BCR-ABL- and Ras-independent activation of Raf as a novel mechanism of Imatinib resistance in CML

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Abstract. Although the BCR-ABL tyrosine kinase inhibitor Imatinib has undoubtedly revolutionized the therapy of chronic myeloid leukemia (CML), acquired drug resistance remains a common problem in CML therapy. Resistance often arises from second-line mutations in BCR-ABL or overexpression of the BCR-ABL protein but in ~20% of CML cases resistance mechanisms do not involve altered BCR-ABL function. Imatinib-resistant CML cell lines have been widely used for comparative proteome/genome-wide expression screens in order to decipher resistance mechanisms but a clearcut molecular mechanism or molecular player in BCR-ABL-independent resistance to Imatinib has not yet evolved from those studies. Here, we report the identification of a novel mechanism for Imatinib resistance in CML cells with unaltered BCR-ABL function. Pharmacological analysis evidenced a constitutive, Imatinib-insensitive activation of the Erk-MAPK pathway in resistant cells. A systematic analysis of pathway constituents illustrated that Ras-GTP accumulation remained fully sensitive to Imatinib but c-Raf activity from serum-fed cultures was largely resistant to the drug's action. Sequencing excluded mutations in either B-Raf or c-Raf as the origin of resistance, indicating that a functional alteration in the regulation of c-Raf activity was responsible for this effect. Collectively, these findings highlight a novel mechanism of acquired Imatinib resistance based on the BCR-ABL and Ras-independent constitutive activation of the Erk-MAPK pathway through activated c-Raf, which could prove helpful for a better functional classification of the causes of Imatinib resistance in CML.

Introduction

Imatinib (STI571, Gleevec®) is a tyrosine kinase inhibitor that can effectively block the progression of chronic myeloid leukemia (CML) by selectively driving CML blasts into apoptosis (1). Imatinib acts as an ATP-competitive inhibitor of BCR-ABL, a chimeric kinase that arises from chromosomal translocations [predominantly t(9;22)(q34;q11)] in hematopoietic precursor cells in essentially in all cases of CML (2,3). Specifically, the BCR-ABL gene(s) encodes constitutively active tyrosine kinases which, depending on the precise translocation breakpoints and RNA splicing can display different molecular weights (p185 BCR-ABL, p210 BCR-ABL and p230 BCR-ABL) and subtle changes in their domain structure. For simplicity we refer here to all BCR-ABL variants collectively as BCR-ABL.

BCR-ABL regulates a large array of signalling and survival pathways that ultimately lead to enhanced proliferation, decreased apoptosis and reduced adhesion of tumor cells (4–6). Among the various pathways addressed by BCR-ABL the Ras/Erk pathway is arguably the one with highest relevance to the transforming potential of BCR-ABL. Numerous cell biological and animal studies coincide in that signal flux through the Ras/Erk pathway is essential for BCR-ABL-driven carcinogenesis (7), a notion that has spurred investigation of the mechanism of Ras/Erk pathway activation by BCR-ABL (8).

Activation of Ras, a small guanine nucleotide binding protein that represents the entry point into the Erk pathway, is the consequence of an increased exchange rate of guanine nucleotides on Ras, which ultimately results in the accumulation of GTP-loaded, active Ras-GTP complexes. Activation of Ras in CML cells probably results from the engagement of the guanine nucleotide exchange factor (GEF) Sos by BCR-ABL (9) via the intermediary action of adapter proteins such as Grb-2 and Gab 2 (10,11). Other studies, however, have evoked...
the mediation of other GEFs (10) and even GEF-independent mechanisms have been proposed (11,12). Thus, while the exact signalling topology linking BCR-ABL to Erk remains undeciphered, it is well established that BCR-ABL feeds into the Erk pathway at the level of Ras, and that activation of this pathway is paramount to the transforming action of BCR-ABL in CML.

Although Imatinib is a very potent and efficient drug in the chronic phase of CML, response rates drop during disease progression (13). Moreover, relapse of the disease is observed in many cases due to the emergence of resistance to Imatinib. It is well recognized that various mechanisms underlie the acquisition of resistance to Imatinib at later stages of CML. In most cases, second line mutations in BCR-ABL can prevent binding of Imatinib to the kinase. Alternatively, overexpression of the BCR-ABL protein can confer partial resistance to therapeutical doses of Imatinib (14). Although resistance to Imatinib is thus mostly attributable to genetic and/or functional alterations in BCR-ABL, in ~20% of cases disease relapse proceeds in the absence of obvious changes in BCR-ABL function. Clearly, the limited availability of material from patients that develop this type of BCR-ABL-independent resistance to Imatinib represents a significant obstacle for the biochemical identification and investigation of resistance mechanisms in primary CML cells. To obtain initial information as to the general features characteristic of Imatinib resistance in CML cells with normal BCR-ABL function we and others have used cell lines that evolved Imatinib resistance in vitro to characterize the lesions responsible for drug resistance (15-17). We report here on a cell clone that acquired resistance through an unexpected constitutive activation of the Ser/Thr kinase Raf, leading to the Imatinib-resistant tonic activation of the Erk-pathway.

Materials and methods

Cell culture and inhibitors. KCL22-S and -R cells were obtained from Junia Melo (Molecular Haematology, Imperial College London) and grown in RPMI (Pan Biotech GmbH) with 10% FCS (Biochrom) in a 5% CO2 atmosphere at 37°C. Cells were reseeded twice a week. Sensitive and resistant clones were isolated from the parental cell line KCL22 using methylcellulose as described (15). The Imatinib resistant cell line KCL22-R+ grows in the presence of 1 µM Imatinib. Imatinib (kindly provided by Novartis) was dissolved in sterile DMSO as a 10 mM stock solution and diluted to a working concentration of 100 µM with sterile PBS. CI-1040, also named PD184352, was kindly provided by Pfizer and a 1 mM working solution was prepared in sterile DMSO. PI3K inhibitors LY294002 (Cell Signaling) and Wortmannin (Upstate) were dissolved in DMSO at 50 and 10 mM concentrations, respectively. A 100-µM Stock in DMSO was used for the mTOR inhibitor Rapamycin (LC Laboratories). Stock solutions were stored at -20°C.

Antibodies. Phospho-Erk (Cell Signaling; no. 9101, Thr202/204), phospho-Mek (Cell Signaling; no. 9154, Ser271/221), pan-Erk (BD Biosciences; no. 610152), phospho-c-ABL (Cell Signaling; no. 2865, Tyr412), BRAF (Santa Cruz; no. sc-6662), C-19), CRAF (BD; no. 610152), β-actin (Sigma; no. A2066), pan-Ras (Calbiochem; no. Ab-4).

Recombinant proteins. Recombinant GST-c-Raf-RBD protein for the analysis of cellular Ras-GTP levels was purified from E. coli as described before (18). Recombinant GST-Mek and GST-Erk-His proteins (expression plasmids kindly provided by R. Marais, London, UK) were expressed and purified in E. coli according to published protocols (19). Protein concentration was determined by the method of Bradford and quality of isolated proteins was analysed by SDS-PAGE and Coomassie staining. MBP protein was purchased from Sigma, Taufkirchen, Germany.

Cell proliferation experiments. Cells were seeded at 5x10^4 cells/ml into 12-well plates and cell proliferation was assayed in the presence of 1 µM Imatinib, 0.1 µM CI-1040, 0.05-0.1 nM Rapamycin, 5-20 µM LY294002 and 100 nM Wortmannin. Media and inhibitors were refreshed daily. Cell growth was measured daily by counting cell number with the CASY device (Model TTC, Schärfe Systems) or by measure-ment of ATP using the ATPlite Luminescence Assay system (Perkin-Elmer). For CASY analysis 50 µl cell suspension was diluted into 10 ml CASY buffer (CASYton) shortly before analysis. Live cells scored 9.90-24.99-µM, while the whole cell score was between 7.50 and 24.99 µM. ATP measurement on 100 µl cell suspension in RPMI medium was performed as described by Hipler et al (20) in triplicates.

Measurement of apoptosis. Apoptosis was examined with the Apo-One Homogeneous Caspase-3/7 assay kit (Promega) after 24-48 h of treatment with 1 µM Imatinib, 0.5 µM CI-1040, 20-50 µM LY294002, 20 nM Rapamycin and 100 nM Wortmannin. Cells were harvested by centrifugation and resuspended in 100 µl PBS. Suspension (20 µl) was mixed with 20 µl Apo-ONE assay solution and incubated for 2-4 h in the dark. Fluorescence was measured with a SpectraMAX Gemini spectrometer (Molecular Devices).

RBD pulldown assay. Analysis of Ras-GTP levels was performed essentially as described (19). Briefly, cells were deprived of serum overnight and reseeded in medium supplemented with 0.5 or 10% FCS at a cell concentration of 1x10^6 cells/ml. Cell suspensions were treated with inhibitors or corresponding carrier molecules for 20 h and cells were harvested by a quick centrifugation. Cell pellets were lysed in ice cold lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM MgCl2, 1 mM EGTA, 1% NP-40) and protease/phosphatase inhibitors supplemented with 25 µg GST-c-Raf-RBD protein/ml and 100 µM GDP. GDP and GST-RBD were included at this point of the procedure to quench post-lytic GTP loading and GAP-dependent Ras-bound GTP hydrolysis, respectively. Lysates were cleared by centrifugation and GST-RBD/Ras-GTP complexes were collected on glutathione-sepharose. Precipitates were washed once with lysis buffer and resolved by SDS-PAGE.

BRAF and CRAF kinase-coupled activity assay. In these assays, immunoprecipitated B-Raf or c-Raf was used to sequentially activate the MAPK cascade constituents MEK1 and
Erk2. The final readout of this assay (the phosphorylation of MBP by Erk) therefore only reflects phosphorylation events on MEK1, that are productive in terms of Erk kinase activity, and which are highly specific for Raf activity. Assays for B-Raf and c-Raf were performed as described by Alessi et al (19). Briefly, cells were treated with 0.1-10 µM Imatinib or vehicle for 20 h, harvested and washed with PBS. Cells were lysed with 20 mM Tris, pH 7.5, 0.27 M sucrose, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100 and protease/phosphatase inhibitors. Anti-B-Raf or c-Raf antibodies (1 µg) was added to the cell lysates and extracts were incubated in the cold for 1 h with mixing (21). Protein-G-Sepharose bead suspension (40 µl) was added to each vial and reactions were incubated at 4°C in a head-to-head mixer for 2 h. Precipitates were washed with lysis buffer supplemented with 0.6 M NaCl. After washing, recombinant Mek and Erk proteins were added to the precipitates and incubation was continued for 20-30 min at 30°C. Part of this kinase reaction mix (5 µl) was added to 45 µl MBP buffer [25 mM Tris, pH 7.5, 0.1 mM EGTA, MBP (0.33 mg/ml), 10 mM Mg acetate, 0.1 mM [γ32P]-ATP] and incubated for 10 min at 30°C. Reactions were terminated by boiling with SDS loading buffer. Samples were separated on a SDS gel overnight, dried and phosphorylated MBP bands were visualized by autoradiography.

**Determination of relative expression levels of B-Raf and c-Raf using quantitative PCR.** Total RNA was isolated using TRI Reagent (Sigma) according to the manufacturer's instructions. Total RNA (2 µg) was used for cDNA synthesis with a mixture of random/oligo-dT primers and the SuperscriptTM First Strand Synthesis system (Invitrogen). Real-time PCR for relative quantification of B-Raf mRNA levels was performed using LightCycler instrument (22) and Quantitect SYBR-Green Kit (Qiagen). Amplification of β-actin as an endogenous control was used to standardize the amount of sample added to the reactions (Table I, for primer sequences).

**Sequencing of B-Raf and c-Raf.** BRAF exons 11 and 15 are known hot-spot regions for mutations in various cancer types (23). For c-Raf no missense mutations associated with constitutive, pathophysiological activation have been described to date. Because of the similarity of protein domains between the two kinases CRAF exons 9/10-14 were also screened. cDNA was amplified using oligos as reported by Rimoldi (24) and Kumagai (25). PCR products were purified using the DNA extraction kit (Fermentas) according to manufacturer's instructions. PCR products were cloned in the pJet1/blunt vector using GeneJet™ PCR cloning kit and transformed to E. coli using TransformAid™ bacterial transformation kit (both Fermentas). Plasmids were isolated according to standard lab methods. Sequencing reaction was performed using pJet infrared-labeled forward (5’-gcctgaacaccatatccatcc-3’) and reverse (5’-gcagctgagaatattgtaggagatc-3) oligos and SequiTherm Excel™II DNA sequencing kit (Epicentre Technologies) with an PTC200 thermocycler (MJ Research, Biozym). Sequencing protocol in brief: initial denaturation at 94°C for 2 min followed by 30 cycles of 94°C for 30 sec, 50°C for 15 sec and 72°C for 60 sec. Sequencing products were separated and analyzed on an automated fluorescence-based DNA sequencer (Li-Cor, Nebraska).

**Results**

**MEK inhibitor CI-1040 blocks the growth of Imatinib-resistant cell line.** The parental BCR-ABL-positive CML cell line KCL22 is resistant to Imatinib but a few sensitive clones were isolated by Mahon and coworkers (15). We have previously characterized the karyotype of an Imatinib-resistant clone derived from KCL22, which we termed KCL22-R (16). As shown in Fig. 1A proliferation rates of KCL22-R are almost fully unperturbed by inclusion of 1 µM Imatinib in the growth medium. Sequencing of BCR-ABL evidenced that resistance was not due to missense mutations in the kinase sequence (data not shown). Accordingly, BCR-ABL autophosphorylation in the resistant KCL22-R cell line exhibited a similar sensitivity to Imatinib as in the parental cell line KCL22-S (Fig. 1B). To investigate which pathway enabled BCR-ABL-independent growth in KCL22-R, we employed pharmacological inhibitors of the Erk and PI3K pathways, two major signalling paths engaged by BCR-ABL (9). Whereas proliferation of KCL22-R, as assayed by automated cell counting, was virtually not affected by PI3K inhibition with LY294002 (data not shown), blockade of the Erk pathway with the MEK inhibitor CI-1040 markedly stalled proliferation if administered in conjunction with Imatinib (Fig. IC). Similar results were obtained using a second proliferation assay based on ATP quantification (data not shown).

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<th>Table I. List of primers used for mRNA quantification.</th>
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<td>Gene</td>
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Erk activity confers Imatinib-resistant proliferative potential rather than protection against apoptosis. Since Imatinib acts largely by reverting the anti-apoptotic effects of BCR-ABL and since aberrant Erk activity can protect against apoptosis in other contexts of oncogenic transformation (26), we investigated whether or not Erk pathway activity protected the resistant KCL22-R cells against apoptosis induction following Imatinib treatment. As shown in Fig. 2, Imatinib administration induced marked apoptosis rates in sensitive cells, but not in the resistant KCL22-R line. Remarkably, the ability of resistant KCL22-R cells to evade Imatinib-induced apoptosis was not contingent on the activity of the MEK/Erk pathway, since CI-1040 did not restore Imatinib-driven apoptosis in KCL22-R. Taken together, these data evidenced that Erk pathway activity contributed to the acquisition of Imatinib resistance by ensuring Imatinib-resistant proliferation rather than by ensuring cell survival in a background of BCR-ABL inhibition.

Erk activity is insensitive to Imatinib treatment in KCL22-R. The data reported above suggested that Erk activity had gained independence from the BCR-ABL input in the resistant KCL22-R cells. To prove this assert, we tested the activity status of Erk in dependence of Imatinib administration. Basal Erk activity was higher in serum-fed KCL22-R cells compared to the Imatinib-sensitive cell line (Fig. 3). Remarkably, whereas Imatinib treatment fully inhibited Erk activity in the sensitive cell line, Erk activity was not affected by BCR-ABL inhibition in KCL22-R cells. Erk phosphorylation/activation was 100-fold less sensitive to Imatinib in KCL22-R cells than in the parental cells (Fig. 3). Taken together with the data shown above, these findings suggested that a lesion other than missense mutations in BCR-ABL had rendered the flux...
Ras activity remains sensitive to Imatinib in KCL22-R cells. To determine the exact point at which the Ras/Raf/MEK/Erk pathway had garnered independency from a BCR-ABL-input we systematically measured the activity of pathway constituents. Activation of the three prototypical Ras G-proteins K-Ras, N-Ras and H-Ras (collectively Ras) represents the entry point into the Erk pathway. Immunodetection with isoform-selective antibodies detected the presence of K-Ras and N-Ras in KCL22 cells while H-Ras was not detectable in KCL22-extracts (data not shown), an expression profile reminiscent of other leukaemia cell lines (26). In order to assess activity levels of Ras we affinity-purified active Ras-GTP complexes from cell extracts. Ras-GTP complexes were readily detected in serum-fed cultures (Fig. 4). In line with the prevailing concept, BCR-ABL inhibition blocked Ras-GTP accumulation in KCL22 S and R cells irrespective of the serum concentration in the medium (27). Importantly, Ras-GTP accumulation in the Imatinib-resistant clone KCL22-R and its sensitivity to Imatinib was essentially indistinguishable from the situation in the sensitive cells, indicating that Ras-activation was still driven by BCR-ABL activity in KCL22-R cells.

c-Raf activity is insensitive to Imatinib in KCL22-R cells. Ras activates the MAP kinase kinase kinase (MAPKKK) Raf in order to propagate the signal towards Erk. The two Raf isoforms B-Raf and c-Raf (collectively referred to as Raf) are ubiquitously expressed and carry most of the cellular MAPKKK activity in detriment of the third member A-Raf (28). To investigate if Raf activity was sensitive to Imatinib we carried out immunoprecipitation-based kinase-coupled Raf activity assays (see experimental section). C-Raf kinase activity in KCL22S was highly sensitive to Imatinib (Fig. 5A) with its dose-dependency fully matching the dose-response of BCR-ABL autophosphorylation and Erk phosphorylation (Figs. 1B and 3). A virtually identical response pattern was obtained for B-Raf (data not shown). Strikingly, both c-Raf and B-Raf activity was not sensitive to Imatinib in the resistant cells, although B-Raf activity data were harder to interpret owing to the very high basal activity of this isoform, a common, previously often appreciated caveat in B-Raf investigations (19,28). To explain whether or not the acquisition of Imatinib-insensitivity was a result of mutations in the Raf sequence, we sequenced all exons in B-Raf (and their counterparts in c-Raf) harbouring hot-spots of oncogenic missense mutations in human cancer (25). Sequencing evidenced that the coding sequence of both Raf genes was intact in KCL22R (data not shown) indicating that B-Raf and c-Raf were not mutated.

To understand if changes in expression intensity contributed to the resistance phenotype we measured c-Raf and B-Raf mRNA levels. Real-time PCR analysis evidenced hardly any differences in the expression of either Raf gene message compared to the reference gene β-actin in sensitive and resistant cells. Also, similar levels of c-Raf and B-Raf protein detected in control Western blots of Raf-immunoprecipitates from KCL22R and KCL22S (Fig. 5A and B) corroborated this conclusion.

Taken together, these findings illustrate that C-Raf and/or B-Raf activity had become independent of BCR-ABL. Since Ras-GTP loading was strictly dependent on BCR-ABL activity in KCL22R cells (Fig. 4), this notion further implied that Raf activity had also become independent from a Ras-GTP input in the resistant cells.
Discussion

Disease relapse in CML after sustained Imatinib therapy often involves mutations in the kinase domain of BCR-ABL that compromise binding of the drug. Several of these mutations have been mapped and a number of second-line drugs obtained by rational derivatization of Imatinib have been developed to target many of these altered BCR-ABL mutants. However, there is currently little prospect on how to treat those 20% or so cases of CML disease relapse that do not feature functional alterations in BCR-ABL and in which insensitivity towards Imatinib is caused by as yet not understood mechanisms. Our finding that the BCR-ABL-independent constitutive activation of Raf kinases can render growth of CML cells insensitive to Imatinib represents a novel mechanism of resistance acquisition that may open up new avenues of investigation for the therapy of relapsed CML. The fact that aberrant activation of a component of the Ras/Erk pathway underlies resistance to Imatinib in CML is not that surprising as such, considering the ever increasing body of experimental data underpinning the critical role of Ras and Ras downstream signalling in BCR-ABL-driven tumorogenesis (27,29). In fact, the synergistic effect of Imatinib and CI-1040 or other inhibitors of the Erk-pathway on the growth of CML-cells, as documented in the present report, is not unprecedented (30,31). It is, however, unexpected and surprising that the gain of Imatinib-resistance in Raf activity reported here does not result from missense mutations or amplification of either Ras or Raf genes, but rather seems to arise from an altered functional regulation of Raf kinase activity. Regulation of the c-Raf and B-Raf kinase activity is complex and our knowledge about the Raf activation process has experienced several major twists in recent times. New findings indicate that the activity of both kinases cannot be considered isolated from each other since B-Raf and c-Raf form homodimers and heterodimers of distinct composition with each other (and with other related Ser/Thr kinases like Ksr) (32,33). Ras is also found in these c-Raf/B-Raf multiprotein complexes along with numerous other constituents such as heat shock proteins and scaffolding proteins (34). These and additional studies have documented that, as a particular and so far unique feature of Ras/c-Raf/B-Raf multiprotein complexes, the inhibition of either Raf kinase isoform with specific inhibitors may result in the antidromic activation of the dimerized partner kinase. It is assumed that the often controversial and paradox effects obtained with Raf inhibitors in clinical trials reflect the particular nature of Ras/Raf multiprotein complexes in the particular tumor entity which can lead to a unique and apparently irrational response to the drug. In the KCL22 CML cells investigated here, both c-Raf and B-Raf as well as Ras genes harbour no activating mutations. Using cross-co-immunoprecipitation of native B-Raf vs. c-Raf we have detected the presence of B-Raf/c-Raf heterodimers in these cells (data not shown), as reported previously for numerous other cell types (35). Owing to this complex regulation and to the existence of B-Raf/c-Raf complexes in KCL22 cells it is difficult to judge which of the Raf kinases is responsible for the Imatinib-independent MEK-phosphorylating activity that we detect in our experiments, since MEK-phosphorylating activity recovered e.g. from c-Raf immunoprecipitates could conceivably arise from co-precipitated B-Raf kinase, and vice versa. To address this issue, we tried siRNA-mediated knock down of B-Raf and C-Raf but these experiments did not yield conclusive results owing to the reduced transfection efficiency of the suspension cell line KCL22. The acquisition of Imatinib resistance could also result from functional alterations in additional constituents of the Ras/Raf multiprotein complex. For example, hsp70, a known constituent of Raf-multiprotein complexes (36), is markedly upregulated in KCL22R cells (our unpublished observations). We are currently investigating whether or not this phenomenon is in some way linked to the reported gain in BCR-ABL-independent Raf activity in KCL22R cells.

It is worth to note at this point, that inhibition of the Erk-pathway with the MEK inhibitor CI-1040 does only effectively suppress the growth of CML cells if administered in combination with Imatinib (Fig. 1C), indicating that one or more BCR-ABL-dependent pathways other than the Ras/Erk cascade do contribute and sustain uncontrolled growth in these cells, even in a background of Erk pathway inhibition. Since Ras activation remained sensitive to Imatinib in KCL22-R cells, this pathway cannot lie downstream of Ras and must therefore bifurcate from the Ras/Erk branch at a level most proximal to BCR-ABL. We are currently devising an unbiased experimental approach to identify the pathway(s) addressed by BCR-ABL that are responsible for this effect.

It remains to be seen whether the C-Raf-mediated Imatinib resistance occurs also in CML patients. However, investigation of patient material can be very arduous because of the multiplicity of Imatinib resistance mechanisms, differences in the course of disease progression and medication as well as intra-individual differences in protein expression patterns.

In conclusion, the presented findings reveal a novel acquired mechanism of Imatinib resistance in CML cells based on an enigmatic, as yet not attributable constitutive activation of Raf. Beyond this appreciation, our findings highlight the importance of conducting biochemical signal transduction analysis in cases of acquired drug resistance that are not readily attributed to common characterized genetic lesions.

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References