IRF5 is a target of BCR-ABL kinase activity and reduces CML cell proliferation

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Interferon regulatory factor 5 (IRF5) modulates the expression of genes controlling cell growth and apoptosis. Previous findings have suggested a lack of IRF5 transcripts in both acute and chronic leukemias. However, to date, IRF5 expression and function have not been investigated in chronic myeloid leukemia (CML). We report that IRF5 is expressed in CML cells, where it interacts with the BCR-ABL kinase that modulates its expression and induces its tyrosine phosphorylation. Tyrosine-phosphorylated IRF5 displayed reduced transcriptional activity that was partially restored by imatinib mesylate (IM). Interestingly, a mutant devoid of a BCR-ABL consensus site (IRF5^{Y104F}) still presented significant tyrosine phosphorylation. This finding suggests that the oncoprotein phosphorylates additional tyrosine residues or induces downstream signaling pathways leading to further IRF5 phosphorylation. We also found that ectopic expression of IRF5 decreases the proliferation of CML cell lines by slowing their S-G, transition, increasing the inhibition of BCR-ABL signaling and enhancing the lethality effect observed after treatment with IM, α-2-interferon and a DNA-damaging agent. Furthermore, IRF5 overexpression successfully reduced the clonogenic ability of CML CD34-positive progenitors before and after exposure to the above-indicated cytotoxic stimuli. Our data identify IRF5 as a downstream target of the BCR-ABL kinase, suggesting that its biological inactivation contributes to leukemic transformation.

Introduction

Interferon regulatory factors (IRFs) are a family of transcription factors involved in the response to viral infection (1), cell growth and differentiation (2,3). Among them, IRF5 transcriptionally regulates type I interferon (IFN)-dependent genes, while also modulating cell cycle progression and cell death (4,5). Indeed, ectopic IRF5 inhibits cell proliferation possibly via one of the multiple targets of its transcriptional activity (6–9). Convincing evidence has demonstrated that serine/threonine phosphorylation regulates IRF5 intracellular localization, dimerization and transcriptional activity (10). In fact, in several recipient cell models, transiently expressed IRF5 was phosphorylated on its

Abbreviations: ANOVA, analysis of variance; ATP, adenosine triphosphate; CFU, colony-forming unit; CML, chronic myeloid leukemia; DAS, dasatinib; DSB, double-strand break; ERK, extracellular signal-regulated kinase; EV, empty vector; IFN, interferon; IRF5, interferon regulatory factor 5; IM, imatinib mesylate; MEF, murine embryonic fibroblasts; NIL, nilotinib; PON, ponatinib; rh, human recombinant; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; STAT5, signal transducer and activator of transcription 5. C-terminal auto-inhibitory region by multiple serine/threonine kinases including TANK-binding kinase-1 and receptor interacting protein 2 (11). Although IRF5 serine and threonine phosphorylation sites have been well characterized, there is only preliminary evidence linking this protein to tyrosine phosphorylation. Hu *et al.* (7) have shown previously that activated IRF5 lacks tyrosine phosphorylation suggesting that this is likely associated with the suppression of IRF5 transactivation.

Several studies have previously linked different members of the IRF family to chronic myeloid leukemia (CML) pathogenesis and progression (12,13). Barnes et al. (8) have investigated the role of IRF5 in human leukemias reporting a lack of IRF5 transcripts that suggests epigenetic silencing of its promoter. However, CML was not included in these studies. CML is a hematological disorder derived from the neoplastic transformation of the hematopoietic stem cell (14). The BCR-ABL oncoprotein is the molecular hallmark of the disease displaying constitutive tyrosine kinase activity and inducing both antiapoptotic and proliferative stimuli that favor the expansion of the malignant clone (15,16). A plethora of protein substrates is associated with BCR-ABL, and their ensuing tyrosine phosphorylation contributes to leukemic transformation (15,17). Evidence generated in murine models has identified extracellular signal-regulated kinase (ERK). AKT and signal transducer and activator of transcription 5 (STAT5) as critical downstream mediators of BCR-ABL-dependent myeloid leukemogenesis (18-20). Pharmacological suppression of BCR-ABL kinase activity inhibits these signaling pathways killing CML cells (21-23). Indeed, tyrosine kinase inhibitors have changed the natural course of the disease generating survival rates comparable with those observed in the healthy population (24).

We investigated IRF5 expression and function in BCR-ABLpositive cells and found that the oncoprotein inactivates IRF5 by modulating its expression and inducing its tyrosine phosphorylation. Imatinib mesylate (IM) treatment decreased IRF5 phosphorylation restoring its ability to transactivate the β -IFN promoter. Ectopic IRF5 reduced the proliferation rate and clonogenic potential of CML cell lines and enhanced the cytotoxic effects of IM, α -2-IFN and etoposide (VP-16), significantly decreasing both ERK and AKT signaling. IRF5 overexpression also decreased the colony-forming ability of CML-CD34-positive cells. Taken together, our data suggest that IRF5 inactivation contributes to BCR-ABL-dependent transformation.

Materials and methods

Cell lines and drug treatments

Human acute myeloid leukemia HL-60 cells or K562 and KYO1-CML cell lines were grown in RPMI 1640. HEK293 and IRF5^{-/-} murine embryonic fibroblasts (MEFs) were cultivated in Dulbecco's modified Eagle's medium 4500 mg/l glucose. All growth media were supplemented with 4 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin (all from Sigma– Aldrich, St Louis, MO) and 10% inactivated fetal bovine serum (Lonza, Basel, Switzerland). HL-60, K562, KYO1 and HEK293 were purchased from DSMZ (Braunschweig, Germany), whereas IRF5^{-/-} MEFs were donated by Tadatsugu Taniguchi (Tokyo, Japan). IM and nilotinib (NIL) were provided by Novartis (Basel, Switzerland); dasatinib (DAS) was donated by Bristol-Myers Squibb (Princeton, NJ) and ponatinib (PON) was bought from Santa Cruz Biotechnology (Dallas, TX). Nocodazole was purchased from Calbiochem (Darmstadt, Germany), and α -2-IFN or VP-16 from Sigma–Aldrich.

Isolation and expansion of human hematopoietic CD34+ progenitors

The CD34+ population was immunomagnetically separated from bone marrow samples derived either from CML patients at diagnosis or from healthy donors according to the manufacturer's protocol (130-046-702; Miltenyi Biotec, Bergisch Gladbach, Germany). After separation, CD34+ cells were analyzed by FACSCalibur (Becton-Dickinson, Franklin Lakes, NJ) using CD34-fluorescein isothiocyanate and CD45-phycoerythrin antibodies (both from Miltenyi Biotec) obtaining >90% purity. For selected experiments, progenitors were grown in StemSpan Serum-Free Expansion medium (SFEM) supplemented with StemSpan Cytokine Cocktail 100× (final concentration, recombinant human

Generation of lentiviral vectors

The *pLEX-pCMV-IRES-PAC* lentiviral vector (pLEX-EV, empty vector; Open Biosystems, Lafayette, CO) was used as a backbone to clone FLAG-IRF5^{WT}, HIS-IRF5^{WT}, FLAG-IRF5^{Y104F}, HIS-IRF5^{Y104F}, FLAG-BCR-ABL^{WT}, FLAG-BCR-ABL^{WT}, FLAG-BCR-ABL^{WT}, FLAG-BCR-ABL^{WT}, and FLAG-MyD88. The FLAG-IRF5^{WT} and HIS-IRF5^{WT} complementary DNAs were amplified from the IRF5 Tag2B vector (a gift of P.M.Pitha, Baltimore, MD) using the following primers:

FLAG-IRF5^{WT}:

Fw SpeI 5'actagtatggattacaaggatgacgacgataag3'

Rv NotI 5'ataagaatgcggccgcttattgcatgccagctgggtg3'

HIS-IRF5^{WT}:

pcDNA3.1-BCR-ABL^{WT} was used as a template to generate pLEX-FLAG-BCR-ABL^{WT} and pLEX-HIS-BCR-ABL^{WT} inserting the FLAG or HIS sequences in the forward primer and using the same reverse oligonucleotide (Rv MluI 5'cgacgcgtctacctctgcactatgtcact3'):

FLAG-BCR-ABL^{WT}:

Fw SpeI 5'gactagtgccacctggattacaaggatgcgacgataagatggtggaccggtggc3'

HIS-BCR-ABLWT:

Fw SpeI 5'gactagtgccaccatgcatcaccatcaccatggtggacccggtgggc3'.

To generate BCR-ABL^{KD} (K271H) or FLAG-IRF5^{Y104F}, we used direct mutagenesis using the following primers by quickchange Site-Direct Mutagenesis Kit XL II (Agilent Technologies, Santa Clara, CA):

IRF5^{Y104F}:

Fw 5'ggacttccgcctcatcttcgacgggcc3'

Rv 5'ggcccgtcgaagatgaggcggaagtcc3'

BCR-ABL^{KD}:

Fw 5'ctgacggtggccgtgcacaccttgaaggaggac3'

Rv 5'gtcctccttcaaggtgtgcacggccaccgtcag3'.

pLEX-MyD88 was cloned from total RNA of leukocytes derived from a healthy donor. Reverse transcription was performed using Superscript III (Invitrogen, Paisley, UK) and the resulting complementary DNA was amplified using MyD88-specific oligonucleotides with the FLAG sequence in the forward primer:

Fw BamHI 5'ggatccatggattacaaggatgacgacgatgaggacgacggcggcggcggcggcggggacggggacagggcctt3'.

Lentivirus production, titering and lentiviral transduction

Recombinant lentiviruses were produced by transient transfection in TLA-HEK-293t according to the protocol provided by Open Biosystems. Titration of the lentiviruses supernatant was performed by antibiotic selection modifying the Open Biosystems protocols as follows. Twenty-four hours before transduction, 1×10^5 TLA-HEK-293t cells were implanted in a 12-well plate. The following day, cells were transduced with a series of 5-fold (from 5 to 390.625) dilutions of lentiviral supernatant in the presence of 4 µg/ml polybrene (Sigma-Aldrich) for 24 h. Viral supernatant was then replaced with fresh medium and, after an additional 24 h, cells were exposed to puromycin (3 µg/ml). This medium was replaced every 72 h for 12-15 days and colonies stained by 0.5% crystal violet in ethanol/phosphate-buffered saline solution. We obtained from 2×10^6 to 4×10^{6} TU/ml as indicated in the Open Biosystems protocol. About 1 ml of unconcentrated viral supernatant was used to transduce immortalized cell lines (K562 or KYO1 cells) or, after a 100-fold concentration step, to infect CD34+ cells (26). K562 and KYO1 cells were spinoculated twice for 90 min at 32°C (1200g) in the presence of polybrene (8 µg/ml). After 48 h of post-transduction, 6 µg/ml of puromycin were added to select resistant clones. CD34+ cells were pre-stimulated in StemSpan Serum-Free Expansion medium supplemented with StemSpan Cytokine Cocktail 100× for 18 h. Cells were then subjected to two rounds of spinoculation as previously reported, in the presence of 4 µg/ml polybrene. At this time, cells were plated for the methylcellulose assay or, after an additional 24 h, used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or immunoprecipitation experiments.

Immunoprecipitation, co-immunoprecipitation and immunoblots

Immunoprecipitations were performed as previously reported (27). Co-immunoprecipitation experiments were carried out by lysing cells with lysis buffer [50 mM Tris pH 7.4, 100 mM NaCl, 0.5% Triton-X, 1 mM ethylenediaminetetraacetic acid pH 7.4, 100 mM NaF, 1 mM Na₃VO₄, 0.1 mM phenylmethylsulfonyl fluoride, 1× protease inhibitor (Roche)] in ice for 30 min. Experiments were then performed as reported for the immunoprecipitation. For immunoblots, whole cell lysates were obtained by resuspending cells in Laemmli buffer. The ensuing cell suspension was sonicated, denatured and separated by SDS-PAGE. Antibodies used were as follows: anti-HIS, anti-ABL (K12; both from Santa Cruz), anti-phosphotyrosine (clone 4G10; Millipore, Billerica, MA), anti-IRF5 (ab2932; Abcam, Cambridge, UK), anti-actin (AC-15) and anti-FLAG-M2-F3165 (both from Sigma), anti-AKT (9272), anti-p44-42 mitogen-activated protein kinase (ERK1/2, 9102), anti-STAT5 (9363) and phospho-specific antibodies anti-pAKT-Ser473 (9271), anti-p44-42 mitogen-activated protein kinase (ERK1/2, Thr202-Tyr204, 9101) and pSTAT5-Y694 (9351; all from Cell Signaling, Beverly, MA). Appropriate horseradish peroxidase-conjugated secondary antibodies were used to detect the intended proteins using the LiteAblot enhanced chemiluminescence reagent (EuroClone, MI, Italy).

Stable HEK293 cell lines

Stable HEK293 cell lines were obtained by transfection with pLEX-EV, pLEX-FLAG-IRF5^{WT} and FLAG-IRF5^{Y104F} constructs using the calcium phosphate method. After 24 h of post-transfection, cells were subjected to a 20-fold dilution and placed in selection medium containing 3 µg/ml puromycin (Sigma–Aldrich). Every 3 days this medium was changed, until single colonies were formed. At this time, six colonies for each condition were expanded, lysed in 1× Laemmli buffer, sonicated, denatured and each lysate loaded on SDS–PAGE. Nitrocellulose membranes were immunoblotted with anti-FLAG and anti-actin antibodies. The colony showing major transgene expression (calculated as FLAG/actin ratio) was further expanded and used for immuno-precipitation and luciferase experiments.

Luciferase assays

HEK293 stably expressing pLEX-EV, pLEX-FLAG-IRF5^{WT} or FLAG-IRF5^{Y104F} were transiently transfected by calcium phosphate with pLEX-FLAG-MyD88, pLEX-FLAG-BCR-ABL^{WT}, BCR-ABL^{KD} and pGL3-IFN-β-promoter-Luc+ (a gift of W.E.Royer Jr, Worcester, MA) constructs. HL-60, K562 and KYO1 cells were electroporated using Ingenio Electroporation Solution (Mirus-Bio, Madison, WI). The pGL2-survivin-promoter was a gift of Bruno Calabretta (Philadelphia, PA). A renilla-encoding vector (pRL-CMV; Promega, Madison, WI) was used to normalize transfection efficiency. Luciferase activity was measured with the Dual-Luciferase Reporter Assay System according to the manufacturer's protocol (Promega).

Soft-agar colony-forming unit assay

A 1.2% water solution of Noble Agar (Sigma–Aldrich) was diluted with RPMI 1640 supplemented with 20% fetal bovine serum at a final concentration of 0.3%. About 15 ml of this solution was used to resuspend 5×10^3 K562 and KYO1 cells in a 100 mm dish. After 15 min, 2 ml of RPMI 1640 supplemented with 20% fetal bovine serum was used as a feeding layer and replaced every 6 days. After 15 days, colonies were counted under an optical microscope (IX71; Olympus).

Trypan blue exclusion, caspase 3 activation and MTS assays

About 1×10^5 /ml K562 and KYO1 cells transduced with pLEX-EV or pLEX-FLAG-IRF5 were seeded in triplicates in 24-well plates. For the following 5 days, 10 µl of the cell culture was diluted in 10 µl of 0.4% Trypan Blue solution, mixed and counted in a hemocytometer.

The same cell lines, treated with IM alone or in combination with α -2-IFN or VP-16, were used to analyze caspase 3 activation (EnzChek Kit; Life Technologies, Grand Island, NY) or were implanted in triplicates in 100 μ l of culture media for MTS assays (CellTiter 96® AQueous One Solution Cell Proliferation Assay; Promega) to quantify cell proliferation. MTS assays were also used to calculate VP-16, NIL, DAS or PON IC_{50} values as follows.

To evaluate the IC₅₀ values by MTS assay, 1×10^{47} K562 and KYO1 or 25 × 10³ CD34+ cells were implanted in triplicates in 100 µl of culture media and exposed to logarithmic dilutions (from 1 nM to 100 µM VP-16) for 24 h. To establish the IC₅₀ of NIL, DAS and PON, 1×10^4 K562, KYO1 and HL-60 were exposed to logarithmic dilutions (from 1 pM to 1 µM) of each tyrosine kinase inhibitor for 48 h. The Prism Software (GraphPad Software, San Diego, CA) was used to determine the IC₅₀.

Protein purification system and in vitro kinase assay

To purify HIS-BCR-ABL^{WT}, HIS-IRF5^{WT} or HIS-IRF5^{Y104F}, we transiently transfected HEK293 cells using calcium phosphate. After 48 h, transfected cells were subjected to a purification step using the Ni-NTA purification

system (Life Technologies). About 2×10^7 cells were sonicated in native or denaturing buffers (both additioned with 5% glycerol, 0.5% Triton-X, 1 mM phenylmethylsulfonyl fluoride, 2 µg/ml aprotinin) to isolate HIS-BCR-ABL (native condition), HIS-IRF5^{WT} or Y104F (hybrid condition) according to the manufacturer's protocol. All proteins were quantified by staining the SDS– PAGE with coomassie blue as previously reported (28).

Approximately, 8 nM of BCR-ABL and 250 nM of IRF5^{WT} or IRF5^{Y104F} were incubated in kinase reaction buffer (40 mM Tris-HCl pH 7.5, 20 mM MgCl₂, 0.1 mg/ml bovine serum albumin) with 50 µM of adenosine triphosphate (ATP), for 1 h at room temperature. The reactions were stopped by adding the same volume of 2× Laemmli buffer, denatured and loaded on SDS–PAGE. For the Michaelis–Menten reaction, ~4 nM of purified BCR-ABL were incubated in kinase reaction buffer with 50 µM ATP. Substrates used were as follows: IRF5^{WT}, IRF5^{Y104F} (from 0.125 to 16 nM) or the ABLTIDE peptide [EAIYAAPFAKKK (SignalChem, British Columbia, Canada), from 1.56 to 200 µM]. All substrates were diluted in kinase reaction buffer, incubated for 1 h at room temperature and the experiments were then continued as reported in the manufacturer's protocol (ADP-GLO; Promega). Data were used to calculate the ATP-ADP conversion and analyzed using the Prism Software to obtain the Michaelis constant (Km) value.

Nocodazole synchronization and cell cycle analysis

About 1 × 10⁶/ml K562-EV, K562-FLAG-IRF5, KYO1-EV and KYO1-FLAG-IRF5 were exposed to 40 ng/ml nocodazole for 12 h. Cells were then harvested, fixed in 70% phosphate-buffered saline/ethanol for 24 h at -20° C and treated with propidium iodide and RNAase for 30 min. Cell cycle distribution was subsequently analyzed with a FACSCalibur flow cytometer (Becton-Dickinson) using the WinMidi2.8 and Cylchred Software packages (Cell Cycle Analysis Software, Cardiff, UK).

Methocult colony-forming unit assays

About 4 × 10³ human CD34+ cells transduced with pLEX-EV or pLEX-FLAG-IRF5 were treated as reported in Figure 5A with IM, IFN and IC₅₀ values for VP-16. After 24 h, cells were implanted in 1 ml of methylcellulose (H4435) additioned with the drugs according to the manufacturer's specified procedure (Stem Cell Technologies). Colonies were counted under an optical microscope (IX71; Olympus) after 15 days of culture. Each colony with >20–30 cells was considered (29).

Statistical analysis, IC50 and Michaelis-Menten constant (Km)

The Prism Software was used to perform analysis of variance (ANOVA) plus Bonferroni's posttests and unpaired one-tailed *t*-tests with 95% confidence intervals as well as to obtain IC_{50} values and Km (28).

Ethics statement

Hematopoietic progenitors were isolated from mononuclear cells starting from the bone marrow of either healthy donors or newly diagnosed CML patients. All patients were in chronic phase (blasts lower than 15%) with >95% of Philadelphia chromosome-positive metaphases at conventional cytogenetic analysis. All specimens (13 CML patients and 5 healthy donors) were collected following an institutionally approved protocol at the Azienda Ospedaliera Policlinico 'Vittorio Emanuele' Hospital, Catania, Italy with the informed consent of each subject.

Results

Suppression of BCR-ABL catalytic activity increases IRF5 expression in CML cells

Barnes *et al.* (8) have shown previously a lack of IRF5 gene expression in both acute and chronic lymphocytic leukemias, suggesting that IRF5 silencing might contribute to the pathogenesis of these hematological malignancies. We wanted to establish if IRF5 could also be involved in the development of CML. To this end, we initially chose to analyze IRF5 protein expression in CD34+ hematopoietic progenitors isolated from healthy individuals, newly diagnosed CML patients or in CML immortalized cell lines. We found that IRF5 was expressed at similar levels in CD34+ progenitors from healthy donors and CML patients as confirmed by a densitometric analysis (Figure 1A, right panel). We also detected IRF5 expression in K562, KYO1 and acute myeloid leukemia cells (HL-60) used as a negative control (Figure 1A, left panel).

Because BCR-ABL catalytic activity is the major force driving CML transformation, we wanted to establish if the oncoprotein modulated IRF5 expression. To this end, we performed a time course experiment on both HL-60 and CML cell lines exposed to 1 μ M IM

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(16) or IC_{50} values of NIL, DAS or PON. We found that suppression of BCR-ABL kinase activity increased IRF5 expression in both K562 and KYO1 cells but not in HL-60 (Figure 1B and C), suggesting that IRF5 could be a downstream target of BCR-ABL.

BCR-ABL interacts with IRF5 and induces its tyrosine phosphorylation

To investigate if IRF5 is a target of BCR-ABL kinase activity, we performed an anti-IRF5 immunoprecipitation followed by an immunoblot with the 4G10 antibody before and after IM treatment. We found that IRF5 is more tyrosine phosphorylated in BCR-ABL-positive cells compared with the HL-60 control. Furthermore, IM treatment reduced IRF5 phosphorylation in K562 and KYO1 (Figure 2A). These data were confirmed by an anti-phosphotyrosine immunoprecipitation followed by an anti-IRF5 immunoblot (Figure 2B). These results indicate that the BCR-ABL oncoprotein causes IRF5 tyrosine phosphorylation. Immunoprecipitation experiments determined that this phosphorylation involves a direct interaction between the two proteins (Figure 2C and 2D). Furthermore, this interaction was reduced by IM treatment indicating that it was at least partially dependent on BCR-ABL catalytic activity. The modest co-immunoprecipitation detected in BCR-ABL-negative cells (HL-60) suggests that IRF5 may also be an ABL substrate.

The IRF5 amino acidic sequence displays a potential ABL kinase consensus motif (30) involving tyrosine 104 (YDGP) in the DNA binding domain. To establish if this residue is a major *in vivo* phosphorylation site, we mutagenized Y104 in phenylalanine, generating a pLEX-FLAG-IRF5^{Y104F} construct. An anti-FLAG immunoprecipitation followed by a 4G10 immunoblot on K562 and KYO1 cells overexpressing IRF5^{WT} or IRF5^{Y104F} unexpectedly showed equal phosphorylation levels (Supplementary Figure 1, available at *Carcinogenesis* Online).

To exclude the possibility that these results may be affected by the use of a transformed CML cell model, we performed further experiments using a non-morphologically transformed cell type (27). HEK293 whole cell lysates overexpressing FLAG-tagged IRF5 (WT or Y104F) and BCR-ABL (WT or KD) were immunoprecipitated using an anti-FLAG antibody and then blotted with 4G10. Interestingly, we failed to detect any tyrosine phosphorylation in either IRF5^{WT} or IRF5^{Y104F} when these constructs were transfected alone or co-transfected with BCR-ABL^{KD}. However, after BCR-ABL co-expression, we found an increased IRF5^{WT} phosphorylation that was modestly reduced by the Y104F substitution but was abrogated by IM treatment (Figure 2E). Although these findings identify IRF5 as a target of BCR-ABL catalytic activity, they also suggest that Y104 is not a major *in vivo* phosphorylation site of the oncoprotein.

BCR-ABL reduces IRF5 transcriptional activity

Because IRF5 transcriptionally regulates several genes that modulate apoptosis and cell cycle progression (7,8,31), we wanted to evaluate whether BCR-ABL-dependent IRF5 tyrosine phosphorylation affected its transcriptional activity. We, therefore, overexpressed IRF5^{WT} or IRF5^{Y104F}—alone or in combination with BCR-ABL^{WT} or BCR-ABL^{KD}—in HEK293 cells and measured IRF5 transcriptional activity on the β -IFN-Luc+ promoter in the presence of the MyD88 co-activator (10). We found that BCR-ABL reduced the transactivation of both IRF5^{WT} and IRF5^{Y104F} and that suppression of the BCR-ABL kinase by IM partially restored the transcriptional activity of both constructs (Figure 3A). Surprisingly, we observed a weak reduction of IRF5 transcriptional activity even in the presence of BCR-ABL^{KD} and this reduction was unaffected by IM treatment. These results support the hypothesis that constitutive BCR-ABL kinase negatively modulates IRF5 transcriptional activity and that the oncoprotein may also inhibit IRF5 in a kinase-independent manner. The latter finding also explains imatinib's inability to fully restore IRF5-dependent transactivation of the β -IFN promoter (Figure 3A).

To establish if endogenous IRF5 was transcriptionally active in BCR-ABL-positive cells, we electroporated K562, KYO1 and HL-60 with the β -IFN-Luc+ promoter. As hypothesized, IRF5 transcriptional activity was lower in CML cell lines compared with the



Fig. 1. Inhibition of BCR-ABL catalytic activity increases IRF5 expression. (**A**) Whole cell lysates from human CD34+ progenitors derived from healthy donors (N33-N44) and CML patients at diagnosis (CML58–CML78), acute myeloid leukemia (HL-60 BCR-ABL-negative) or CML (K562 and KYO1) cell lines were separated by SDS–PAGE. Immunoblots were then performed to assess the expression of both IRF5 and BCR-ABL with actin used as a loading control. IRF5^{-/-} MEFs, before and after electroporation with FLAG-IRF5^{WT}, were used as a negative (Ctrl–) and positive (Ctrl+) control, respectively. Histograms show the densitometric values of the IRF5/actin ratio in primary cells. One-way ANOVA was used to calculate the statistical significance. (**B** and **C**) To evaluate if IRF5 expression was affected by BCR-ABL kinase activity, we treated the specified cell lines with 1 μ M IM (B) or IC₅₀ values for NIL, DAS and PON (shown in parenthesis) (C), for the indicated time points. Protein lysates were subjected to SDS–PAGE and nitrocellulose membranes were blotted using anti-IRF5 and 4G10 antibodies. Histograms show the densitometric analysis of IRF5 expression normalized for actin. Bars indicate the standard deviation from three independent experiments. For negative and positive controls, see Figure 1A. HL-60 cells were exposed to the highest drug concentration determined for CML cell lines. One-way ANOVA was used to calculate the statistical significance.



BA KD

BCR-ABL-negative control (Figure 3B, left columns). When we overexpressed IRF5 in K562 and KYO1 and evaluated its activity comparing it to the EV condition, we found that the exogenous protein activated the β -IFN promoter in BCR-ABL-positive cells (Figure 3B, middle columns). These data suggest that endogenous IRF5 is inactivated in the presence of BCR-ABL and that its overexpression restores its transcriptional ability.

The pGL2 Survivin-Luc+ promoter was used as an internal control and, as expected, showed increased activity in the presence of BCR-ABL (Figure 3B, right columns (16)).

IRF5 Y104 is a target of BCR-ABL catalytic activity

To evaluate if IRF5 was a direct target of BCR-ABL catalytic activity, we performed an *in vitro* kinase assay and a Michaelis–Menten reaction.

Purified HIS-tagged BCR-ABL, IRF5^{WT} or IRF5^{Y104F} were incubated in the presence of ATP and subjected to SDS–PAGE. 4G10 or anti-HIS antibodies were then used to evaluate IRF5^{WT} and IRF5^{Y104F} phosphorylation levels showing that IRF5 is a substrate of BCR-ABL catalytic activity. The Y104F substitution partially reduced IRF5 phosphorylation levels, suggesting that this residue is a direct target



Fig. 3. BCR-ABL reduces IRF5 transcriptional activity and phosphorylates it on Y104. (A) HEK293 cells transfected with the specified constructs were used to perform luciferase experiments. pGL3-β-IFN-Luc+ was used to analyze the transcriptional activity of IRF5^{WT} and Y104F alone or in the presence of kinase-proficient (BA) or kinase-deficient (BA^{KD}) BCR-ABL before and after 10 µM IM. Luciferase values were normalized using the pRL-CMV renilla vector. Bars indicate standard deviation from three independent experiments carried out in triplicates. Two-way variance analysis (ANOVA plus Bonferroni's posttests) was used to evaluate statistical significance for untreated (UNT) versus IM treated conditions. One-way ANOVA (plus Bonferroni's posttests) was used to obtain *P*-values for IRF5^{WT} and mutant alone versus co-expression with BA and BA^{KD} (**P* < 0.05, ***P* < 0.001). (**B**) HL-60, K562 or KYO1 cells were electroporated with the indicated constructs. Cell lysates were then used to determine luciferase activity. *P*-values (**P* < 0.05, ***P* < 0.001) were calculated using the *t*-test for pGL2 Survin-Luc+ and one-way ANOVA (plus Bonferroni's posttests) for the β-IFN-Luc+ promoter. (C) *In vitro* kinase assays were performed incubating purified BCR-ABL and IRF5^{WT} or IRF5^{Y104F}. The reaction mixture was then subjected to SDS–PAGE, transferred to a nitrocellulose membrane and immunoblotted with the indicated antibodies. (**D**) A Michaelis–Menten reaction shows the phosphorylation rate of purified IRF5^{WT} or IRF5^{Y104F}.

of the BCR-ABL kinase but also implying the presence of additional ABL consensus motif(s) in the IRF5 sequence (Figure 3C).

To measure BCR-ABL affinity for IRF5, we used the Michaelis– Menten enzymatic reaction. The low Km value obtained for IRF5^{WT} (0.22 nM) if compared with the ABLTIDE positive control (14.99 μ M) (28), indicates a rapid saturation of the reaction suggesting that BCR-ABL has high affinity for IRF5. Moreover, when we performed the same experiment using IRF5^{Y104F}, we obtained an increased Km value (0.41 nM), indicating a reduction in BCR-ABL affinity for IRF5 (Figure 3D). These data confirm that IRF5^{Y104F} is a substrate of the BCR-ABL kinase and also suggest the presence of further residues phosphorylated by BCR-ABL.

IRF5 shows antiproliferative activity in BCR-ABL-positive cells

Previous studies have demonstrated that IRF5 inhibits the growth of the BJAB cell line both *in vitro* and *in vivo* (8). To ascertain the biological role of IRF5 in CML cells, we evaluated both its antiproliferative and anticlonogenic activity. We found that IRF5 expression was associated with slower proliferation rates (***P < 0.001; Figure 4A) and induced a strong reduction in colony number formation (***P < 0.001; Figure 4B). As it has been previously reported that IRF5 transcriptionally induces the p21 promoter (8), we hypothesized that p21 could contribute to these effects. However, an anti-p21 immunoblot failed to demonstrate an increase in p21 levels in K562 and KYO1 (data not shown). As IRF5 has also been shown to induce G₂ arrest (8), we analyzed the cell cycle distribution of K562 and KYO1 lentivirally transduced with IRF5 detecting minor differences compared with the EV control (Figure 4C and D). Because the reduction in cell proliferation observed in K562 and KYO1 became evident after 72 h, we hypothesized that IRF5 may increase overall cell cycle length. Indeed, when we synchronized these cells in G₂ by nocodazole exposure, we found that IRF5 overexpression was associated with a residual S phase that was significantly higher than that detected after EV transduction (K562-IRF5 S phase 16% versus K562-EV S phase 2% ***P < 0.001; KYO1-IRF5 S phase 25% versus KYO1-EV S phase 12% **P < 0.01). Likewise, CML cells overexpressing IRF5 displayed a significantly lower G2 phase than their EV-transduced counterpart (K562-IRF5 G₂ phase 70% versus K562-EV G₂ phase 98% ***P < 0.001; KYO1-IRF5 G₂ phase 47% versus KYO1-EV G₂ phase 68% **P < 0.01; Figure 4E and F). These data imply that IRF5 slows the S-G₂ cell cycle transition thereby negatively affecting CML cell growth.



Fig. 4. IRF5 reduces CML cell proliferation. (**A**) Trypan blue exclusion assay was used to assess the viability of the specified cell lines. Bars indicate standard deviation derived from three independent experiments performed in triplicates. One-way variance analysis (ANOVA plus Bonferroni's posttest) was applied to evaluate statistical significance (***P < 0.001). (**B**) The same cells were used to carry out soft-agar colony-forming unit (CFU) assays to evaluate IRF5 anticlonogenic activity in a BCR-ABL-positive background. Histograms show CFU numbers as counted under an optical microscope after 15 days of culture with standard deviation calculated from three independent experiments performed in triplicates. *t*-test was used to calculate statistical significance (***P < 0.001). (**C**–**F**) The indicated K562 and KYO1 transduced cells were either left untreated (C and D) or exposed to nocodazole (E and F) to induce G₂ synchronization. Cells were subsequently analyzed by fluorescence-activated cell sorting analysis to assess their cell cycle distribution. Results are representative of three independent experiments guide for subG₀-G₁ to normalize cell death induced by nocodazole. Histograms show the effect of IRF5WT on the S-G₂ transition compared with EV condition. Bars indicate standard deviation from three independent experiments. Two-way variance analysis (ANOVA plus Bonferroni's posttest) was used to calculate statistical significance (**P < 0.001, **P < 0.001).

Published evidence has suggested that IRF5 sensitizes a colon cancer cell model to the apoptotic effect of DNA double-strand break (DSB)-inducing agents, either alone or in combination with β -IFN (31). As previously reported in CML patients progressing to blast crisis, we used VP-16 as a cause of DSB (32). Furthermore, we substituted β -IFN with α -2-IFN that was considered first-line treatment for CML before the advent of the IM era (33,34). K562 and KYO1 overexpressing IRF5 were exposed to IM alone or in combination with α -2-IFN and/or VP-16 according to the treatment schedule indicated in Figure 5A. We observed that cells overexpressing IRF5 presented a lower proliferation rate compared with their EV-transduced counterpart after exposure to IM or to IM and VP-16 (Figure 5B, ***P < 0.001). However, the addition of α -2-IFN did not increase the cytotoxic effect observed with the combination of IM and VP-16. Interestingly, we found high levels of caspase 3 activation in CML cells expressing ectopic IRF5 and exposed to the triple combination of IM, VP-16 and α -2-IFN (Figure 5C). These data indicate that IRF5 increases the apoptotic rate of CML cells after exposure to different cvtotoxic stimuli.

IRF5 strengthens the inhibition of ERK and AKT signaling observed after treatment with IM, α -2-IFN and VP-16

We wanted to investigate the molecular mechanisms underlying the increased cytotoxicity of IM in combination with α-2-IFN and VP-16 observed after IRF5 overexpression. We, therefore, evaluated the canonical signaling pathways activated in BCR-ABL-transformed cells. Whole cell lysates from K562 expressing ectopic IRF5 and treated as reported in Figure 5A were blotted with antibodies recognizing ERK^{T202, Y204}, AKT^{S473} and STAT5^{Y694} (Figure 6A). In the presence of ectopic IRF5, a densitometric analysis showed a significant reduction in ERK phosphorylation as compared with K562 transduced with an EV (**P < 0.01). Exposure of the latter cells to IM, α -2-IFN and VP-16 resulted in a progressive reduction of phospho-ERK. These reductions were significantly increased in the presence of IRF5, resulting in a complete abrogation of ERK phosphorylation (***P < 0.001). When we analyzed AKT, we found a significant reduction in its phosphorylation only when IM was combined with α -2-IFN and VP-16 in the presence of IRF5 (*P < 0.05). IM exposure completely suppressed STAT5 phosphorylation, regardless of IRF5 expression.

When we repeated the same experiments using KYO1 cells, IRF5 overexpression was associated with a significant decrease in ERK phosphorylation in all treatment conditions (Figure 6B, *P < 0.05, ***P < 0.001). A similar result was found when we assessed phospho-AKT levels (**P < 0.01, ***P < 0.001), with the exception of IM alone and IM plus α -2-IFN conditions. As previously reported for K562 cells, IM-dependent suppression of BCR-ABL catalytic activity abolished STAT5 phosphorylation. Taken together, these data suggest that IRF5 increases the cytotoxic effects of IM and a DSB-inducing agent by negatively modulating the ERK and AKT pathways.

IRF5 suppresses the clonogenicity of human CML hematopoietic progenitors

To confirm the high IRF5 phosphorylation observed in CML cell lines (Figure 2A and 2B), we performed an immunoprecipitation experiment in human myeloid progenitors derived from two CML patients and one healthy donor. Limited cell numbers allowed us to repeat the experiment in the presence of IM only in the N51 and CML91 samples. We found that IRF5 was more phosphorylated in CML progenitors as compared with the control cells. Moreover, this phosphorylation was reduced by IM in CML91 but not in N51 suggesting that this event is strictly dependent on BCR-ABL catalytic activity (Figure 7A).

To determine if IRF5 reduces the clonogenic activity of CML CD34+ cells, we isolated hematopoietic progenitors from four CML patients at diagnosis and—after lentiviral transduction with IRF5—we observed a significant reduction in their colony-forming ability (Figure 7B). We next wished to establish if IRF5 overexpression

would also increase the cytotoxic effects of IM, α-2-IFN and VP-16 on BCR-ABL-positive human progenitors. We, therefore, isolated CD34+ cells from three further patients and calculated their VP-16 IC_{50} (Figure 7C). These progenitors were then lentivirally transduced with IRF5 (Figure 7D) and left untreated or exposed to the drugs specified in Figure 5A. Cells were then plated in triplicates in methylcellulose to assess their colony-forming potential. IRF5 significantly decreased colony numbers, confirming its antiproliferative activity in human CML progenitors (Figure 7E, ***P < 0.001). IM treatment generated a further significant reduction in colony formation (***P < 0.001), whereas the combination with α -2-IFN provided only a modest additional benefit (***P < 0.001). On the contrary, the association of IM and VP-16 strongly decreased the number of colonies in the two responsive patients (patients 45 and 55, ***P < 0.001, **P < 0.01). Again, minimal benefits were evident when α -2-IFN was added to the combination of IM and VP-16 (patients 51 and 55, *P > 0.05). This data indicate that the IRF5 antiproliferative effect observed in immortalized cell lines is also detectable in CD34+ progenitors derived from CML patients.

Discussion

Malignant transformation by the BCR-ABL oncoprotein is critically dependent on its tyrosine kinase activity that favors cell growth and antiapoptotic signaling (15). On the contrary, the role of IRF5 in tumorigenesis remains controversial. In fact, initial reports suggested that IRF5 negatively regulates cell proliferation (8,31,35,36). However, we have recently shown that—in thyroid cancer cells—IRF5 displays tumor-promoting properties (37). In this study, we demonstrate that IRF5 is expressed in CML cells, where it presents antiproliferative activity that is compromised by BCR-ABL-dependent tyrosine phosphorylation.

Extensive evidence suggests that different members of the IRF family contribute to myeloid leukemogenesis (38-40). IRF4 overexpression in murine CD34+ progenitors reduces BCR-ABL-dependent transformation (12). Likewise, genetic data demonstrates that mice devoid of IRF8 develop a myeloproliferative CML-like syndrome (41). Furthermore, IRF8 overexpression reduces the levels of both c-Myc and Bcl-2, thereby downregulating the mitogenic and transforming activity of the BCR-ABL oncoprotein (13,42). IRF5 expression and function have never been investigated in CML cells. We found IRF5 in both immortalized cell lines and human CML hematopoietic progenitors. We also found that IRF5 is highly tyrosine phosphorylated in both primary and immortalized cell lines and that the extent of this phosphorylation is reduced by IM. Hu et al. (31) have previously reported that tyrosine phosphorylation is associated with IRF5 transcriptional inactivation. Hence, our findings support the notion that BCR-ABL-dependent IRF5 phosphorylation compromises its biological function. Indeed, suppression of the BCR-ABL kinase by IM partially restored IRF5-dependent transactivation of the β-IFN promoter. These results are in agreement with previous findings indicating that tyrosine phosphorylation is a crucial determinant of IRF function. In fact, IRF8 tyrosine phosphorylation is required for its biological activation, and BCR-ABL-dependent induction of the SHP2 phosphatase antagonizes this event favoring leukemogenesis (43). Our data-generated both in a morphologically untransformed cell type and in a cell-free system-indicate that IRF5 tyrosine 104 is a direct target of the BCR-ABL kinase. However, the persistent tyros-ine phosphorylation of IRF5^{Y104F} in both experimental models implies that the oncoprotein (i) could activate additional tyrosine kinases that recognize IRF5 as a downstream substrate and (ii) phosphorylates other tyrosine residues in the IRF5 sequence.

The increased IM sensitivity of K562 and KYO1 cells lentivirally transduced with IRF5 established that this protein could also reduce CML cell viability in response to BCR-ABL kinase inactivation. Previous evidence has shown that, in colon cancer cells, IRF5 strengthens the cytotoxic effect of a DSB-inducing agent associated with β -IFN (31). We combined each of these agents with IM and found that IRF5 further reduced the viability of leukemic cells exposed to VP-16



Fig. 5. IRF5 increases the cytotoxic effect induced by IM and VP-16. (A) Treatment schedule followed for the described experiments. Cells were either left untreated or exposed to 1 μ M IM (solid arrow). Two hours after IM addition, cells were treated with either α -2-IFN 1000 U/ml (dashed arrow) or their VP-16 IC₅₀ concentration (K562 30 μ M, KYO1 5 μ M, small dashed arrow), or with a combination of both drugs for an additional 22 h. (**B**) The specified cells transduced with the indicated constructs were subjected to MTS proliferation assays to determine if IRF5 modulates the cytotoxic effect of IM, alone or in combination with α -2-IFN and VP-16. Histograms show relative percentage of metabolically active cells with the untreated condition arbitrarily set at 100%. Bars represent standard deviation of three independent experiments carried out in triplicates. Two-way analysis of variance (ANOVA plus Bonferroni's posttests) was used to evaluate statistical significance (****P* < 0.001). (**C**) Histograms show the increase in caspase 3 activity in K562 and KYO1 cells transduced with EV or IRF5 after exposure to the pharmacological treatments summarized in panel A. Fold increase was calculated for each condition using the untreated EV value arbitrarily set at 1. Immunoblots using FLAG and 4G10 antibodies were performed to confirm transgene expression and IM-dependent inhibition of BCR-ABL kinase, respectively. Bars indicate standard deviation for three independent experiment performed in duplicates. Two-way analysis of variance (ANOVA plus Bonferroni's posttests) was used to evaluate statistical significance (**P* < 0.05, ***P* < 0.01).



Fig. 6. IRF5 strengthens the reduction in pERK-p44/42^{Thr202,Tyr204} and AKT^{Ser473} phosphorylation observed after treatment with IM and VP-16. (**A** and **B**) Total protein lysates from K562 (A) and KYO1 (B) cells transduced with EV or IRF5 and treated as indicated in Figure 5A were separated by SDS–PAGE. Immunoblots for the specified proteins were then performed to establish if IRF5 affects the expression and/or phosphorylation of canonical BCR-ABL signaling. Histograms represent a densitometric analysis of pERK-p44/42^{Thr202,Tyr204} and AKT^{Ser473} phosphorylation derived from the immunoblots. The densitometric value of each protein was initially normalized for actin. The final relative densitometric units were obtained by calculating the ratio between phosphorylated versus total protein fractions. Two-way analysis of variance (ANOVA plus Bonferroni's posttests) was used to evaluate statistical significance (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

used as a source of DSB. On the contrary, the addition of α -2-IFN did not improve the cytotoxic effect observed with IM and VP-16. Yet, α -2-IFN increased caspase 3 activation in CML cells overexpressing IRF5, suggesting a drug-dependent modulation of IRF5 intracellular signaling. The modest effects that α -2-IFN displayed on CML cells were unexpected in the light of prior data generated in a solid tumor model. These differences may be attributable to a diverse cellular background or to β -IFN substitution with α -2-IFN. However, using the former drug would have been inappropriate because α -2-IFN was the approved first-line treatment for CML before the IM era and still represents a viable therapeutic option 33,34).

When we investigated if IRF5 modulates the canonical BCR-ABLdependent signaling, we found a strong reduction in ERK phosphorylation after treatment with IM, alone or in combination with VP-16 and α -2-IFN. These results reflected the reduced proliferation rates observed after IRF5 overexpression (Figure 5B). In the presence of these drugs, IRF5 also appeared to decrease AKT phosphorylation, albeit at a lower level than that seen for ERK. Again, these findings are in agreement with the moderate increase in caspase 3 activation mediated by ectopic IRF5. Our results argue against an IRF5-dependent transcriptional regulation of ERK and AKT (Figure 6A and B). We, therefore, hypothesize that IRF5 negatively regulates the different kinases involved in ERK and AKT phosphorylation after exposure to different cytotoxic stimuli. As IRF5 induces B-Raf in thyroid cancer cells (37), it is tempting to speculate that—in a different cellular background—IRF5 may elicit the opposite effect downregulating B-Raf and thus reducing ERK phosphorylation. There is currently no evidence linking IRF5 to the PI3K/AKT pathway. Hence, it is possible that the reduction in AKT phosphorylation observed after IRF5 overexpression may require the crosstalk between the RAS/Raf/ERK and the PI3K/AKT pathways (44–46). However, we cannot exclude alternative/additional transcriptional effects directly mediated by IRF5.

In summary, we provide evidence suggesting an antileukemogenic role for another member of the IRF family. Our results are in line with D







IP:IRF5

N 51

IM

IRF!

CML91

+

CML95



Ε



Fig. 7. IRF5 shows high phosphorylation levels, anticlonogenic activity and increases the cytotoxic effects of IM, IFN and VP-16 in CD34+ progenitors from CML patients. (**A**) CD34+ cells derived from the indicated specimens were left untreated (–) or exposed to 1 μ M IM (+). Total cells lysates were subjected to an anti-IRF5 immunoprecipitation followed by immunoblotting with 4G10 (IB:4G10). Total IRF5 (IB:IRF5) was used to confirm similar amounts of immunoprecipitated proteins in each lane. (**B**) CD34+ progenitors were transduced with the indicated constructs and their whole cell lysates were separated by SDS–PAGE. Immunoblot with a FLAG antibody confirmed FLAG-IRF5 expression. The same CD34-positive cells were used to perform CFU assays. After 15 days, colonies were secored under a microscope. Histograms show the number of colonies in the presence of IRF5 compared with the EV condition. Bars indicate standard deviations for each experiment performed in triplicates. One-way analysis of variance (ANOVA plus Bonferroni's posttests) was used to evaluate statistical significance (**P* < 0.05, ***P* < 0.01). (C) Proliferation curves of CD34+ cells isolated from three indicated CML patients after exposure to VP-16 logarithmic dilutions. The Prism Software® was used to assess the IC₅₀ value for each patient. (**D**) CD34+ progenitors were transduced with the indicated constructs and their whole cell lysates blotted as reported in panel A. (**E**) The same cells were also used to perform CFU assays under the treatment conditions reported in Materials and methods using IC₅₀ values shown in Figure 7C. Histograms show the increased cytotoxicity of IM, IFN and VP-16 in the presence of IRF5. Bars indicate standard deviations for each specimen plated in triplicates. Two-way analysis of variance (ANOVA plus Bonferroni's posttests) was used to conditions reported in Materials and methods using IC₅₀ values shown in Figure 7C. Histograms show the increased cytotoxicity of IM, IFN and VP-16 in the presence of IRF5. Bars in

what has previously been reported for IRF8, suggesting that the biological inactivation of multiple IRF members by different oncogenic alterations is required for myeloid malignant transformation. Indeed, we demonstrate—for the first time—that IRF5 is highly tyrosine phosphorylated in primary CML progenitors and that its transcriptional activity is negatively regulated by this phosphorylation. In addition, we report that IRF5 exerts antiproliferative effects and increases the cytotoxicity of IM, VP-16 and α -2-IFN in both immortalized and primary CML cells. Mechanistic investigation of these biological events revealed that ectopic IRF5 negatively modulates ERK and AKT activity in response to drug treatments, antagonizing BCR-ABL signaling. Although further investigations will be required to clarify the molecular mechanisms linking IRF5 to CML pathogenesis, our findings point to IRF5 as a novel player in BCR-ABL-dependent transformation.

Supplementary material

Supplementary Figure 1 can be found at http://carcin.oxford journals.org/

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