BCR-ABL Affects STAT5A and STAT5B Differentially

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Abstract

Signal transducers and activators of transcription (STATs) are latent cytoplasmic transcription factors linking extracellular signals to target gene transcription. Hematopoietic cells express two highly conserved STAT5-isofoms (STAT5A/STAT5B), and STAT5 is directly activated by JAK2 downstream of several cytokine receptors and the oncogenic BCR-ABL tyrosine kinase. Using an IL-3-dependent cell line with inducible BCR-ABL-expression we compared STAT5-activation by IL-3 and BCR-ABL in a STAT5-specific manner. RNAi targeting of STAT5B strongly inhibits BCR-ABL-dependent cell proliferation, and STAT5B but not STAT5A is essential for BCL-XL-expression in the presence of BCR-ABL. Although BCR-ABL induces STAT5-tyrosine phosphorylation independent of JAK2-kinase activity, BCR-ABL is less efficient in inducing active STAT5A:STAT5B-heterodimerization than IL-3, leaving constitutive STAT5A and STAT5B-homodimerization unaffected. In comparison to IL-3, nuclear accumulation of a STAT5A-eGFP fusion protein is reduced by BCR-ABL, and BCR-ABL tyrosine kinase activity induces STAT5A-eGFP translocation to the cell membrane and co-localization with the IL-3 receptor. Furthermore, BCR-ABL-dependent phosphorylation of Y682 in STAT5A was detected by mass-spectrometry. Finally, RNAi targeting STAT5B but not STAT5A sensitizes human BCR-ABL-positive cell lines to imatinib-treatment. These data demonstrate differences between IL-3 and BCR-ABL-mediated STAT5-activation and isoform-specific effects, indicating therapeutic options for isoform-specific STAT5-inhibition in BCR-ABL-positive leukemia.

Introduction

Signal transducers and activators of transcription (STATs) are a family of proteins involved in signal transduction from multiple cytokine or growth factor receptors with a similar modular composition (STAT1, 2, 3, 4, 5A, 5B, and 6) [1,2]. Inactive STATs are believed to exist either as monomers or pre-formed dimers in an anti-parallel conformation. Upon receptor activation STATs are recruited to activated receptors, and tyrosine phosphorylation of a critical C-terminal residue leads to dimerization or to conformational changes of pre-formed dimers into a parallel orientation involving reciprocal phosphotyrosine-SH2-domain interactions. Active dimers translocate to the nucleus and initiate target gene transcription which may occur via tetrmeric STAT-complexes [3].

STAT5 has a critical role within the hematopoietic system: it is activated by the receptors for Epo, GM-CSF, G-CSF, TPO, IL-2, IL-3, IL-5, IL-7, and IL-15 [1,4]. STAT5 exists in two isoforms with high sequence homology, STAT5A and STAT5B, which are encoded by two different genes. Generation of STAT5A and/or STAT5B null mice has demonstrated redundant and differential functions for these 2 isoforms in predominantly non-hematopoietic cells [5-8]. However, development, proliferation and differentiation of hematopoietic progenitors are also affected by inactivation of STAT5 genes [9-12]. STAT5 activation appears to involve a similar molecular event including phosphorylation of Y694 (STAT5A) and Y699 (STAT5B) [13]. Substitution of Y694 and Y699 with phenylalanine results in dominant-negative STAT5 mutants which inhibit cell proliferation and induce apoptosis. One well characterized STAT5 target is the anti-apoptotic BCL-XL, gene essential for fetal erythropoiesis [14-16].

The oncogenic fusion gene BCR-ABL results from the reciprocal translocation t(9;22)(q34;q11) characteristic for chronic myeloid leukemia (CML) and BCR-ABL-positive acute lymphoblastic leukemia (ALL). BCR-ABL is a constitutively active cytoplasmic tyrosine kinase which activates many intracellular signalling cascades largely overlapping with those activated by cytokine receptors [17-19]. STAT5 is activated by BCR-ABL and is required for induction and maintenance of BCR-ABL-positive leukemia in mice [20-22]. However, we have shown that BCR-ABL is less effective than cytokines to induce proliferation of cells with reduced STAT5 expression using an RNAi-approach targeting STAT5A and STAT5B simultaneously [23]. To compare STAT5 activation by IL-3 with that by BCR-ABL, we used the TonB cell line with inducible BCR-ABL-expression and analyzed STAT5A- and STAT5B-specific loss- and gain-of-function phenotypes in the presence and absence of IL-3 and BCR-ABL. This approach allows direct comparison of IL-3 function with that of BCR-ABL under identical expression and
were cloned into the U3 region of the (LTR) of pHR-SIN-SR- to resulted in a loss of RFP from the lentiviral transgene plasmid.

or DNA sequencing. The preparation of recombinant lentiviral supernatants and lentiviral transductions were performed as according to the manufacturer guidelines, Stratagene, La Jolla, CA. Murine the RFP gene by site-directed mutagenesis (according to the holtz Centre for Infection Research, Braunschweig, Germany). To SR- into STAT5A-eGFP was excised with STAT5-cDNAs were excised with 5' Sal and 3' HindIII (STAT5A) and 5'Sal and 3'SpeI (STAT5B), blunted and inserted into BanHI-digested pHR-SIN-SIEW-SnaBl [25].

A STAT5A-eGFP expression plasmid (pN1-S5a-E-GFP) was kindly provided by Hansjoerg Hauser and Mario Koster (Helmholtz Centre for Infection Research, Braunschweig, Germany). To insert the STAT5A-eGFP cassette into the lentiviral pHR-SIN-SR-SnaBl vector [26] a KpnI restriction site was created upstream the RFP gene by site-directed mutagenesis (according to the manufacturer guidelines, Stratagene, La Jolla, CA). Murine STAT5A-eGFP was excised with 5'BglII and 3'KpnI and inserted into 5'BanHI/3' KpnI-digested pHR-SIN-SR-SnaBl. This strategy resulted in a loss of RFP from the lentiviral transgene plasmid.

For lentiviral expression of shRNAs the H1-shRNA cassettes were cloned into the U3 region of the A3' long terminal repeat (LTR) of pHR-SIN-SR-SnaBl as described before [24]. For construction of epitope-tagged STAT5 isoforms see Information S1.

All molecular modifications were verified by control digestions or DNA sequencing. The preparation of recombinant lentiviral supernatants and lentiviral transductions were performed as described earlier [23].

Proliferation Assay

Cell proliferation was analyzed by the Trypan-blue dye exclusion assay or the MTS Assay. For the Trypan-blue dye exclusion assay, TonB cells were cultured in 24-well plates as follows: 1 x 10^5 cells/mL in the presence of IL-3 and/or BCR-ABL, and 3 x 10^5 cells/mL in the absence of IL-3 (Starvation). Human cell lines were cultured at 1.5 x 10^5 cells/mL in 24-well plates and the number of viable cells was determined 96 hours later by Trypan-blue exclusion. For the MTS Assay TonB cells were cultured for 48 hours with Ruxolitinib and proliferation was measured using MTS (Promega, Mannheim, Germany). Therefore 20 μL of MTS substrate was added to 200 μL cell suspension in a 96 well plate, incubated for 3–4 hours at 37°C in a humidified, 5% CO2 atmosphere and absorbance was recorded at 490 nm using an ELISA plate reader (Mithras LB 940, Berthold Technologies, Bad Wildbad, Germany).

Immunoblotting and Immunoprecipitation

TonB cells cultured with IL-3 or Dox (BCR-ABL) were either starved by removal of IL-3 or BCR-ABL tyrosine kinase activity was inhibited by addition of 1 μM imatinib mesylate (IM) overnight. Whole cell lysates were prepared with either RIPA buffer (50 mM Tris- HCl, pH 7.5; 150 mM NaCl; 1% Triton X-100; 0.5% sodium-deoxycholate; 0.1% SDS; 5 mM EDTA) supplemented with appropriate inhibitors (25 mM NaF; 5 mM Na2VO4; 10 mM AEBSF; 25 μM ALLN; 40 μM MG-132; 20 μM Protein Tyrosine Phosphatase Inhibitor Set IV and 1/ 50th volume of Protease Inhibitor Cocktail Set III - all purchased from Calbiochem, La Jolla, CA, USA) or by direct lysis in NuPAGE LDS Sample Buffer (Invitrogen, Karlsruhe, Germany).

For preparation of subcellular extracts see Information S1. Protein concentrations were determined using the Bradford protein assay (Bio-Rad, Munich, Germany).

Immunoprecipitations (normally 2–3 mg of total protein) were performed overnight in RIPA buffer supplemented with inhibitors, specific antibodies (3–5 μg) and Protein A/G Plus-Agarose Beads (Santa Cruz Biotechnology, Heidelberg, Germany). Proteins were denatured by resuspension in NuPAGE LDS Sample Buffer plus NuPAGE Reducing Agent (Invitrogen, Karlsruhe, Germany) followed by incubation for 10 minutes at 95°C. Lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to OptiTrran BA-S83 reinforced nitrocellulose membranes (Whatman, Dassel, Germany). Membranes were incubated with following antibodies: STAT5A (L-20), STAT5B (G-2), pTyr (PY99), ERK2 (C-14), anti-mouse-IgG-HRP, anti-rabbit-IgG-HRP from Santa Cruz Biotechnology; α-Tubulin (DM1A) from Calbiochem; STAT5A (611835), BCL-xL (610211) from BD Transduction; STAT5B from BD Pharmingen (556517) or from R&D Systems (AF1584) and phospho-STAT5 (Tyr694) (C11C5), phospho-JAK2 (Tyr1007/1008) (C80C3), phospho-GAB2 (Tyr452) (C35G1), BCL-2 (#2186), HA-Tag (6E2), MYC-Tag (9B11), and β-Actin (13E5) from Cell Signaling.

Visualization was obtained by chemiluminescence using the ECL Western Blotting Detection Reagents (GE Healthcare, Munich, Germany). Densitometry was performed using a VersaDoc-4000MP imaging system equipped with QuantityOne quantification software (Bio-Rad, Munich, Germany).

Immunofluorescence

TonB cells were transferred to glass slides by spin-occulation and fixed in 4% [w/v] PFA for 10 minutes. Permeabilization was performed with 0.05% Triton X-100 for 4 minutes. Cells were blocked in PBS supplemented with 10% [v/v] FCS (Biochrome, Berlin, Germany) for 1 hour and stained with PE Rat anti-mouse CD131 (559920) from BD Pharmingen and DAPI (0.5 μg/mL) for 2 hours. Samples were embedded in Mowiol plus DABCO (Roth, Karlsruhe, Germany).
Intracellular localization of STAT5AeGFP-expressing TonB cells was analysed in living cells. Cells were concentrated by centrifugation and resuspended in a small volume of medium. Cells were transferred to poly-L-lysine-coated glass slides and covered with poly-L-lysine-coated cover slides. Both were sealed with silicone and analyzed immediately.

Microscopic analyses were run on a Leica DM IRB laser scanning microscope equipped with a TCS SP2 AOBS scan head, a 405 nm light source for excitation of blue dyes and Leica LCS Lite software (Leica, Wetzlar, Germany).

Selected Reaction Monitoring of STAT5 Isoforms

For MS/MS analysis STAT5 isoforms were immunoprecipitated in larger scales. At least 25 mg of total protein were precipitated with either 50 μg STAT5A (L-20) or STAT5B (G-2) antibodies, then resolved by SDS-PAGE and visualized using a colloidal Coomassie staining (2% [v/v] phosphoric acid, 5% [w/v] Al2(SO4)3 × (H2O)17, 10% [v/v] ethanol, and 0.05% Coomassie Brilliant Blue G-250). A protein band at the correct molecular weight (90-95 kDa) was excised, destained with repeated incubation in 200 mM ammonium bicarbonate, 40% [v/v] acetonitrile. Gel pieces were dried with three washes in 100% acetonitrile and then trypsinised (Trypsin resuspended in 100 mM ammonium bicarbonate, 40% [v/v] acetonitrile, 0.1% [v/v] formic acid) overnight at 37°C. Peptides were extracted from the gel pieces by incubation in 50% [v/v] acetonitrile, 0.1% [v/v] formic acid, peptides were desiccated and resuspended in 5% [v/v] acetonitrile, 0.1% [v/v] formic acid, 20 mM citric acid; pH 2.7. For each analysis, 10% of the peptide sample was loaded onto a nanoACQUITY UPLC Symmetry C18 Trap (5 μm, 180 μm×20 mm) and flow was set to 15 μL/min of 3% [v/v] acetonitrile, 0.1% [v/v] formic acid and 20 mM citric acid for 5 minutes. Analytical separation of the peptides was performed using nanoACQUITY UPLC BEH C18 Column (1.7 μm, 75 μm×250 mm). Briefly, peptides were separated over a 91 minutes solvent gradient from 3% [v/v] acetonitrile, 0.1% [v/v] formic acid to 40% [v/v] acetonitrile, 0.1% [v/v] formic acid on-line to a LTQ Orbitrap Velos (Thermo). Data was acquired using an information dependent acquisition (IDA) method where, for each cycle one full MS scan of m/z 300–1700 was acquired in the LTQ. Each full scan was followed by the selection of the most intense ions, CID and MS/MS analysis was performed in the LTQ. Selected ions were excluded from further analysis for 60 seconds. Ions with an unassigned charge or a charge of +1 were rejected.

Selected reaction monitoring (SRM) analysis of STAT5A phosphotyrosine 682/683 was performed; the liquid chromatography conditions were as described above. For each cycle, one full MS scan was acquired in the LTQ. Each full scan was followed by MS/MS analysis of parent ions selected in the LTQ with an m/z 477.77 (unphosphorylated Y682 peptide YYITYPVLAK), 517.75 (singly phosphorylated) and 557.73 (doubly phosphorylated). Product ions of m/z 216.04, 527.36, 791.47 and 871.43 were scanned for.

SRM analysis was also performed using a LC Packings Ultimate liquid chromatography system and 4000 QTrap (AB Sciei). Typically 30% of the peptide sample was loaded onto a C18 trap column using 2% [v/v] acetonitrile in 0.1% [v/v] formic acid (Buffer A) at a flow rate of 30 μL/min and washed for 4 minutes to remove salts. After desalting the flow was reduced to 320 nL/min and diverted to a 15 cm×75 μm i.d. PepMap, C18, 3 μm column. Gradient conditions; the percent of buffer B (80% [v/v] acetonitrile, 0.1% [v/v] formic acid) was increased from 8% to 40% over 21 minutes and then increased to 60% over the following 10 minutes. The flow was held with 60% buffer B content for 2 minutes before being reduced to 8% for equilibration. Separated peptides were eluted from the analytical column directly into the 4000 Q TRAP which was instructed to scan for the parent and product ions described above.

IDA data was analysed using Mascot (Matrix Sciences) the parameters were; Uniprot database, taxonomy Homo Sapiens; trypsin with up to 1 missed cleavage allowed, variable modification were oxidised methionine, phosphorylated serine, threonine and tyrosine and the peptide tolerance of 0.025 Da and 0.03 Da for MS/MS tolerance.

Determination of Imatinib IC50-values in Human CML Cell Lines

K562 and LAMA-84 cells were lentivirally transduced with control or isoform-specific human STAT5 shRNAs and plated 4 days after transduction in 24-well plates at 1.5×10⁴ cells/mL in the presence of increasing concentrations of imatinib mesylate (0, 0.025, 0.05, 0.1, 0.15, 0.20, 0.25 μM IM). The number of viable cells was determined 96 hours later by Trypan-blue dye exclusion. The number of viable cells was plotted against the concentration of imatinib mesylate and the concentration which reduces this number by 50% of controls was calculated.

Results

TonB Cells and Generation of Isoform-specific Anti-STAT5 shRNAs

In the first instance we characterized a system for the study of STAT5 activation by IL-3 or BCR-ABL or both using TonB cells, a murine IL-3-dependent pro-B-cell line with doxycycline inducible BCR-ABL-expression [27]. Induction of BCR-ABL protein-expression reaches up to 87% of that seen in the human K562 CML cell line in a dose- and time-dependent manner (Figure S1). The TonB model allows direct comparison between IL-3 and BCR-ABL signalling at identical expression levels of STAT5 isoforms and/or relevant cofactors. It was determined that optimal proliferation of these cells is observed in the presence of IL-3 whereas BCR-ABL-mediated proliferation is only about 25% of this IL-3 effect. IL-3 plus BCR-ABL achieved about 70% of the optimal levels of proliferation observed (Figure 1A). Interestingly, inhibition of BCR-ABL tyrosine kinase activity by Imatinib (IM) further improved TonB cell proliferation in the presence of IL-3 and BCR-ABL (Figure 1A). These data demonstrate some kind of interference of BCR-ABL- with IL-3R- signalling in TonB cells most likely by competition and differential impact on common signalling components.

We next designed isoform-specific anti-STAT5 shRNAs to discriminate between the actions of the A- and B-isoforms. The most effective shRNA was selected out of 5 as previously described [24] and western blot analysis revealed isoform-specific reduction of STAT5A and STAT5B expression (>90% with very low cross-reactivity, ≤5%, by the respective shRNAs) (Figure 1B).

Isoform-specific STAT5 Loss-of-function Phenotypes in TonB Cells

TonB cells grown in the presence of IL-3 or maintained by BCR-ABL action were subjected to isoform-specific anti-STAT5 shRNA treatment. We observed that anti-STAT5A and anti-STAT5B shRNAs reduced the number of viable cells by 33% and 23%, respectively, (IL-3 as stimulus) and by 40% and 85%, respectively, (BCR-ABL) compared to control shRNAs (Figure 2A). This decrease in number of viable cells is due to apoptosis as...
shown by an increase in Sub-G1-fraction (Figure S2). Protein expression of BCL-XL, a known target of STAT5, is reduced in BCR-ABL- but not in IL-3-supplemented cultures with reduced STAT5B (sh-muS5B) but not STAT5A (sh-muS5A) expression (Figure 2B). In contrast, BCL-2 expression is only slightly affected by reduction of STAT5 expression under both culture conditions. Similarly, BCL-XL mRNA expression is significantly diminished only in BCR-ABL-expressing but not IL-3 supplemented cultures of TonB cells with reduced STAT5B expression as compared to controls (sh-muS5B 43.7% +/- 19.4%, p = 0.03; Figure 2C). These data demonstrate different STAT5A activity in the presence of IL-3 and BCR-ABL and suggest specific functions of STAT5B in TonB cells in the presence of BCR-ABL.

Isoform-specific STAT5 Gain-of-function Phenotypes in TonB Cells

To investigate these differential effects further, TonB cells were transduced to over-express STAT5A or STAT5B. Increased expression was confirmed by western blotting (Figure 3A). Four days after transduction IL-3 was removed and the number of viable cells was determined over time. Over-expression of STAT5B increased cell survival and proliferation much more efficiently than that of STAT5A with intermediate cell proliferation upon over-expression of both STAT5A and STAT5B (Figure 3B). Furthermore, tyrosine phosphorylation of STAT5B is detectable in TonB cells over-expressing STAT5B in the absence of IL-3 and doxycycline (Figure S3). Interestingly, STAT5B-dependent proliferation was reduced about 20-fold in the presence of imatinib although cells were cultured without doxycycline.
Parental BaF3 cells were not transduced to factor-independent growth by over-expression of either STAT5A or STAT5B under these conditions (data not shown). These data suggest a low basal BCR-ABL expression level in non-induced TonB cells and enhancement of BCR-ABL signalling by over-expression of STAT5B. In addition, they correlate with the functional role of STAT5B in maintenance of anti-apoptotic BCL-XL levels in the presence of BCR-ABL (Figure 2B and C).

STAT5 Phosphorylation by IL-3 Receptor/JAK2 and BCR-ABL Signalling

We next attempted to analyze STAT5 tyrosine phosphorylation by IL-3 and BCR-ABL respectively, using pharmacological JAK2 and BCR-ABL inhibitors. Since JAK2 constitutively binds to the IL-3 receptor β-chain we first analyzed the role of JAK2 kinase activity on BCR-ABL-induced STAT5 tyrosine phosphorylation in the presence or absence of ruxolitinib, a specific JAK2/JAK1-inhibitor (for review see [28]). Ruxolitinib inhibits IL-3 induced cell proliferation more efficiently than that driven by BCR-ABL (Figure 4A). In addition, IL-3 induced STAT5 tyrosine phosphorylation is inhibited by ruxolotinib (Figure 4B). In contrast, STAT5 tyrosine phosphorylation in the presence of BCR-ABL is only inhibited by imatinib but not by ruxolitinib. These data demonstrate tyrosine phosphorylation of STAT5 by BCR-ABL even in the absence of JAK2 kinase activity. However, JAK2 itself can be tyrosine phosphorylated by BCR-ABL in the absence of cytokine stimulation and may thereby propagate BCR-ABL signals. Thus...
oncogenic signalling through JAK/STAT pathway is different to that mediated by cytokines in this model.

Hetero- and Homod-(oligo)merization of STAT5A and STAT5B

There is data on STAT molecules as monomers, pre-formed or active dimers [2] and the interplay between STAT5A and STAT5B, we therefore next considered physical association between these two proteins. STAT5A and STAT5B were immunoprecipitated from whole cell lysates of native TonB bulk cultures under different conditions using isoform-specific antibodies. When BCR-ABL was active, imatinib blocked STAT5A:S-STAT5B heterodimerization as indicated by reduced co-immunoprecipitation of STAT5A and STAT5B (Figure 5). Thus this process is clearly BCR-ABL tyrosine kinase sensitive. This may be linked to tyrosine phosphorylation and indeed tyrosine phosphorylation of STAT5A and STAT5B is detectable if either cells are cultured in IL-3 and/or BCR-ABL, STAT5A and STAT5B heterodimerization as indicated by reduced co-immunoprecipitation of STAT5A and STAT5B (Figure 5). Thus this process is clearly BCR-ABL tyrosine kinase sensitive. This may be linked to tyrosine phosphorylation and indeed tyrosine phosphorylation of STAT5A and STAT5B is detectable if either cells are cultured in IL-3 and/or BCR-ABL, tyrosine kinase activity is present. However, tyrosine phosphorylation of STAT5B appeared to be relatively weaker compared to STAT5A under steady state conditions (Figure 5, right panel). Co-immunoprecipitation of STAT5A and STAT5B was also observed in the presence but not in the absence of IL-3 and again correlated to STAT3 tyrosine phosphorylation. The situation was more complex in the presence of BCR-ABL and IL-3. STAT5A immunoprecipitates in BCR-ABL-expressing cells have about two third less STAT5B present compared to BCR-ABL plus IL-3-treated cells, although tyrosine phosphorylation levels were similar (Figure 5, left panel). In the complementary experiment with STAT5B immunoprecipitates, there was at most only a marginal increase in co-immunoprecipitated STAT5A detectable in the presence of IL-3 and BCR-ABL as compared to BCR-ABL-only cultures (Figure 5, right panel). Finally, BCR-ABL kinase activity induced co-immunoprecipitation of several tyrosine phosphorylated proteins with STAT5A and STAT5B, respectively, and this was independent of the presence of IL-3. IL-3 alone did not achieve such an effect. Thus there is a clear demonstration that phosphotyrosine-dependent STAT5A:S-STAT5B-heterodimerization or -oligomerization is affected by BCR-ABL.

One potential inference from the above data is that BCR-ABL alters the formation of homo- or heteromeric STAT5 complexes by favouring tyrosine phosphorylated STAT5 in monomeric or inactive homomeric conformation and/or heterologous complexes with other signalling molecules. We therefore analyzed homodi- or oligomerization of STAT5 isoforms using epitope-tagged STAT5A and STAT5B variants and found constitutive and phosphotyrosine-independent homodi- or oligomerization of both STAT5A and STAT5B (Figure S4A). These non-functional
homodi- or oligomers of both STAT5A and STAT5B are mostly in the cytoplasm and only about 20% in the nucleus independent of the presence or absence of IL-3 (Figure S4B).

**Intracellular Localization of STAT5A in the Presence of BCR-ABL**

To investigate subcellular localization of STAT5 isoforms in the presence and absence of IL-3 and BCR-ABL, TonB cells were stained with isoform-specific anti-STAT5 antibodies and analyzed by confocal microscopy. STAT5B is mainly found within the nucleus of TonB cells in the presence of either IL-3 or BCR-ABL (Figure 6A). In contrast to STAT5B we could not visualize intracellular STAT5A distribution by this approach. We therefore expressed a STAT5A-eGFP fusion protein in TonB cells which accumulates in the nucleus in almost all cells in the presence of IL-3. In contrast, only approximately 50% of cells show nuclear accumulation of STAT5A-eGFP in the presence of BCR-ABL. Release of tyrosine kinase inhibition by washing out imatinib induced rapid translocation of STAT5A-eGFP to the cell membrane within minutes in a fraction of cells (Figure 6B, middle). Here STAT5A-eGFP co-localizes with the IL-3 receptor β-chain (Figure 6C) before it eventually accumulates within the nucleus. These data demonstrate specific effects of STAT5 Isoforms and BCR-ABL.
BCR-ABL on intracellular localization of STAT5A in line with defective activation by this oncogene. We could not perform corresponding experiments with a STAT5B-RFP transgene since over-expression of STAT5B-RFP is transforming in TonB cells.

Aberrant Tyrosine Phosphorylation of STAT5A in the Presence of BCR-ABL

The above data show that BCR-ABL and IL-3 differentially affect STAT5A and STAT5B. This could be achieved via different tyrosine phosphorylation by the BCR-ABL tyrosine kinase activity (or its downstream activation of other protein kinases). A potential novel phosphorylation site on STAT5A or STAT5B was therefore investigated using a mass spectrometry approach. From an initial discovery based proteomic analysis it was determined that either STAT5A Y682 or Y683 was phosphorylated. Using immunoprecipitated STAT5A from TonB cells treated with IL-3 or doxycycline, selected reaction monitoring (SRM) analysis was used to verify the site of phosphorylation from the peptide YYTPVLAK. As shown in Figure S5 the site of phosphorylation can be determined due to the presence of the product ions at 791.4662Th or 871.4325Th from the parent ion 517.77Th (the doubly charged, phosphorylated YYTPVLAK). Also the immonium ion generated at 216.04Th by decomposition of phosphotyrosine present in the peptide is diagnostic for tyrosine phosphorylation substantiating the likely tyrosine phosphorylation. Transfected STAT5A was immunoprecipitated from TonB cells, and SRM was performed, this demonstrated that Y682 was phosphorylated (Figure 7A). In Figure 7A (lower right panel) ions at 791.47Th and 216.04Th were detected, indicative of a phosphorylation event on Y682 in the presence of BCR-ABL. There was no signal seen at 871.4325Th (diagnostic for phosphorylation on the second tyrosine residue; Y683), thus further indicating correct assignment of the site of phosphorylation being Y682. Interestingly in the top right panel (TonB cells in the presence of IL-3) the phosphorylated Y682 site was not observed. A final validation of the site of phosphorylation was seen when Y682 and/or Y683 were mutated to phenylalanine (Figure S6A–C). Although a product ion was seen in both single mutants at 492.16Th (phosphorylated Y at either 682 or 683) no product ion was detected at 527.352Th for the Y682F, thus the 492.16Th ion in this case could be a contaminant. However when the Y683F mutant was analyzed (Figure S6B) both ions at 527.352Th and 492.16Th were seen (Figure S7).

It was also attempted to identify this phosphorylation event in human K562 cells (Figure 7B) and in primary CML cells (Figure S6D). In both cases a signal of the product ion at 791.47Th from the appropriate parent ion was detected, indicating that the pY682 event is present in these samples.

Phosphorylation of the well characterized tyrosine residues Y694 in STAT5A and Y699 in STAT5B was observed in the presence of both IL-3 and BCR-ABL. However, analysis of STAT5B led to no identification of any phosphorylation from the homologous site on STAT5B (Y682).

Finally, we over-expressed STAT5A Y682F, Y683F and Y682/683F mutants in TonB cells and analyzed cell proliferation as well as intracellular localization and complex formation of STAT5A mutants. So far, we could only detect reduced complex formation with heterologous proteins in particular for STAT5A Y682/683F double mutants (Figure S8) but no specific effects on cell proliferation or cellular localization of STAT5A (data not shown).

Function of STAT5 Isoforms in Human Cells

To study the different functions of STAT5A and STAT5B in human cells shRNAs targeting human STAT5A and STAT5B were generated as described above. Again isoform-specific shRNAs specifically inhibit protein expression of human STAT5 isoforms with low cross-reactivity (Figure 8A). Upon lentiviral transduction both anti-STAT5A and anti-STAT5B shRNAs inhibit cell proliferation and survival of human BCR-ABL-positive cell lines to a similar extent (Lama-84) or slightly stronger in the presence of anti-STAT5B shRNA (K562, EM-2) (Figure 8B). In addition, depletion of STAT5B but not STAT5A sensitizes K562 and Lama-84 cells to imatinib with a 2.4- to 3.2-fold reduction of IC50 (Figure 8C). In contrast, anti-STAT5A RNAi slightly increases the IC50 in both cell lines studied, and the combination of both shRNAs was not superior to anti-STAT5B shRNA alone. In EM-2 cells anti-STAT5A and anti-STAT5B shRNAs alone reduced viability to more than 40% so IC50 could not reliably be determined. In contrast, depletion of STAT5A or STAT5B did not differentially affect cytokine-stimulated colony formation of chronic phase CML CD34+ cells (Figure S9). However, these data demonstrate specific biological effects of STAT5B for BCR-ABL-
driven proliferation in human cell lines in line with biochemical study described above.

**Discussion**

Here we have considered differential effects of IL-3 and BCR-ABL on STAT5 isoforms. Our data demonstrate differences between IL-3- and BCR-ABL-mediated STAT5-activation with STAT5A activation by BCR-ABL being partially deficient. This defective activation of STAT5A may explain the only partial rescue of BCR-ABL-dependent proliferation by the addition of IL-3 which can be further enhanced by inhibition of BCR-ABL tyrosine kinase activity (Figure 1A). Accordingly, depletion of STAT5B has more impact on BCR-ABL-expressing than on IL-3-supplemented TonB cells indicating that the remaining STAT5A function can only partially rescue loss of STAT5B in the presence of BCR-ABL (Figure 2A). Similarly, BCL-XL expression is only affected in BCR-ABL but not in IL-3 cultures by depletion of STAT5B (Figure 2B and C). Finally, over-expression of STAT5B but not STAT5A can expand TonB cells in the absence of IL-3, but this transformation still depends most likely on low level BCR-ABL tyrosine kinase activity (Figure 3B and C and Figure S3). These and the related data on reduced IC50-values for imatinib upon depletion of STAT5B but not STAT5A in human cell lines (Figure 8C) indicate a crucial role of STAT5B/BCR-ABL interactions for BCR-ABL-induced cell proliferation.

The mechanism of differential STAT5 activation by IL-3R/JAK2 and BCR-ABL is not yet known. BCR-ABL can induce tyrosine phosphorylation of STAT5, JAK2 as well as GAB2 even in the absence of both cytokine stimulation and JAK2 kinase activity (in AG490 supplemented cultures). Since only imatinib but neither ruxolitinib nor AG490 inhibits BCR-ABL-dependent tyrosine phosphorylation of JAK2 (Figures 4A and B) JAK2 that constitutively binds to the IL-3R b-chain [29,30] is phosphorylated either directly by BCR-ABL or by another kinase not inhibited by AG490. Similarly, STAT5 (and GAB2) may be phosphorylated either directly by BCR-ABL and/or by JAK2 (e.g. upon activation by BCR-ABL) and/or by other kinases such as HCK [31]. Our observations are in line with and expand recently reported data that BCR-ABL uncouples JAK/STAT signalling and may directly induce STAT5 tyrosine phosphorylation [32].
Different mechanisms of phosphorylation by IL-3R/JAK2 and BCR-ABL may affect STAT5 conformation and function since heterodimerization of STAT5A with STAT5B is more efficient upon stimulation by IL-3 compared to BCR-ABL. IL-3R and STAT5A are initially found to be phosphorylated on Y682 in the presence of BCR-ABL but not in the presence of IL-3. This suggests that the appropriate parent mass/charge ratios were selected for tandem mass spectrometric analysis. Product ions 527.3527Th (identifying the peptide), 871.4325Th (indicative for pY683), 791.4662Th (indicative for pY682), and 216.04Th (indicative for phosphorylated tyrosine) are labelled. A list of all product ions is shown in Figure S5. (B) STAT5A was precipitated from K562 cells and analyzed as described above.

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Figure 7. Mass spectrometry-based analysis of aberrant STAT5 tyrosine phosphorylation. For each part of the figure there are 2 panels, the left hand panel the parent ion transition is 477.77Th (unphosphorylated STAT5A) and the right hand panel the parent ion transition is 517.75Th (phosphorylated STAT5A). (A) STAT5 was isomod-specific immunoprecipitated from TonB cells in the presence of IL-3 or BCR-ABL. A peptide (YYPVLAK) in STAT5A was initially found to be phosphorylated on Y682 in the presence of BCR-ABL but not in the presence of IL-3. This suggests that the appropriate parent mass/charge ratios were selected for tandem mass spectrometric analysis. Product ions 527.3527Th (identifying the peptide), 871.4325Th (indicative for pY683), 791.4662Th (indicative for pY682), and 216.04Th (indicative for phosphorylated tyrosine) are labelled. A list of all product ions is shown in Figure S5. (B) STAT5A was precipitated from K562 cells and analyzed as described above.

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STAT5 Isoforms and BCR-ABL

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Finally, our MS analyses do not allow quantification of aberrant STAT5A-Y682 phosphorylation by BCR-ABL.

What of the functional role of defective STAT5A activation by BCR-ABL in human cells? Firstly, it may explain the presence of tyrosine phosphorylated STAT5 in the cytoplasm as reported by several groups [36,38,45]. Furthermore, loss-of-function of STAT5B reduces the IC50 for imatinib about 3-fold in K562 and LAMA-84 cells, whereas RNAi targeting STAT5A slightly increases it (Figure 8C). These data point to specific STAT5B-BCR-ABL interactions required for proliferation in human cells too. In cytokine-stimulated colony assays of CML CD34+ cells, however, we could not detect isoform-specific effects of anti-STAT5 shRNAs (Figure S9). These data point to specific STAT5B-BCR-ABL interactions required for proliferation in human cells too.

In summary, our data suggest the following model. Receptor linked tyrosine phosphorylation favours the generation of active STAT5 dimers in parallel conformation which translocate into the nucleus [2] whereas cytoplasmic phosphorylation favors the formation of inactive and even heterologous STAT5 complexes retained in the cytoplasm [36]. Upon phosphorylation by BCR-ABL, this cytoplasmic retention is more pronounced for STAT5A shifting STAT5-transcriptional activity towards STAT5B. The precise contribution of the aberrant Y682 phosphorylation in STAT5A, however, remains to be determined. In addition to aberrant phosphorylation, the abundance of STAT5 isoforms and that of other signalling molecules and their tyrosine phosphorylation status may impact on the formation of cytoplasmic pSTAT5-complexes in a cell line and cell-lineage specific manner.
Such cytoplasmic pSTAT5 complexes may induce specific effects such as an increase in ROS production. Walsch et al. recently described a STAT5α and ABL-tyrosine kinase dependent increase in ROS production and a highly significant and clinically relevant correlation between STAT5α expression and mutation status of BCR-ABL [37]. Although the functional impact of cytoplasmic pSTAT5 complexes remains to be precisely characterized the data suggest different functions for STAT5α and STAT5β in the context of BCR-ABL which are, however, both suitable for specific therapeutic intervention.

Supporting Information

Figure S1 Time and dose dependent expression of BCR-ABL in TenB cells.

Figure S2 TenB cell apoptosis in the presence of STAT5α and STAT5β specific shRNAs.

Figure S3 Tyrosine phosphorylation of STAT5β in TenB cells upon STAT5β over-expression.

Figure S4 Homodi-(oligo)merization of STAT5α and STAT5β in TenB cells.

Figure S5 Overview of putative mass to charge ratios of the identified STAT5α phosphopeptide.

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Figure S6 Mutant analysis of YYTPVLAK and identification of the phosphorylation event in PBMCs from a patient with first diagnosed CML.

Figure S7 Putative mass spectrometric values for tandem MS-generated product ions from the tryptic peptides YYTPVLAK, YYTPVLAK emanating from mutated STAT5α.

Figure S8 Coimmunoprecipitates with wildtype and mutant STAT5α.

Figure S9 Effects of isoform-specific STAT5 shRNAs on primary CD34+ cells.

Information S1 Supplementary Materials and Methods.

Acknowledgments

Conceived and designed the experiments: MS-S AKW JRG ADW MS ME. Performed the experiments: MS-S DB AKW JRG ID KB. Analyzed the data: MS-S DB AKW JRG AG ADW MS ME. Wrote the paper: MS-S DB AKW JRG ADW ME.
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