressing cells, human fibrosarcoma cell lines 2C4, y2A, U4C and 11.1, and the retroviral bicistronic vectors pRex-TYK2-IRESCD2 and pRex Jak1-IRESCD2. We thank Marieke van Linden for helpful discussions and critical reading of the manuscript prior to submission.

FUNDING
This work was supported by the Dutch Cancer Society, KWF kankerbestrijding [grant number EMCR 2006-3585] and the Austrian Science Foundation through Erwin-Schrödinger fellowship [grant number J2536-B05 (to A.W.)].

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