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Mechanisms of transformation by the *BCR-ABL* oncogene: new perspectives in the post-imatinib era

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Abstract

Since its introduction less than 3 years ago, imatinib mesylate (STI571) has altered the entire approach to the therapy of patients with chronic myeloid leukemia (CML). In addition to its impact on clinical practice, imatinib has also increased the focus of basic and translational CML research on enhancing the cellular effects of imatinib and preventing and overcoming resistance to the drug. Here, I summarize some recent advances in our understanding of the regulatory and signaling mechanisms of Bcr-Abl, with an emphasis on therapeutic implications.

Keywords: Oncogene; Imatinib mesylate; Chronic myelogenous leukemia; Gleevec; Tyronsine kinase

Because of its discovery over 15 years ago and the early recognition of its role in human cancer, *BCR-ABL* is one of the most highly studied oncogenes. Yet, there are still many gaps in our knowledge. For instance, we do not understand the precise basis for the selectivity of imatinib for those tyrosine kinases (Abl, c-Kit, PDGFβ and α receptors) that it targets, nor do we have an appreciation of the exact molecular pathways thorough which Bcr-Abl induces myeloproliferative disease. In this review, I will focus on two areas of Bcr-Abl regulation and signaling that have implications for the clinical use of imatinib. First, recent studies of Bcr-Abl have shown that, like c-Abl, Bcr-Abl is autoinhibited via its SH3 domain. This form of the enzyme is uniquely sensitive to imatinib and mutations that disrupt the autoinhibited conformation may contribute to imatinib resistance. Second, while Bcr-Abl activates a myriad of signaling pathways in cultured cells, only a subset of these pathways is likely to be critical for initiating and maintaining leukemia in patients. Defining these pathways is important because they may provide additional molecular targets for therapy of chronic myeloid leukemia (CML), and animal models of CML can play an essential role in identifying and validating such targets. As an example, I will summarize recent studies of the signaling pathway downstream of Tyr177 in Bcr-Abl.

1. The sensitivity of Abl to imatinib is dictated by the regulatory conformation of the enzyme

To appreciate the dynamics of inhibition of Abl by imatinib it is necessary to understand the regulatory mechanisms governing the catalytic activity of the different forms of the enzyme. It is instructive to consider first the normal c-Abl protein, which has two isoforms (types Ia and Ib) that result from expression of two small alternative first exons. Type Ib c-Abl contains a C_{14} myristoyl fatty acid moiety covalently linked to the N-terminus and is expressed at higher levels than type Ia, which is not myristoylated. In the inactive state, the c-Abl SH3 domain binds intramolecularly to a single proline (Pro242 in type Ib c-Abl) in the linker region between the SH2 and catalytic domains (SH2-CD linker), a regulatory mechanism shared with Src family kinases. Mutations in SH3 that block ligand binding or mutation of the Pro242 SH3-binding site dysregulate c-Abl and increase its catalytic activity in vivo and in vitro [1–3]. Enzymological studies of purified c-Abl have shown that Abl can undergo intermolecular autophosphorylation at two distinct regulatory tyrosines, Tyr412 and Tyr245 [4]. Phosphorylation of Tyr412 in the kinase domain activation loop stimulates Abl kinase activity and induces rapid phosphorylation at Tyr245, which displaces the SH3 domain from the adjacent Pro242 site and results in a highly active enzyme with activity equivalent to SH3-mutated c-Abl. Activation of c-Abl in response to physiological stimuli such as oxidative stress or growth factors may involve auto- or trans-phosphorylation of Tyr412,

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for example by a Src family kinase [5], followed by loss of self-inhibition by the SH3 domain.

The relevance of Abl regulation to the pharmacology of imatinib has been revealed recently through a series of structural and enzymological studies. The original crystal structure of Abl complexed with a homologue of imatinib included only the Abl catalytic domain [6]. In this structure, the configuration of the activation loop and Tyr412 (named Tyr393 in this paper, which used the c-Abl Ia numbering) resembled that of inactive Src kinases [7,8], with Tyr412 unphosphorylated and lying in the catalytic cleft in the position of a pseudosubstrate. In most tyrosine kinases, phosphorylation of the activation loop tyrosine induces a conformational change of the loop and reorients this residue to point outside the catalytic cleft. While there is as yet no structure of the Abl catalytic domain in the phosphorylated state, extrapolation from the structure of activated Lck [9] suggested that the conformation of activated Abl would be unfavorable for binding imatinib.

In agreement with this structural prediction, phosphorylation of Tyr412 in the isolated Abl catalytic domain by the Src kinase Hck or autophosphorylation of purified c-Abl results in relative resistance to imatinib [6,10]. In the case of purified c-Abl, autophosphorylation increases the IC_{50} for imatinib 5.4-fold from 1.56 to 8.51 μM (Fig. 1). Interestingly, enzymatic studies of the isolated Abl catalytic domain in the unphosphorylated state [6,11] consistently find a much lower IC_{50} value for imatinib, around 25–34 nM. This difference is likely to be real, and probably reflects a more closed conformation of the Abl catalytic domain in the context of the full-length autoinhibited protein that impairs drug binding [12]. Indeed, disruption of the inhibited conformation of c-Abl by SH3 mutation [10] or addition of phosphopeptide ligands for the Abl SH2 domain [13] significantly increases the sensitivity of Abl to imatinib (Fig. 1).

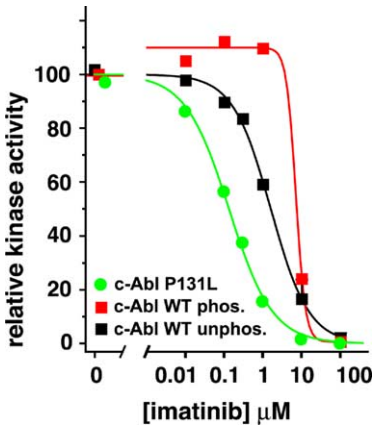

Fig. 1. The sensitivity of Abl to imatinib is influenced by the regulatory state of the enzyme. Plot of enzyme activity versus imatinib concentration for purified c-Abl wild-type (WT) enzyme in the unphosphorylated state (black squares) or after autophosphorylation (grey squares), and for unphosphorylated c-Abl with a dysregulating mutation in the SH3 domain (P131L, circles), adapted from [10]. Note that dysregulation of Abl increases and autophosphorylation decreases the sensitivity of the enzyme to imatinib.

The main clinical implication of these observations is that small molecule inhibitors that bind to both the inactive and active conformations of Abl might be superior to imatinib. A recent study of another ATP mimetic tyrosine kinase inhibitor, the pyrido-2,3-d pyrimidine compound PD173955 (Parke–Davis), found that it co-crystallized with the Abl catalytic domain in a conformation resembling the active state, and biochemical data suggest the compound is equally active against the unphosphorylated and phosphoylated Abl kinase [14]. PD173955 has an IC_{50} for Abl that is in the nanomolar range, significantly lower than imatinib, but this likely reflects its basic binding affinity for Abl and is not a consequence of its ability to inhibit both active and inactive conformations of Abl. Nonetheless, such a dual inhibitor could be significantly more effective than imatinib in a clinical setting and the further development of this class of inhibitors is warranted.

2. Mechanism of dysregulation of Abl by fusion of Bcr

While the catalytic activity of c-Abl is tightly controlled within the cell, the Bcr-Abl fusion protein has constitutively high tyrosine kinase activity in vivo and in vitro compared to c-Abl [15]. However, the exact mechanism of dysregulation of Abl kinase activity upon fusion with Bcr has remained unclear despite nearly two decades of intensive research. Relative to c-Abl, the Bcr-Abl fusion protein retains the Abl SH3 domain but lacks Abl first exon sequences and myristoylation and has gained sequences from the N-terminus of Bcr, including *BCR* first exon-derived sequence common to all Bcr-Abl isoforms. Theoretically, dysregulation of Abl could be due to either loss of the Abl first exon or gain of the Bcr first exon polypeptides. In type Ib c-Abl, recent studies demonstrated that sequences N-terminal to the SH3 domain (the N-terminal “cap”) bind to the Abl catalytic domain [16] and are required along with the myristoyl group [13] for proper regulation of Abl kinase activity upon overexpression in vivo. In agreement, a crystallographic study demonstrated a physical interaction of the myristoyl group with the C-terminal lobe of the Abl catalytic domain [17]. Binding of the myristoyl group induces a conformational change that permits the docking of the SH2 domain with the non-catalytic face of the C-lobe in a fashion that closely resembles the inactive conformation of Src [7]. These observations suggest that loss of the myristoly group and the portion of the cap donated by the Abl first exon might contribute to dysregulation of Bcr-Abl. However, the c-Abl Ia isoform and several c-Abl mutants are not constitutively active despite lacking the type Ib cap and myristoyl group, indicating that Abl dysregulation is not an inevitable consequence of deletion of the first exon [18].

On the other side of the Bcr-Abl fusion protein, there is definitive evidence that Bcr contributes directly to Abl

dysregulation. The N-terminus of Bcr contains a region predicted to form an amphipathic α-helix that tetramerizes as a peptide in vitro [19]. In a recent crystal structure of the Bcr coiled-coil domain [20] the monomer forms a left-handed α-helix characterized by a heptad repeat with apolar residues in the first (a) and fourth (d) positions of the repeat. Two monomers associate in an antiparallel dimer that stacks to form a tetramer, consistent with experimental observations. Deletion of the initial 63 amino acids of Bcr or insertion of proline-containing peptides into the α-helical domain produced Bcr-Abl fusion proteins with decreased in vivo tyrosine kinase activity and impaired induction of anchorage-independent growth in Rat1 fibroblasts [19]. By analogy to receptor tyrosine kinases that are dimerized by extracellular ligands [21], a simple model suggested that fusion of Bcr activates the Abl kinase by dimerization-induced intermolecular autophosphorylation. Fibroblast transformation by Bcr-Abl lacking the coiled-coil domain can be restored by replacement with the leucine zipper domain from GCN4 [22] demonstrating that the transforming properties of Bcr-Abl, like c-Abl [23], can be activated by dimerization. Transformation by Bcr-Abl lacking the coiled-coil domain can also be partially restored by deletion of the SH3 domain [24]. However, there was no direct evidence that oligomerization is the critical function of Bcr sequences in transformation and leukemogenesis by Bcr-Abl, and mechanistic roles for autophosphorylation and the SH3 domain in Bcr-Abl catalytic and biological activity have not been defined.

3. The imatinib-sensitive state of Bcr-Abl is monomeric, unphosphorylated, and autoinhibited via the SH3 domain

A recent study has clarified the mechanism of Bcr-Abl regulation by demonstrating that the fusion protein, like c-Abl, is negatively regulated through its SH3 domain [25]. Substitution of alanine for the hydrophobic amino acids at the “a” and “d” positions across the Bcr coiled-coil domain abolishes oligomerization of Bcr-Abl in vivo, and greatly impairs kinase activity in vivo as assessed by phosphotyrosine levels. The low but detectable in vivo tyrosine kinase of monomeric Bcr-Abl may reflect the loss of a portion of the Abl N-terminal cap and the myristoyl group. Transformation of primary bone marrow B-lymphoid cells in vitro and induction of CML-like myeloproliferative disease by Bcr-Abl are likewise greatly impaired by alanine substitution or deletion mutations of the Bcr coiled-coil domain, but restored by an SH3 point mutation (P1013L) that blocks ligand binding or a complementary mutation at the proline in the SH2-CD linker region (P1124 in p210 Bcr-Abl) that is the site of intramolecular SH3-binding as defined in c-Abl. In addition, phosphorylation of tyrosines in the activation loop of the Bcr-Abl catalytic domain (Tyr1294, homologous to Tyr412 in c-Abl) and the SH2-CD linker (Tyr1127, homologous to Tyr245 in c-Abl) is dependent on oligomerization and required for leukemogenesis [25].

Taken together, these observations suggest that an elegant and simple mechanism governs Bcr-Abl catalytic activity (Fig. 2). The results imply that Bcr-Abl can assume an inactive state where the enzyme is monomeric and unphosphorylated, with the SH3 domain engaged intramolecularly to Pro1124 in the SH2-CD linker. The sole function of the Bcr coiled-coil domain is to disrupt the autoinhibited conformation through oligomerization and intermolecular autophosphorylation. The primary consequence of oligomerization of Bcr-Abl is autophosphorylation at Tyr1294 in the activation loop of the catalytic domain. As in c-Abl [4], Tyr1294 phosphorylation may lead to secondary phosphorylation events, including phosphorylation of Tyr1127, the homologue of c-Abl Tyr245, which results in displacement of the SH3 domain from the SH2-CD linker. Other phosphorylation sites may contribute to leukemogenesis by Bcr-Abl independent of any role in autoregulation, as is the case with Tyr177, which forms a binding site for the SH2 domain of Grb2 and is required for induction of CML-like leukemia by Bcr-Abl [26]; (see further).

The autoinhibited state of Bcr-Abl described above is very likely to be the form that is highly sensitive to imatinib. The monomeric autoinhibited and oligomerized phosphorylated forms of Bcr-Abl depicted in Fig. 2 are in equilibrium in leukemic cells, and imatinib acts to trap the enzyme in the inactive state. Oligomerization of Bcr-Abl and activation of the enzyme through intermolecular autophosphorylation would force the catalytic domain into the open, activated conformation that is unfavorable for drug binding. This model predicts that mutations in Bcr-Abl that impair its ability to assume the autoinhibited conformation, such as those in the SH3 and SH2-CD linker regions, would tend to drive the enzyme towards the activated state in vivo and could be associated with clinically significant resistance to imatinib. In agreement with this, a recent random, unbiased screen for mutations in Bcr-Abl that confer imatinib resistance recovered mutations in the SH3 domain (including P1013Q) that are predicted to abolish ligand binding and in the SH2-CD linker adjacent to Pro1124 [27], although these mutations have not yet been identified in imatinib-resistant CML patients. In theory, the same class of mutations might contribute to the progression of chronic phase CML to blast crisis by increasing the in vivo kinase activity of Bcr-Abl.

4. Bcr-Abl activates many signaling pathways in cell lines, but not all are likely to be relevant to leukemogenesis

Since its discovery over 15 years ago, Bcr-Abl has been intensively studied in cell lines, including human Ph^+ cell lines such as K562, and other hematopoietic and non-hematopoietic (e.g. fibroblast) cell lines into which the *BCR-ABL* gene has been transferred. Because Bcr-Abl is

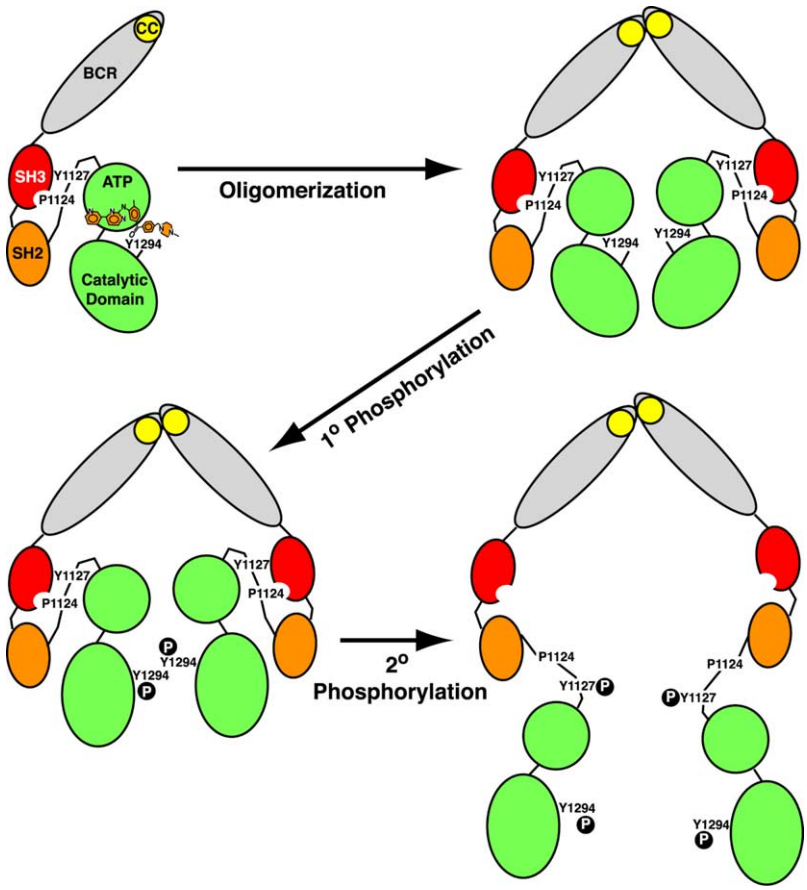

Fig. 2. A model for the regulation of Bcr-Abl. The Bcr sequences, SH3, SH2, and catalytic domains of Abl are depicted. In its autoinhibited state, Bcr-Abl is monomeric, unphosphorylated and the Abl SH3 domain interacts Pro1124 in the SH2-CD linker region. This form of the enzyme binds efficiently to imatinib (shown interacting with the ATP–binding lobe of the Kinase domain). After oligomerization, a primary phosphorylation event at Tyr1294 is rapidly followed by secondary phosphorylation at Tyr1127, which disrupts the SH3-linker interaction and results in full catalytic activity. In the absence of oligomerization via the coiled-coil domain, kinase and transformation activities can be restored by mutations in the SH3 domain that disrupt ligand binding or by complementary mutation of the SH3 ligand in the SH2-CD linker region. For simplicity, other phosphorylation sites including Tyr177 in Bcr are not shown. Adapted from [25].

a constitutively active tyrosine kinase, expression of this fusion protein causes activation of a myriad of signaling pathways within the cell, and this has been the subject of several recent and comprehensive reviews [28–32]. Pathways activated by Bcr-Abl include Ras [33], MAPK [34], JNK/SAPK [35], phosphatidylinositol-3 kinase [36,37], NF-kB [38], and STAT pathways [39–41]. In fact, it is not an exaggeration to say that nearly every known cell signaling pathway has been shown to be affected by Bcr-Abl in one publication or another. Studies with inhibitors and dominant-negative mutants have suggested that several of these pathways contribute to transformation of fibroblasts or hematopoietic cells by Bcr-Abl in vitro [33,37,38,42–45]). However, there is a major concern about how faithfully biochemical results obtained in cell lines, hematopoietic or otherwise, reflect signaling events that are critical in Bcr-Abl-expressing primary stem/progenitor cells. As an example, a recent study of conditional Bcr-Abl expression in embryonic stem (ES) cells undergoing hematopoietic differentiation did not identify activation of the Stat or Akt pathways but instead found downregulation of the p38 MAPK pathway [46].

Why should we care about Bcr-Abl signaling pathways now that we have imatinib, which should block all downstream events? One reason is the emergence of acquired resistance to imatinib in CML patients, something that is observed very frequently in advanced CML and is also seen in up to 7% of chronic phase patients when treatment is initiated at diagnosis [47]. Here, it may be useful to make an analogy to therapy of HIV infection with reverse transcriptase (RT) inhibitors (Fig. 3). Monotherapy of HIV patients with RT inhibitors is associated with initial anti-viral responses in the majority of patients but nearly all patients relapse with resistant infection that is principally due to mutations in the drug target. Superior results are obtained by simultaneously inhibiting RT and another essential step in the viral life cycle such as the viral protease. Like HIV, CML is a dominant, acquired malady of the hematopoietic system where the disease has a high intrinsic frequency of genetic evolution. Also like HIV, a major mechanism of

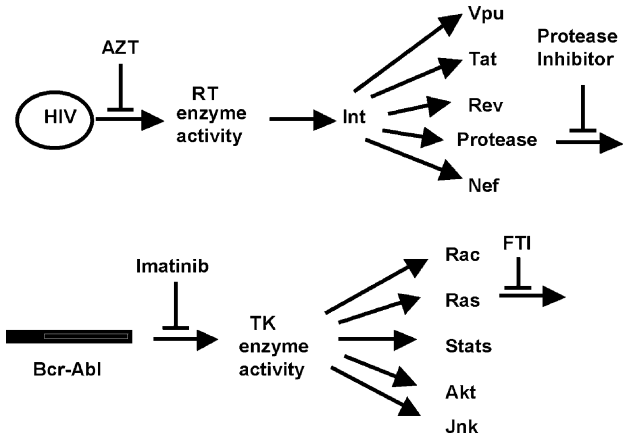

Fig. 3. Combination molecularly targeted drug therapy in HIV infection and CML. Monotherapy of HIV infection with AZT results in clinical resistance due to mutations in the target (RT), and such resistance can be delayed or prevented by simultaneously targeting a critical downstream enzyme, the viral protease. By analogy, imatinib resistance in CML might be prevented or overcome by inhibiting Abl and a critical downstream effector; here the use of a farnesyltransferase inhibitor (FTI) targeting the Ras pathway is depicted.

acquired resistance to targeted CML therapy (imatinib) is mutations in the target, Bcr-Abl [48]. By analogy, imatinib resistance may be prevented, delayed, or overcome by combining imatinib treatment with drugs targeting pathways downstream that are essential to initiating or maintaining the leukemia. In this regard, several recent studies have identified synergy against Bcr-Abl-expressing cell lines between imatinib and drugs targeting the Ras [49] or phosphatidylinositol 3-kinase (PI3K) [50] pathways. In order to optimize this strategy clinically, it is necessary to first identify the pathways that are critical for leukemogenesis. In this regard, animal models of Ph^+ leukemia can play an important role.

5. The Grb2–Gab2 connection: an essential signaling pathway downstream of Bcr Tyr177

A good example of the importance of animal models in identifying and validating molecular targets for therapy of CML downstream of Bcr-Abl is the pathway emanating from Tyr177. This residue is highly tyrosine phosphorylated in the active form of Bcr-Abl as a consequence of autophosphorylation and possibly via trans-phosphorylation by Src kinases such as Fps [51]. Phosphorylated Tyr177 forms a high-affinity binding site for the SH2 domain of the adapter protein Grb2 [52,53]. Mutation of Tyr177 to Phe (Y177F) blocks the ability of Bcr-Abl to bind Grb2 directly, but the biochemical and transforming properties of the Bcr-Abl Y177F mutant are conflicting and do not allow any inference to be drawn about the importance of this binding site to leukemogenesis. There is general agreement that the p210 Y177F mutant is completely defective for transformation of fibroblasts to anchorage-independent growth, and the original report found the Bcr-Abl Y177F mutant unable to stimulate transcription of a Ras-responsive promoter in fibroblasts [52]. This observation suggested that the principal pathway downstream of Grb2 was Ras, presumably mediated by binding of the guanine nucleotide exchange factor Sos by the Grb2 SH3 domain. However, the Bcr-Abl Y177F mutant is capable of transforming IL-3 dependent hematopoietic cell lines such as Ba/F3 and 32D to become independent of IL-3 for survival and proliferation [54,55], and in these hematopoietic cells the Y177F mutant stimulates Ras as efficiently as wild-type (WT) Bcr-Abl [54]. Finally, there were conflicting data on the ability of Bcr-Abl Y177F to transform primary bone marrow B-lymphoid progenitors in vitro, as this mutant was originally reported to be completely defective in this assay [52] but subsequently found to be no different from wild-type Bcr-Abl [55]. Based on these observations, it is difficult to predict the importance of the Tyr177 signaling pathway to CML or identify the downstream effectors involved.

Fortunately, there is an accurate and quantitative mouse model of CML available that employs retroviral transfer of the *BCR-ABL* gene into murine bone marrow followed by transplantation into syngeneic recipients. Recipients of *BCR-ABL*-transduced marrow uniformly develop fatal CML-like myeloproliferative disease characterized by overproduction of maturing myeloid cells and infiltration of spleen, liver, and lungs (for review, see [56]). However, the myeloproliferative disease induced by Bcr-Abl Y177F in this model is greatly attenuated and nonfatal, with recipients succumbing to T-cell lymphoma instead after long latent periods [26,57,58]. These results demonstrate that the Tyr177 signaling pathway plays a major role in the pathogenesis of CML. This conclusion was supported by subsequent studies that employed cell-permeable peptides to block the Grb2 SH3 domain and impair proliferation of primary human CML cells in vitro [59].

Recently, progress has been made in defining the signaling pathway downstream of Grb2 in CML, which is an important effort as Grb2 lacks enzymatic activity and is not a good drug target. Although the main effectors of Grb2 were assumed to be Sos and Ras, phosphorylated Tyr177 also recruits the scaffolding adapter protein Gab2 to Bcr-Abl via a Grb2/Gab2 complex. Ba/F3 cells expressing Bcr-Abl Y177F exhibit markedly reduced Gab2 tyrosine phosphorylation and association of PI3K and the tyrosine phosphatase Shp2 with Gab2 and Bcr-Abl, and display decreased PI3K/Akt and Ras/Erk activation, cell proliferation and spontaneous migration relative to cells expressing wild-type Bcr-Abl [60]. In addition, Bcr-Abl-induced PI3K/Akt and Erk/MAPK activation also are impaired in primary myeloid and lymphoid cells derived from mice with homozygous null mutations in the *Gab2* gene. Bone marrow myeloid progenitors from *Gab2*−/− mice are absolutely resistant to transformation by Bcr-Abl (Fig. 4), while lymphoid transformation is diminished as a consequence of markedly increased apoptosis [60]. These results identify Gab2 and its associated proteins

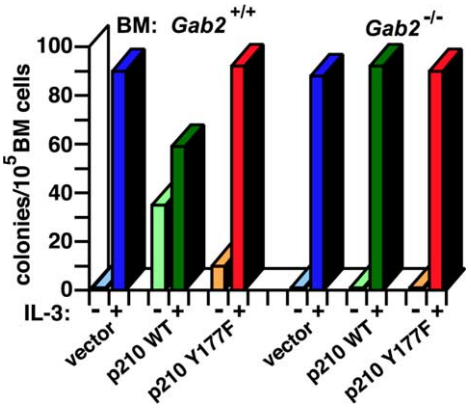

Fig. 4. In vitro transformation of primary bone marrow myeloid progenitors by Bcr-Abl requires Gab2. Bone marrow from mice with homozygous null mutations in Gab2 (right) or wild-type littermates (left) was transduced with empty vector, or with retrovirus expressing p210 Bcr-Abl wild-type (WT) or the Y177F mutant, and myeloid colony formation in methylcellulose assayed in the presence or absence of interleukin-3 (+ or −). p210 Y177F induces significantly fewer IL-3-independent colonies than p210 WT in wild-type BM, and all transformation is abolished in the absence of Gab2. Adapted from [60].

as critical determinants of transformation and leukemogenesis by Bcr-Abl.

There are several clinical implications of these findings. The Gab2 pathway may represent a very good target for drug development in CML, because mice lacking Gab2 are viable, fertile, and have essentially normal baseline hematopoiesis [61]. Therefore, it might be anticipated that inhibition of this pathway would be well-tolerated in patients. Second,

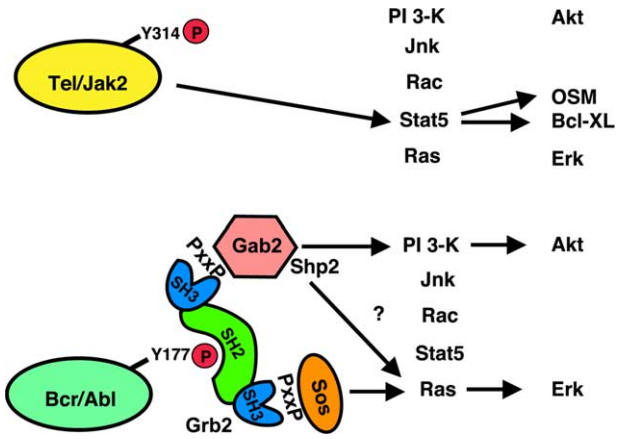

Fig. 5. Distinct leukemogenic signaling pathways utilized by different fusion tyrosine kinases. Schematic depiction of signaling pathways required by the Tel-Jak2 fusion kinase (top) and Bcr-Abl (bottom) to induce myeloproliferative disease in the murine bone marrow transduction/transplantation model system. Leukemogenesis by Tel-Jak2 is completely defective in bone marrow lacking Stat5a and b [63] but does not require Gab2 even though it contains a Grb2 binding site at Tyr314; other proteins downstream on the Stat pathway include Bcl-XL and oncostatin M (OSM). In contrast, Bcr-Abl does not absolutely require Stat5 for leukemogenesis [62] but does depend on Tyr177 and Gab2 [60], with potential downstream effectors PI3K and Shp2.

Bcr-Abl transformation and leukemogenesis requires Gab2 [60] but not the transcription factor Stat5 [62]. In contrast, the myeloproliferative disease induced in mice by the Tel-Jak2 fusion kinase depends absolutely on Stat5 [63]. Thus, studies that employ animal models of leukemia suggest that molecular therapies will need to be individually tailored to each disease, because oncogenic tyrosine kinases may utilize different pathways to induce phenotypically similar leukemias (Fig. 5).

6. Summary

The past several years have been marked by extraordinarily rapid progress in the biology and treatment of CML. Imatinib is now the paradigm for molecularly targeted cancer therapy. In reality, imatinib and similar drugs are both therapeutic agents and valuable tools for understanding the molecular pathogenesis of CML. The challenge for the future is to improve upon current clinical results with kinase inhibitor therapy in CML and develop treatment strategies that result in eradication and cure of this leukemia. In addition, CML is perhaps the best understood human malignancy, and the lessons learned should be valuable in developing molecularly targeted drugs for the therapy of other leukemias and solid tumors.

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