The transcription factor STAT5 (signal transducer and activator of transcription 5) is frequently activated in hematological malignancies and represents an essential signaling node downstream of the BCR-ABL oncogene. STAT5 can be phosphorylated at three positions, on a tyrosine and on the two serines S725 and S779. We have investigated the importance of STAT5 serine phosphorylation for BCR-ABL-induced leukemogenesis. In cultured bone marrow cells, expression of a STAT5 mutant lacking the S725 and S779 phosphorylation sites (STAT5SASA) prohibits transformation and induces apoptosis. Accordingly, STAT5SASA BCR-ABL+ cells display a strongly reduced leukemic potential in vivo, predominantly caused by loss of S779 phosphorylation that prevents the nuclear translocation of STAT5. Three distinct lines of evidence indicate that S779 is phosphorylated by group I p21-activated kinase (PAK). We show further that PAK-dependent serine phosphorylation of STAT5 is unaffected by BCR-ABL tyrosine kinase inhibitor treatment. Interfering with STAT5 phosphorylation could thus be a novel therapeutic approach to target BCR-ABL-induced malignancies.

Keywords: BCR-ABL; STAT5; serine phosphorylation; nuclear translocation

INTRODUCTION
Janus kinase/signal transducer and activator of transcription (JAK/STAT) molecules are key players in a number of highly conserved signaling pathways involved in cell-fate decisions such as differentiation, proliferation and apoptosis. Mounting evidence pinpoints a role for JAK/STAT signaling in human cancer and STAT proteins are attracting increasing interest as potential molecular targets for cancer therapy. Constitutively active forms of JAK2 have been identified as drivers of myeloid and T lymphoid leukemia. Studies in STAT5a/b-deficient mice have revealed that STAT5a/b are essential effectors for JAK2-triggered leukemogenesis. In other malignancies, STAT5 signaling is activated downstream of oncogenic tyrosine kinases and contributes to transformation and tumor maintenance. An example of a tyrosine kinase that exerts its oncogenic function via STAT5 is the Abelson (BCR-ABL) oncogene, generated by a reciprocal translocation t(9;22) and found in leukemic cells of human chronic myeloid leukemia and acute lymphoid leukemia patients. Fusion with the BCR protein turns the Abelson kinase into a constitutively active tyrosine kinase capable of transforming hematopoietic cells. Deletion of STAT5 during induction or maintenance of BCR-ABL+ leukemia leads to abrogation of the disease.

STAT proteins are phosphorylated on tyrosine and serine residues and phosphorylation is generally necessary for full transcriptional activity, although there is mounting evidence that unphosphorylated STAT1 activates a certain subset of target genes. Tyrosine phosphorylation allows dimerization of STAT molecules that is believed to be a prerequisite for nuclear translocation. The importance of phosphorylated STAT5 for hematopoietic malignancies is underlined by observations in lymphoid, myeloid and erythroid leukemias that have constitutive STAT5 phosphorylation. The introduction of constitutively active STAT5a mutants into murine hematopoietic cells suffices to induce multilineage leukemia in mice.

Although the role of serine phosphorylation in transcriptional control has been intensively investigated, only limited information is available about its importance in STAT5a/b function. Serine phosphorylation of STAT1 is required for cytotoxic T-cell responses and/or interferon-γ-mediated innate immunity. Phosphorylation of STAT3 on S727 is needed for Ras-mediated tumor formation. Consistently, serine phosphorylation of STAT3 has been linked to the growth of solid tumors such as prostate or skin cancer. Moreover, STAT3 and STAT1 are constitutively phosphorylated on serine residues in a subset of acute myeloid leukemia as well as B-cell chronic lymphocytic leukemia, although the significance of the modification is still unclear.

The causal link between serine phosphorylation of STAT5a and leukemogenesis has only recently been established. Using bone marrow (BM) transplantations, we described a critical role for STAT5a serine phosphorylation in STAT5a-driven leukemogenesis (using a constitutively active murine STAT5a as driver oncogene). The importance of this result was underlined by the finding that both serine residues of STAT5a (S726 and S780, corresponding to murine S725 and S779) are phosphorylated in human myeloid malignancies including acute myeloid leukemia and BCR-ABL chronic myeloid leukemia. This study provided the first indication.
that serine phosphorylation of STAT5a might play a part in myeloid leukemia driven by constitutively active STAT5a, indirectly implying that serine phosphorylation of STAT5a might be required in other naturally occurring malignancies that depend on STAT5. We thus investigated whether STAT5 serine phosphorylation is downstream of oncogenic tyrosine kinases, using BCR-ABL-induced disease as a model system. We report here that serine phosphorylation of STAT5a is necessary for nuclear localization of STAT5 in BCR-ABL+ cells. We identify group I p21-activated kinases (PAKs) as upstream regulators and suggest that they might represent an attractive therapeutic point of attack independent of BCR-ABL kinase activity.

MATERIALS AND METHODS

Mouse strains

*Mx-1Cre*26, Stat5a/b−/−27, C57Bl/6J and NSG (NOD.Cg-Pkdcl299Ighm1S勉强/SJ L); The Jackson Laboratory, Bar Harbor, ME, USA) were maintained under pathogen-free conditions at the University of Veterinary Medicine Vienna (Vienna, Austria). All animal experiments were approved by the institutional ethics committee and conform to Austrian laws (license BMWF-68.205/0218-I/3b/2012).

Site-directed mutagenesis

Site-directed mutagenesis was performed using the QuikChange site-directed Mutagenesis Kit from Stratagene (La Jolla, CA, USA) according to the manufacturer’s instructions (using pmMSCV-STAT5a-ires-GFP as parental vector).

Generation of leukemic cell lines and in vitro deletion of endogenous STAT5

The following leukemic cell lines were used: murine BCR-ABLp185−/−, v-ABLp190−/−, Ba/F3-p190−/− and Ba/F3-p185−/− pro-B cells as well as the human K562 and KU812 cell lines (both myeloid; BCR-ABLp190−/−). To generate v-ABLp190−/− and BCR-ABLp185−/− cell lines, fetal liver cells of a *Stat5a/bfl/fl* mouse were transduced with a viral vector expressing the *Mx-1Cre* cross were transformed and maintained in RPMI supplemented with 10% fetal calf serum, 50 U/ml penicillin, 100 μg/ml streptomycin (PAA), and 50 U/ml GMCSF (PAA, Pasching, Austria) as previously described.30 For Stat5 deletion, stable Stat5a/bfl/fl/Mx-1Cre BCR-ABLp185−/− cell lines were incubated for 48 h in 1000 μl recombinant interferon-β (PBL Interferon Source, Piscataway, NJ, USA). After 2 weeks, deletion efficiency was verified by genotyping PCR as described before.11

Transfection of leukemic cell lines

Stat5a/bfl/fl/Mx-1Cre BCR-ABLp185−/− cell lines were transduced with pmMSCV-ires-GFP-based constructs encoding individual STAT5 variants by co-culture with gp-116 ectopic retroviral producer cells as described previously.31 Vector-positive (GFP+) cells were sorted using a fluorescence-activated cell sorting (FACS) Aria III device (BD Biosciences, San Jose, CA, USA).

Transplantation studies in mice

A total of 2500 BCR-ABLp185−/− cells were injected via the tail vein into nonirradiated NSG mice. Mice were monitored daily. Sick mice were killed and analyzed for spleen weight, white blood cell count and the presence of STAT5a-vector-positive leukemic cells (GFP+) in BM, spleen and peripheral blood (PB) by flow cytometry. Differential hemograms were assessed using a VetABC Blood Counter (Sci Animal Care, Viernheim, Germany). The Hemacolor staining kit (Merck Millipore, Billerica, MA, USA) was used for hematocytin and eosin staining.

Flow cytometry and cell sorting of leukemic cells

A total of 5 × 105 cells were stained and analyzed by a FACS Canto II flow cytometer equipped with 488, 633 and 405 nm lasers using the FACS Diva software (Becton-Dickinson, Franklin Lakes, NJ, USA) as described before.11 High-purity FACS sorting was performed on a FACS Aria III equipped with a 488 nm laser at 4°C (Becton-Dickinson).

Transfection and immunofluorescence staining of HEK cells

HEK 293T cells were transfected with a pcDNA 3.1-based vector expressing BCR-ABL185 using PolyFect (Qiagen, Hilden, Germany). Cells were cultured with Dulbecco’s modified Eagle’s medium (PAA) high glucose supplemented with 10% fetal calf serum (PAA), 50 μg 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), 100 U/ml penicillin, 100 μg/ml streptomycin (PAA) and 1000 μg/ml G418 (InvivoGen, San Diego, CA, USA) to select for stable BCR-ABL185-expressing cells. The localization of yellow fluorescent protein (YFP)-tagged STAT5 protein was examined by immunofluorescent laser scanning microscopy (Olympus IX71, 20-fold magnification) using a 530/550 nm filter (U-MNG2 filter, Olympus, Tokyo, Japan).

Cell extracts and immunoblotting

Whole-cell extracts and cellular fractionations were performed as previously described.10,12 For immunoblotting, proteins (50–100 μg) were separated on a 7% SDS polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were probed with antibodies from Santa Cruz (Dallas, TX, USA) against STAT5a/b (N-20; C-17), α-tubulin (DM1A), β-actin (C-15), H5C07 (B-6) and pERK (E-4); Lamin-B (ab45848-100) was purchased from Abcam (Cambridge, UK). The pSTAT5(727,25) and pSTAT5(727,29) specific antibodies were generated by immunization of rabbits (Eurogentec, Liège, Belgium). The following antibodies were purchased from Cell Signaling (Danvers, MA, USA): PKA (P2602), PKA (P2608), pPKA (P*P2602) (2601), pCrk (P*P2601) (3181) and STAT1 (9172). pSTAT5 (61964) and Rac1 (610650) were obtained from BD Transduction Laboratories (San Jose, CA, USA). Immunoreactive bands were visualized by chemiluminescence (20X Lumiglo Reagent and 20X Peroxide, Cell Signaling).

Semiquantitative real-time PCR

RNA was isolated from murine BCR-ABLp185−/− cells expressing wild-type or mutant STAT5 variants using prepGold TriFast reagent (Peqlab, Erlangen, Germany). RNA (1 μg) was transcribed by employing the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Real-time PCR was performed on a MyQ2 cyber (Bio-Rad) with SsoAdvanced SYBR GreenSupermix (Bio-Rad) and Primers for B22 (forward) 5′-ATCAGATACCTGCCAGGCATC-3′, reverse (rev) 5′-GGAGAAATCAAACAGAGGTCA-3′, Cish (fwd 5′-AGACGACCCCTCTACCTCCCG-3′, rev 5′-TGACACATCCTGGAGGCGC-3′) and Gapdh (fwd 5′-TGTCCTCGTCTGTGGACTGA-3′, rev 5′-CGTCTCCCACCCTTCTGA-3′). Target gene expression was normalized to Gapdh.

Proliferation assays and focused compound screen

K562 and murine BCR-ABLp185−/− cells expressing wild-type or mutant STAT5 variants were seeded in 384-well plates (Corning, Corning, NY, USA) at a concentration of 10,000 cells per well in 100 μl medium. Kinase inhibitor libraries (Tocris Kinase Inhibitor Toolbox from Tocris (Bristol, UK), Merck Kinase Inhibitor Library I and Merck Kinase Inhibitor Library II both from Merck-Millipore) were added at a screening concentration of 10 μM. Dapsatinib at 1 μM was used as a positive control and all wells contained a final dimethyl sulfoxide concentration of 0.1%. After 24 h of incubation, CellTiter-Glo (Promega, Fitchburg, WI, USA) was added and luminescence measured with an Envision plate reader (Perkin Elmer, Waltham, MA, USA). Data were normalized to internal controls by linear regression to the mean of the 32 dimethyl sulfoxide wells (set to 100% of control) and the mean of the 32 positive control wells (set to 0% of control) using Pipeline Pilot (Accelrys, San Diego, CA, USA). Screening data were visualized with Spotfire (Spotfire Inc., Cambridge, MA, USA) software. Screening was performed in duplicate.

The methythiazolyltetrazolium bromide (MTT) assay was conducted in 96-well plates with 20,000 cells per well in 100 μl medium. Kinase inhibitors (Kinase Inhibitor Toolbox, Tocris) were added at 10 μM concentration followed by 24 h of incubation. Positive and negative controls were included as above. Cells were incubated for 3 h with 10 μM MTT (5 mg/ml MTT; Sigma-Aldrich). Upon addition of 100 μM of the following compounds: 4-methylthiazolyltetrazolium, 0.1% nonidet P-40; Sigma-Aldrich), absorbance was measured at 590 nm on an EnSpire multimode plate reader (Perkin Elmer).

Kinase inhibitor studies

K562, KU812, BCR-ABLp185−/− and v-ABLp190−/− leukemic cell lines were seeded in a six-well dish at a concentration of 105 cells per ml. Kinase inhibitors were added and after incubation at 37°C and 5% CO2, cells were harvested, washed twice with ice-cold phosphate-buffered saline and subjected to immediate lysis as described previously.53 The following inhibitors were purchased from Calbiochem (Billerica, MA, USA): KN-93, H-89, PD98059, TDZ-D-8, roscovitine and olomoucine. PIM1 kinase inhibitor,
BIO and IPA-3 were purchased from Tocris. Flavopiridol, CAL-101 and foretinib were purchased from Selleck Chemicals (Houston, TX, USA). Anisomycin was purchased from Sigma-Aldrich; olomoucine II from Alexis Biochemical (San Diego, CA, USA) and SB 203580 from Jena-Bioscience (Jena, Germany). All inhibitors were dissolved in dimethyl sulfoxide. As negative control 0.1% dimethyl sulfoxide was used.

Immunoprecipitation studies
STAT5- or PAK1-specific antibodies were incubated with lysates for 1.5 h at room temperature, and then for 1 h with magnetic beads conjugated to proteins A and G (Bio-Adembeads PAG, Ademtech, Pessac, France). Beads were pelleted with a magnet and washed 3 times. Protein complexes were eluted by incubation with PAG elution buffer (Ademtech) and analyzed by immunoblotting.

In vitro kinase assays
Recombinant mouse Tat-TAT5sa and Tat-TAT5sb were produced and purified as described previously. Recombinant mouse PAK1 and Cdc42/PAK1 were purchased from SignalChem (Richmond, BC, Canada) and recombinant Rac1 from Cytoskeleton (Denver, CO, USA). Recombinant Tat-STAT5 proteins (20 ng) were incubated with PAK1 kinase (100 ng) in buffer containing 25 mM Tris-HCl (pH 7.5), 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na3VO4, 10 mM MgCl2, 2 mM MnCl2 and 100 μM ATP for 30 min at 37°C. Kinase reactions were stopped by adding Laemmli buffer and STAT5 phosphorylation was detected by immunoblotting with an anti-pSTAT5S725 antibody (Affinity Bioreagents, Golden, CO, USA).

Expression of STAT5SASA in leukemic cells enhances disease latency
It is conceivable that the strong inhibitory effect that we observed in vitro is overcome by cytokines and growth factors in vivo that act in synergy with BCR-ABL. The role of STAT5 serine phosphorylation in in vivo leukemogenesis was tested using the BCR-ABLp185+ cells. The maternal cell line harbors Stats5Mx1Cre alleles and therefore allows the deletion of endogenous Stat5 by a single interferon-β treatment. The resulting cell lines express only the retroviral Stat5 construct and are either STAT5SASA or Stat5wt positive.

The cells were transplanted into nonirradiated NSG mice (scheme in Figure 2a). We observed a significant increase in disease latency upon transplantation of STAT5SASA-expressing leukemic cells compared with the cohort that had received Stat5wt cells (Figure 2a). In a subsequent experiment, all animals were killed on day 18 (scheme in Figure 2b). We noticed a significant attenuation of the severity of the disease upon transplantation of STAT5SASA leukemic cells as shown by significantly reduced spleen weight (Figure 2c) and white blood cell count (Figure 2d). Blood smear revealed lower numbers of tumor cells in the PB (Figure 2e). Histological spleen sections substantiated these findings and showed less infiltration of leukemic cells (Figure 2f). Differences in disease severity were also obvious when we monitored leukemic cells in PB (Figure 2g) and spleens (Figure 2h) by FACS analysis. Although we observed no differences in leukemic infiltration in the BM on day 18 (12.4 ± 8.2 vs 12.3 ± 5.3 for the Stat5wt and Stat5SASA groups, P = nonsignificant, data not shown), there were profound differences in the PB and in the spleen. In summary, the leukemic cell load was significantly reduced upon transplantation of BCR-ABLp185+ STAT5SASA cells.

Single mutation of STAT5S779 prolongs disease latency
To investigate whether leukemic progression in vivo is modulated by phosphorylation of STAT5 on S725 or on S779 or on both positions, we transduced murine Stat5ΔΔMx1Cre BCR-ABLp185+ cells with a Stat5S725A or a Stat5S779A (a diagram of the STAT5 mutants is provided in Supplementary Figure 1). Whereas the expression of Stat5wt conferred a slight survival advantage, the percentage of cells expressing STAT5SASA declined within 8 days till no cells were detectable (Figure 1a). Similar results were obtained when GFP+ cells were sorted 2 days after infection; expression of STAT5SASA provoked a rapid decrease in GFP+ cell numbers (Figure 1b). Cell cycle analysis (propidium iodide) and apoptosis staining (Annexin V) were performed 2, 3 and 6 days after sorting. STAT5SASA cells accumulated in the sub G0/G1 phase after 2 days (Figure 1c) and at day 3 the number of Annexin V-positive cells increased and reached 100% on day 6 (Figure 1d). This indicates that the expression of STAT5SASA induces apoptosis.
Survival of mice in the STAT5S779A and STAT5SASA groups was further enhanced. Remarkably, the combined loss of both serine phosphorylation sites further delayed disease latency compared with single loss of STAT5S725 phosphorylation (Figure 3a). FACS analysis of leukemic cell infiltrates in the BM (Figure 3b) revealed significant levels of leukemic cells expressing STAT5wt, STAT5S725A or STAT5S779A, whereas only low numbers of STAT5SASA cells were detected. A comparable picture was found in PB (Figure 3c). Numbers of STAT5SASA cells were significantly reduced compared with other experimental groups. In contrast, FACS analysis of spleens (Figure 3d) showed decreased numbers of GFP$^+$ cells in mice of both the STAT5S779A and STAT5SASA groups when compared with the STAT5S725A and the STAT5wt. These data indicate that STAT5S779 phosphorylation is dominating leukemogenesis in vivo.

STAT5S779 phosphorylation is required for nuclear localization

To investigate how serine phosphorylation modulates leukemogenesis, we analyzed the subcellular localization of the modified proteins. Mouse STAT5 serine mutants were introduced into HEK 293T cells stably expressing BCR-ABL$^{p160}$+. The mutants were C-terminally tagged with YFP to monitor subcellular localization. As expected, BCR-ABL$^{p160}$+ activates STAT5 and causes nuclear accumulation of STAT5wt-YFP (Figure 4a). Similarly, STAT5S725A-YFP was primarily found in the nucleus. In contrast, the STAT5S779A and STAT5SASA proteins failed to accumulate in the nucleus, instead being evenly distributed throughout the cells (Figure 4b, upper panel). To investigate whether STAT5S779A and STAT5SASA proteins regain the ability to move to the nucleus upon dimerization with a STAT5wt partner, we additionally transfected untagged STAT5wt proteins (Figure 4b, lower panel) into the cells. The co-transfection caused increased nuclear accumulation of the STAT5S779D,YFP and STAT5SASA,YFP proteins, suggesting that serine phosphorylation of one partner of the STAT5 heterodimer suffices for nuclear localization. The phospho-mimetic variants (S$^D$) STAT5S$^{725D}$, STAT5S$^{779D}$, STAT5S$^{S725D}$ served as controls and efficiently accumulated in the nucleus (Figure 4c and Supplementary Table 2).

To examine whether the findings are relevant to the situation in leukemic pro-B cells, we prepared cytoplasmic and nuclear fractions of BCR-ABL$^{p160}$+ cells expressing STAT5wt, STAT5S725A, STAT5S779A, STAT5SASA or the corresponding phospho-mimetic mutants and performed immunoblotting with anti-STAT5a/b antibodies (Figure 4d), using Lamin B and α-tubulin to control for the quality of our nuclear and cytoplasmic fractions. Consistent with the findings in HEK 293T cells, there were barely detectable amounts of STAT5 proteins in the nuclei of BCR-ABL$^{p160}$+ cells expressing STAT5S779A or STAT5SASA. This indicates that STAT5S779 phosphorylation of at least one STAT5 molecule within a homodimer is required for translocation to the nucleus. In line with this observation, the STAT5 target genes Cish and

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**Figure 1.** STAT5SASA expression does not support transformation in vitro. (a) The wt fetal liver cells were simultaneously infected with v-ABL$^{p160}$+ and STAT5wt, STAT5SASA or the empty vector (pMSCV-IRE-GFP; n = 3, n = 3 and n = 6, respectively). GFP$^+$ cells were monitored via FACS analysis. (b) Growth curve of GFP$^+$ cells. Three days after co-infection, vector-positive cells were sorted. The experiment was performed in triplicate; one representative experiment is depicted. Post sorting, propidium iodide (PI) cell cycle (c) and Annexin V (d) stainings were performed at indicated time points. (e) Stably transformed wt leukemic cells were infected with STAT5wt, STAT5SASA, STAT5S$^{749}$ or the empty vector (n = 8, n = 3, n = 12 and n = 13, respectively). Outgrowth of GFP$^+$ cells was monitored via FACS analysis.
Figure 2. Expression of STAT5SASA impairs leukemogenesis in vivo. (a) STAT5SASA- or STAT5wt-expressing BCR-ABLp185+ cells were injected intravenously (i.v.) into NSG mice (2500 cells/mouse; n = 8 each). Survival curves of recipients are depicted. The median survival was 15 and 22 days for STAT5wt and STAT5SASA group. (b) Scheme depicting experimental setup of data shown in (c–h). Transplantation was performed as described in (a) (n = 7 each). All animals were killed on day 18. (c) Spleen weights of the STAT5SASA group were 1.6-fold reduced (0.28 ± 0.09 and 0.16 ± 0.06 g for the STAT5wt and STAT5SASA groups, respectively, P < 0.05). Data represent mean ± s.d. (d) White blood cell counts (WBCs) were 2.9-fold reduced in mice of the STAT5SASA group (18.7 ± 7 × 10^3/mm^3 and 6.4 ± 5.5 × 10^3/mm^3, for the STAT5wt and STAT5SASA groups, P < 0.01). Data represent mean ± s.d. (e) Blood smears show reduced lymphocyte load in mice of the STAT5SASA group (4.3 ± 0.8% vs 0.7 ± 0.8% of blasts relative to red blood cells for the STAT5wt and STAT5SASA groups, respectively, P < 0.001). One representative example per group is depicted. (f) Hematoxylin and eosin (H&E)-stained spleen sections. One representative example per group is depicted. (g, h, left panels) Representative FACS plots showing infiltration of GFP+ cells in (g) PB (36.1 ± 9.9% vs 5.9 ± 2.2% GFP+ cells in STAT5wt and STAT5SASA groups, P < 0.0001) and (h) spleen (19.3 ± 2 vs 11.1 ± 3.5 for STAT5wt and STAT5SASA groups, P < 0.001). Data obtained from entire cohorts are summarized. Reduced numbers of GFP+ cells in PB and spleens of the STAT5SASA group (PB: 6.1-fold; spleen: 1.7-fold). Data represent mean ± s.d. Asterisks denote statistical significance (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001).
Figure 3. Expression of STAT5^{S725A}, STAT5^{S779A} or STAT5^{SASA} suppresses the leukemic potential of BCR-ABL^{p185}^{+} cells in vivo. (a) Kaplan–Meier plot of NSG mice upon transplantation of STAT5^{wt}, STAT5^{S725A}, STAT5^{S779A} or STAT5^{SASA}-expressing leukemic cells (n = 4, n = 7, n = 5 and n = 4, respectively). The median survival for the STAT5^{wt}, STAT5^{S725A}, STAT5^{S779A} and STAT5^{SASA} groups was 15, 17, 21, 23.5 days, respectively. Table summarizes statistical significances between indicated experimental groups. (b) FACS analysis of BMs for infiltration of GFP^{+} cells (56.4 ± 22.3%, 74.3 ± 10.9%, 58.7 ± 10.3% and 6 ± 2.5% GFP^{+} cells for groups STAT5^{wt}, STAT5^{S725A}, STAT5^{S779A} and STAT5^{SASA}, respectively). (c) In PB, 32.8 ± 10.4%, 49.1 ± 9.1%, 62.3 ± 5% and 2.4 ± 0.5% GFP^{+} cells in the STAT5^{wt}, STAT5^{S725A}, STAT5^{S779A} and STAT5^{SASA} groups were detected. (d) Analysis of spleens resulted in 37.4 ± 8.4%, 55.4 ± 10.7%, 16.3 ± 3.7% and 1.5 ± 0.1% GFP^{+} cells in the STAT5^{wt}, STAT5^{S725A}, STAT5^{S779A} and STAT5^{SASA} groups. One representative FACS plot per experimental group is depicted. Data in right panels represent mean ± s.d. Three mice of the STAT5^{S779A} display censored events and were excluded from FACS analysis. Asterisks denote statistical significances as determined by a (a) logrank test or (b, c, d) a one-way analysis of variance (ANOVA) followed by Tukey's test (*P < 0.05; **P < 0.01; ***P < 0.001).
The transcription of Bcl2 was not transcribed in cells expressing STAT5 SASA and STAT5S779A but within normal range in cells expressing STAT5SDSD and STAT5S779D (Figure 4e).

**Group I PAK kinases as upstream regulators of STAT5S779 in BCR-ABLp185⁺ cells**

Blocking STAT5S779 phosphorylation and thereby nuclear translocation of STAT5 might represent a way to inhibit the transcriptional activity of STAT5, which is essential for maintenance of BCR-ABL-driven disease. To identify the kinase(s) upstream of STAT5S779 we performed *in silico* screens employing group-based phosphorylation scoring, KinasePhos, NetPhosK, prediction of protein kinase-specific phosphorylation site, PredPhospho, Scansite and PhosphoMotif finder. Results for potential candidates are summarized in Figure 5a. As both murine STAT5S779 and human STAT5S780 are flanked by prolines, we focused on hits that represent proline-directed serine/threonine kinases. Mitogen-activated protein kinases (MAPKs) and cyclin-dependent kinases (CDKs) were consistently identified. Protein kinases A, B and C (PKA, PKB and PKC), glycogen synthase kinase-3β, Ca²⁺/calmodulin-dependent protein kinases, PAKs and mammalian target of

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**Figure 4.** STAT5S779 phosphorylation is a prerequisite for nuclear translocation in BCR-ABLp185⁺ cells. (a, b) Immunofluorescence of HEK 293T cells stably expressing BCR-ABLp185-transfected with YFP-tagged STAT5 variants. Scale bars 10 μm. (a) STAT5wt and STAT5S725A translocate to the nucleus. (b) STAT5S779A and STAT5SASA fail to translocate to the nucleus (upper panel). The concomitant expression of untagged STAT5wt alters the nuclear localization of STAT5S779A and STAT5SASA proteins (lower panel). (c) Immunofluorescence of HEK 293Tp185⁺ cells transfected with phospho-mimetic mutants (STAT5S725D, STAT5S779D or STAT5SDSD, all YFP-tagged). Scale bars 10 μm. (d) Immunoblotting for STAT5a/b of nuclear and cytoplasmic fractions of BCR-ABLp185⁺ cells. Lamin B and α-tubulin served as controls of nuclear and cytoplasmic fractions. (e) mRNA expression of Cish and Bcl2 normalized to Gapdh in murine BCR-ABLp185⁺ cells expressing exclusively STAT5a mutant variants was determined by real-time PCR.
rapamycin were predicted with low frequency. In parallel, we initiated a drug screen in the human cell line K562 and in a murine BCR-ABL\(^{+}\) cell line. To control for hits that lead to apoptosis without interfering with STAT5 serine phosphorylation, we included cells expressing a phospho-mimetic variant of STAT5\(^{S779}\) (STAT5\(^{S779D}\)) and excluded positive hits obtained in these cells. Compounds that induced loss of cell viability in 45\% of the cells were further tested. Figure 5a summarizes two individual rounds of experiments. The target profiles of hit compounds were largely consistent with the \textit{in silico} predictions. In a next step, we used \textit{western blot} analysis for validation and found that two independently acting group I PAK kinase inhibitors (IPA-3 and PF-3758309) suppress STAT5\(^{S779}\) phosphorylation in both human and murine BCR-ABL\(^{+}\) cells (Figures 5b and c).

CDK inhibitors were also analyzed in more depth as CDK8 has previously been defined as upstream kinase for STAT proteins.\(^{12,35}\) We treated BCR-ABL\(^{+}\) cells with CDK inhibitors including flavopiridol and analyzed levels of pSTAT5\(^{S779}\) by immunoblotting. As depicted in Figure 5c and Supplementary Figure 2, we failed to detect any reduction of pSTAT5\(^{S779}\) upon inhibition of CDKs.

Figure 5. Inhibition of group I PAK kinases diminishes STAT5\(^{S779}\) phosphorylation and nuclear localization of STAT5. (a) Summary of candidate kinases obtained from \textit{in silico} predictions and screening hit compounds targeting serine/threonine (Ser/Thr) kinases. For screening, K562 and BCR-ABL\(^{+}\) cell lines were incubated with kinase inhibitor libraries. Reduction of cell viability was assessed via CellTiter-Glo assay. (b) Immunoblotting of KU812 cells treated with IPA-3 (group I PAK kinase inhibitor) at 25 or 50 \(\mu\text{M}\) for up to 4 h. (c) Immunoblotting of BCR-ABL\(^{+}\) cells incubated for 5 h either with flavopiridol or PF-3758309 at indicated concentrations. (d) Immunoblotting of nuclear and cytoplasmic fractions of BCR-ABL\(^{+}\) cells treated with PF-3758309 (5 \(\mu\text{M}\); 5 h). Lamin B and \(\alpha\)-tubulin served as controls of nuclear and cytoplasmic fractions. (e) Densitometric analysis of immunoblotting of nuclear and cytoplasmic fractions of KU812 cells treated with PF-3758309 (5 and 10 \(\mu\text{M}\); 5 h). As negative controls in (a–e), cells were treated with 0.1\% dimethyl sulfoxide (DMSO).

**Table 1.** Summary of candidate kinases obtained from \textit{in silico} predictions and screening hit compounds targeting serine/threonine (Ser/Thr) kinases.

| Kinase group | Predicted kinases | In \textit{silico} prediction* | Screened hit compounds |
|--------------|------------------|-----------------------------|------------------------|
| MAPK         | 7/7              | 5-Iodotubercidin, JNK Inhibitor V, MEK Inhibitor I, p38 MAP Kinase Inhibitor, JX401, PD 198306, SB 220025, SC-68376 |
| CDK          | 7/7              | BIO, bohemine, Cdc7/Cdk9 Inhibitor, Cdk1 Inhibitor, Cdk2 Inhibitor II, CR8 (R)-Isomer, CR8 (S)-Isomer, olomoucine II, roscovitine, SU9516 |
| GSK3\(^{\beta}\) | 3/7              | BIO, GSK-3b Inhibitor VIII |
| AGC          | 5/7              | KT5720 |
| CAMK         | 1/7              | -- |
| PIM          | 0/7              | PIM1 Kinase Inhibitor IV |
| STE          | 1/7              | p21-activated Kinase Inhibitor III (IPA-3) |
| mTOR         | 1/7              | Compound 401 |
| PI3-K, ATM,  | 0/7              | ATM kinase Inhibitor, DNA-PK Inhibitor III, TGX-221, PI3-Ky Inhibitor |
| DNA-PK       |                  | |

* number of positive predictions / screened databases

**Figure 5.** Inhibition of group I PAK kinases diminishes STAT5\(^{S779}\) phosphorylation and nuclear localization of STAT5. (a) Summary of candidate kinases obtained from \textit{in silico} predictions and screening hit compounds targeting serine/threonine (Ser/Thr) kinases. For screening, K562 and BCR-ABL\(^{+}\) cell lines were incubated with kinase inhibitor libraries. Reduction of cell viability was assessed via CellTiter-Glo assay. (b) Immunoblotting of KU812 cells treated with IPA-3 (group I PAK kinase inhibitor) at 25 or 50 \(\mu\text{M}\) for up to 4 h. (c) Immunoblotting of BCR-ABL\(^{+}\) cells incubated for 5 h either with flavopiridol or PF-3758309 at indicated concentrations. (d) Immunoblotting of nuclear and cytoplasmic fractions of BCR-ABL\(^{+}\) cells treated with PF-3758309 (5 \(\mu\text{M}\); 5 h). Lamin B and \(\alpha\)-tubulin served as controls of nuclear and cytoplasmic fractions. (e) Densitometric analysis of immunoblotting of nuclear and cytoplasmic fractions of KU812 cells treated with PF-3758309 (5 and 10 \(\mu\text{M}\); 5 h). As negative controls in (a–e), cells were treated with 0.1\% dimethyl sulfoxide (DMSO).
Similarly, inhibitors of MAPK, PKA, PKC, Ca²⁺/calmodulin-dependent protein kinases, mammalian target of rapamycin, glycogen synthase kinase-3β and PIM1—that were predicted as potential upstream kinases—failed to exert any effect (Supplementary Figures 3 and 4).

These data lead to a testable prediction: if PAK kinases phosphorylate STAT5, PAK kinases should prevent the accumulation of nuclear STAT5. In line with the results in STAT5⁻/⁻, mutant cells, we indeed observed a reduction of nuclear STAT5 upon treating murine BCR-ABL⁺ cells and KU812 cells with the PAK inhibitor PF-3758309 (Figures 5d and e). This led us to conclude that group I PAK kinases are direct or indirect upstream regulators of STAT5 phosphorylation, controlling the nuclear localization of STAT5.

PAK kinases directly phosphorylate STAT5

Whereas PAK1 and PAK2 are expressed in human BCR-ABL⁺ cells, it appears that only PAK2 is found in murine leukemic cells: we failed to detect any PAK1 protein in murine BCR-ABL⁺ cells (Figure 6a). Co-immunoprecipitation experiments revealed complexes of STAT5, PAK1, PAK2 and Rac1 in KU812 cells (Figure 6b). STAT5 was consistently associated with PAK1, PAK2 in K562 (Supplementary Figure 5) and in murine BCR-ABL⁺ cells (Supplementary Figure 6). The interaction was specific for STAT5 as no complexes were detectable upon immunoprecipitation of STAT1 in K562 or murine BCR-ABL⁺ cells (Supplementary Figures 5 and 6). Thus, group I PAK kinases directly interact with STAT5.

To investigate whether PAK kinases phosphorylate STAT5, we performed in vitro kinase assays, incubating recombinant PAK1 kinase with recombinant TAT-STAT5a protein. Only in the presence of Rac1—which is required to activate PAK kinases—did phosphorylation of STAT5 become apparent (Figure 6c). As negative controls we used recombinant mouse STAT5b protein that is highly homologous to STAT5a as well as a truncated version of STAT5a (STAT5Δ779) (Supplementary Figure 7a). STAT5b does not harbor a serine at position 779 but at position 778. However, STAT5b778 is not flanked by prolines. We failed to detect any signal using these constructs. Identical results were obtained when we used recombinant PAK2 (Supplementary Figure 7b). The data support the conclusion that group I PAK kinases directly phosphorylate STAT5. To further substantiate the link between PAK1/2 and STAT5 phosphorylation, we performed knockdown experiments against PAK2 in murine BCR-ABL⁺-transformed cells (Supplementary Figures 8a and b). We focused on PAK2 here as murine BCR-ABL⁺ cells do not express PAK1 (see Figure 6a). Despite the successful knockdown of PAK2, the remaining PAK2 proteins displayed increased kinase activity. Accordingly, STAT5 phosphorylation was enhanced indicating a so far unrecognized pronounced feedback loop that was independent of mitogen-activated protein kinase kinase inhibitor U0126 remained without effect (Supplementary Figure 8c).

STAT5 phosphorylation is independent of BCR-ABL kinase activity

As STAT5 phosphorylation depends on BCR-ABL kinase activity, we investigated whether phosphorylation of STAT5 and STAT5 requires BCR-ABL kinase activity. BCR-ABL⁺ cells were treated with imatinib (2 μM) and the kinetics of STAT5 and STAT5 phosphorylation monitored. As expected, STAT5 phosphorylation decreased within 15 min and was hardly detectable after 60 min. In contrast, the level of STAT5 and STAT5 phosphorylation remained unaffected for 6 h (Figure 7a). This indicates that STAT5 serine phosphorylation is independent of BCR-ABL kinase activity and does not require concomitant STAT5 phosphorylation. We reasoned that—if independent of BCR-ABL kinase activity—activation of PAK kinases should not be impaired by imatinib treatment. We used antibodies that recognize phosphorylated STAT5 and that—if independent of BCR-ABL kinase activity—are activated forms of the proteins, after incubation of KU812 cells with 2 μM imatinib. As expected, STAT5 phosphorylation rapidly declined (Figure 7b). However, there was no change to the levels of activated PAK1 and PAK2 and the extent of STAT5 phosphorylation also remained unaltered upon imatinib treatment, suggesting that STAT5 is phosphorylated independently of BCR-ABL.

Support came from an experiment with cells overexpressing a Tyr-phosphorylation mutant of STAT5 (STAT5Y694F), STAT5 or STAT5 without STAT5. STAT5 phosphorylation remained unaffected even in the absence of tyrosine phosphorylation (Figure 7c).

In summary, the data indicate that there are two distinct and independent pathways that control the phosphorylation and thus the intracellular localization of STAT5: phosphorylation of STAT5 controls the protein’s dimerization, whereas STAT5 phosphorylation directly regulates its intracellular localization (Figure 7e).
DISCUSSION

The JAK/STAT pathway has been shown to be among the most important signaling pathways in the development and maintenance of tumors. Inhibitors of individual JAKs and STATs are currently in development and are thought to hold promise for treating a wide variety of tumors. A number of JAK inhibitors are undergoing clinical trials and first compounds have been approved by the Food and Drug Administration (FDA). Nevertheless, there have not yet been any convincing demonstrations of STAT inhibitors that are both safe and sufficiently specific to be appropriate for use in humans. The critical STAT molecules are STAT3 and STAT5, both of which are constitutively activated in a broad range of solid and hematopoietic tumors. Targeting these molecules directly via their dimerization domains has proven especially difficult. We present a different approach to inhibiting the activity of STAT5 in tumor maintenance, based on blocking an upstream serine kinase and consequently preventing nuclear translocation. It is likely that the mechanism we describe will turn out to be relevant to other types of tumor.

Figure 7. STAT5\textsuperscript{5725} and STAT5\textsuperscript{5779} phosphorylations are independent of BCR-ABL\textsuperscript{p185} kinase activity and STAT5\textsuperscript{Y694} phosphorylation. (a) BCR-ABL\textsuperscript{p185} cells were treated with imatinib (2 \textmu M) and STAT5 phosphorylation on residues Y694, S725 and S779 monitored by immunoblotting. (b) Immunoblotting of K562 cells after treatment with 2 \textmu M imatinib for indicated time points. Levels of active versions of PAK1 and PAK2 (pPAK1\textsuperscript{T423}; pPAK2\textsuperscript{T402}) do not alter upon inhibition of BCR-ABL kinase activity. (c) Immunoblotting of Stat5\textsuperscript{fl/fl Mx-1Cre} BCR-ABL\textsuperscript{p185} cells expressing indicated STAT5 mutants. Endogenous Stat5 was deleted via interferon-\textbeta (IFN-\textbeta) treatment 4 weeks before use. STAT5 proteins harboring a Y694 mutation maintain phosphorylations on S725 and S779. (d) Dose–response curves of PAK2 shRNA- or random shRNA-expressing BCR-ABL\textsuperscript{p185} cells toward PF-3758309 (left panel) or imatinib (right panel). (e) Scheme summarizing activation of STAT5 via PAK1/2 kinases independently of BCR-ABL kinase activity.

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Although a number of signaling pathways cooperate to support cell viability, the role of STAT5 in BCR-ABL-induced disease is key.\textsuperscript{28,39} The deletion of STAT5\textsubscript{a/b} is incompatible with cell survival\textsuperscript{10,11} and STAT5 is essential for initial transformation as well as for leukemia maintenance. Loss of STAT5 signaling causes leukemic cell death even in imatinib-resistant cells. Furthermore, deletion of STAT5 is well tolerated by the adult host organism—at least in the murine system.\textsuperscript{10,11} STAT5 thus fulfills all the criteria of a therapeutic target.

STAT proteins lack a catalytic domain but can be targeted by inhibiting critical post-translational modifications. Most attention has been paid to Y694, the phosphorylation of which permits dimerization and nuclear translocation. In nontransformed cells, JAK kinases are responsible for tyrosine phosphorylation, whereas in BCR-ABL\textsuperscript{+} cells the fusion kinase itself phosphorylates STAT5\textsuperscript{Y694}.\textsuperscript{40} Treatment of BCR-ABL\textsuperscript{+} cells with imatinib or any of the other tyrosine kinase inhibitors abolishes STAT5\textsuperscript{Y694} phosphorylation. All BCR-ABL kinase inhibitors thus indirectly target STAT5\textsuperscript{Y694} phosphorylation, and hence all of them essentially represent a single therapeutic avenue against STAT5.

STAT5 is additionally phosphorylated on highly conserved serine residues in the transactivation domain.\textsuperscript{39} We show here that this phosphorylation is independent of STAT5\textsuperscript{Y694} phosphorylation: treatment of BCR-ABL\textsuperscript{+} cells with imatinib reduces phosphorylation of STAT5\textsuperscript{Y694} but not of STAT5\textsuperscript{S725} or STAT5\textsuperscript{S779}. Confirmation comes from experiments using a STAT5\textsuperscript{Y694} phosphorylation mutant, that reveals phosphorylation on STAT5\textsuperscript{S725} and STAT5\textsuperscript{S779}. STAT5\textsuperscript{a} serine phosphorylation has recently been implicated in leukemogenesis. BM transplantation studies using a constitutively active version of STAT5\textsuperscript{a} (cS5a) caused leukemia with STAT5\textsuperscript{a} itself as the driving oncogene but mutation of serine residues (STAT5\textsuperscript{S725A} and STAT5\textsuperscript{S779A}) abrogated disease. This indicated the importance of STAT5 serine phosphorylation but did not address its relevance in forms of leukemia driven by human transforming tyrosine kinases. We provide initial evidence that serine phosphorylation is important for kinase-driven STAT5 hyperactivation. Fetal liver cells could not be transformed with BCR-ABL\textsuperscript{+} on co-infection with STAT5\textsuperscript{SASA}, whereas transfection of stable leukemic cell lines with STAT5\textsuperscript{variants led to only an initial decrease of STAT5\textsuperscript{wt. This indicates that the deletion of STAT5 serine phosphorylation in leukemia may enhance reactive oxygen species levels and the inhibitor PF-3758309, which prevents ATP binding, concentration of which allosterically blocks the group I PAK kinases at concentrations that make it unsuitable for use in human patients, and the inhibitor PF-3758309, which prevents ATP binding, signifying reduced viability of both cell lines, accompanied by decreases in the levels of STAT5\textsuperscript{S779} phosphorylation and of nuclear STAT5. The findings are in line with observations in STAT5\textsuperscript{S779A} mutant cells, in which nuclear accumulation of the protein and transcription of target genes is significantly impaired. Second, PAK kinases are able to phosphorylate STAT5\textsuperscript{S779} in vitro. Co-immunoprecipitation experiments identified complexes containing both STAT5 and group I PAK kinases. Finally, knockdown experiments revealed the tight connection between STAT5\textsuperscript{S779} phosphorylation and PAK kinases, although in an unexpected manner. Reduced expression of PAK2 in murine BCR-ABL\textsuperscript{+} cells is associated with an enhanced activity of the remaining protein and paralleled by increased STAT5\textsuperscript{S779} phosphorylation. This observation might point at a so far unknown feedback loop that tightly adjusts protein expression and activation status. As PAK kinases regulate mitogen-activated protein kinase kinase kinase 1 activation via phosphorylation on S298,\textsuperscript{49} this represents an obvious candidate. However, no effects were observed if we blocked mitogen-activated protein kinase kinase 1 in our cellular system. Nevertheless, reduced PAK protein levels render the cells more susceptible to treatment with PAK inhibitors but not to BCR-ABL tyrosine kinase inhibitors, providing further support for our concept.

Group I PAK kinases 1–3 are known to possess auto-inhibitory phosphotyrosine interaction domains and to require activation by a p21 GTPase, either Rac or Cdc42.\textsuperscript{50} There is convincing evidence that Rac GTPases are key regulators of BCR-ABL-induced malignancies,\textsuperscript{51,52} but the underlying mechanism has remained obscure. Our results suggest that the effect is at least partially mediated by inhibition of nuclear accumulation of STAT5. It is currently unknown which signaling pathways activate PAK kinases in nonsolid tumors, although integrin signaling was recently shown to be crucial for acute myeloid leukemia.\textsuperscript{53} The constitutive activation of PAK kinases may also result from the accelerated cell cycle progression in leukemic cells: PAK kinases are active when cells initiate mitosis and rapidly dividing cells might not have sufficient time to deactivate them. PAK kinases have been reported to be overexpressed in human cancers and are considered promising therapeutic targets.\textsuperscript{54} They have a wide variety of downstream targets, such as c-Raf and MAPK signaling that contribute to a tumorigenic state, and hence the therapeutic potential of inhibiting PAK kinases is considerable.\textsuperscript{54} To the best of our knowledge, there has not been any previous study of PAK kinases in transformed cells, although there is a report that PAK1 regulates lobuloalveolar development in a STAT5\textsuperscript{S779-dependent manner.\textsuperscript{55} Using BCR-ABL\textsuperscript{+} disease we now show that PAK kinases act via STAT5 in an oncogenic setting, thereby adding STAT5 to the list of signaling mediators downstream of PAK kinases. STAT5 may be one of the key downstream targets of PAK kinases in certain types of tumors, mediating their protooncogenic effects.

The importance of STAT5 serine phosphorylation for transformation and its independence from the BCR-ABL-STAT5\textsuperscript{Y694} axis offers a therapeutic opportunity that is distinct from that afforded by current tyrosine kinase inhibitors. Inhibiting PAK1 and/or PAK2 may prevent nuclear localization of STAT5 and as a consequence its oncogenic activity. Targeting PAK kinases may represent a
feasible way to circumvent the difficulties in developing effective direct inhibitors of STATs and might provide a promising strategy for treating cancers with hyperactivated STATs.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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