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ABL: MECHANISMS OF REGULATION AND ACTIVATION

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1. ABSTRACT

The Abl non-receptor tyrosine kinase has been implicated in a wide variety of cellular processes, yet its function and regulation remain poorly understood. Abl has resisted complete understanding not due to lack of interest, but due to the complexity of its overall structure and the corresponding complexity and diversity of its biological activities in the cell. Although Abl consists of many familiar modules with well-understood activities, the ways in which these modules interact are manifold and defy simple categorization. A picture now emerges in which Abl can be potentially regulated in many ways: by phosphorylation, by intramolecular interaction, by interaction with a variety of other proteins, by subcellular localization. Far from being a simple on-off switch, it appears that Abl is better understood as existing in a complex and dynamic equilibrium of states, an equilibrium that can be affected by many signaling inputs. In this review we will discuss the various ways in which the kinase activity of Abl can be regulated; other recent reviews have discussed the larger issue of possible biological roles of Abl (1-3).

2. INTRODUCTION

Abl is a nonreceptor tyrosine kinase that closely resembles the Src family of kinases in its N-terminal region (Figure 1). This portion contains three distinct functional domains: (1) a tyrosine kinase catalytic domain (the Src Homology domain 1, or SH1), (2) a domain that binds tyrosine-phosphorylated peptides (SH2), and (3) a domain that binds to proline-rich peptides (SH3). Like Src, some forms of Abl are also myristoylated at the N-terminus. What defines Abl and its close relative Arg, however, is a long unique C-terminal extension that contains a bewildering array of functionalities. Immediately following the catalytic domain is a proline-rich region that binds the SH3 domains of a variety of adaptor proteins, including Crk, Grb2, and Nck (4,5). Also in the C-terminus are binding regions for the DNA damage response kinase ATM (6), the tumor suppressors p53 (7) and Rb (8), RNA polymerase II carboxy terminal domain (9,10), DNA (11), and both F- and G-actin(12,13). In addition, there are three nuclear localization signals (14) as well as a single nuclear export signal (15).

Abl first came to our attention in the guise of v-Abl, the virally encoded oncogene of Abelson murine leukemia virus (16). Abl was subsequently implicated in human cancer through two naturally occurring oncogenic forms generated by chromosomal translocation: (1) a fusion protein with Bcr (called Bcr-Abl) which is found in >95% of chronic myelogenous leukemias and 10% of acute lymphocytic leukemias (17,18), and (2) a fusion protein with Tel (called Tel-Abl) which has been rarely observed in some forms of leukemia (19). c-Abl, the unmutated endogenous form of Abl, is ubiquitously expressed (20-22). It comes in two forms due to two alternative 5' exons, Type I and Type IV for murine c-Abl and type Ia and Ib for

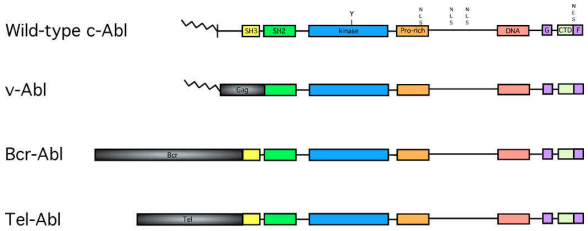

Figure 1. Schematic representation of the domain structure of c-Abl and its oncogenic variants, v-Abl, Bcr-Abl, and Tel-Abl. *SH*, Src homology domains 2 and 3; *DNA*, DNA binding domain; *G*, G-actin binding region; *CTD*, RNA Polymerase II Carboxy Terminal Domain binding region; *F*, F-actin binding region; *zigzag line*, myristoyl group found on Type IV and Type 1b isoforms; *Y*, Tyr-412, the main site of autophosphorylation; *NLS*, nuclear localization signal; *NES*, nuclear export signal.

human c-Abl, the main difference being that Type IV/Type 1b is N-myristoylated and therefore associated with membranes. In vertebrates, there is a single close relative of c-Abl called Arg (Abl-related gene); Arg is very similar to c-Abl at its N-terminus (94% kinase domain, 90% SH2 & SH3 domain), but is quite divergent in its C-terminus (29% overall, but 56% in the last 60 amino acids, and functional domains such as SH3-binding and NLS sequences are conserved)(23).

Early studies focused on the differences between wild-type c-Abl and its oncogenic counterparts. Expression of mutated, oncogenic forms such as v-Abl led to high levels of tyrosine phosphorylation both of Abl itself and of other cell proteins, and to transformation. In contrast, when wild-type c-Abl was overexpressed in mammalian cells not only was transformation not observed, but there was also no increase in phosphotyrosine, suggesting that the activity of c-Abl was tightly regulated ((24,25) ; for review, see (1)). Simply stated, this is the fundamental issue of Abl regulation: what is the mechanism that normally holds the activity of c-Abl in check, and how is it activated in response to normal signals, or in diseases such as leukemia?

3. ABL KINASE DOMAIN

Before discussing the Abl kinase domain, it is worth considering general models of kinase regulation. There are over 500 identified protein kinases (and doubtless many more yet to be identified) and a variety of activation mechanisms (26). These include binding of another molecule (i.e. second messenger, other protein subunits), dissociation from an inhibitor, or phosphorylation/ dephosphorylation by kinases/phosphatases. For many kinases, these activation events are associated with a common outcome – the phosphorylation of a residue on a mobile segment near the catalytic cleft termed the "activation segment" (27). Structural and biochemical data suggest that kinase activation involves a two-step process – (1) a structural transition from a "closed" to "open" conformation, involving rearrangement of structural elements important for substrate binding and catalysis, followed by (2) phosphorylation of the activation segment, which stabilizes the open conformation (28). The first step must occur spontaneously at some basal level, so that the opened and closed states are in an equilibrium that normally favors the closed conformation. However, once the second step occurs, the equilibrium shifts to favor the open conformation, resulting in full activation of the kinase. Mechanisms that maintain the inactive structure must therefore stabilize the closed conformation and/or prevent phosphorylation of the regulatory site.

The X-ray crystal structure of the kinase domain of Abl bound to the inhibitor STI-571 was recently solved (29). In general, the structure of the Abl kinase domain is very similar to those of other protein tyrosine kinases (Figure 2a). In its inactive state, the activation segment is unphosphorylated and blocks the mouth of the catalytic domain, preventing substrate binding. This conformation is held in place by a number of interactions, including a key hydrogen bond between the sole phosphorylatable activation loop tyrosine, Tyr-412, and Asp-382, which is a residue critical for ATP hydrolysis (using murine Type IV Abl numbering). The position of the activation loop tyrosine is almost identical to that of the phosphorylatable tyrosine of a bona fide substrate, as seen in the insulin receptor catalytic domain (30); catalysis cannot occur, however, because the highly conserved Glu-Phe-Gly triad of residues required for Mg^{2+} ligation are displaced by the activation loop. Although the structure of Abl in the active conformation has yet to be solved, to date all activated tyrosine kinases are quite similar in that the phosphorylated activation segment is swung out from the catalytic site, serving as a platform for substrate binding and allowing the catalytic residues to assume an arrangement that allows productive binding of ATP and Mg^{2+} (Figure 2b).

A particularly interesting and informative aspect of the Abl-STI-571 structure is that binding of the inhibitor is seemingly incompatible with the open, active conformation of the catalytic domain; consistent with this structural prediction, the K_i for the unphosphorylated enzyme is ~200-fold lower than for the tyrosine-phosphorylated active form (29). In light of its efficacy in inhibiting Abl activity both *in vitro* and *in vivo*, this observation seems paradoxical. How can the inhibitor inhibit oncogenic forms of Abl, which are constitutively active, when it greatly favors binding to the *inactive* form of the kinase? One likely possibility is that even in the activated state, the conformation of the catalytic domain is not fixed but instead flips between open and closed forms (most likely when the activation segment site is transiently dephosphorylated). The inhibitor would thus draw the equilibrium toward the inactive form by stably binding to and sequestering it. This illustrates the more general concept that, rather than thinking of Abl activity in black-and-white terms, it is more accurately considered in terms of a dynamic equilibrium between active and inactive forms.

4. SRC- MODEL FOR ABL REGULATION?

Although the crystal structure of the Abl catalytic domain provides important clues regarding Abl regulation,

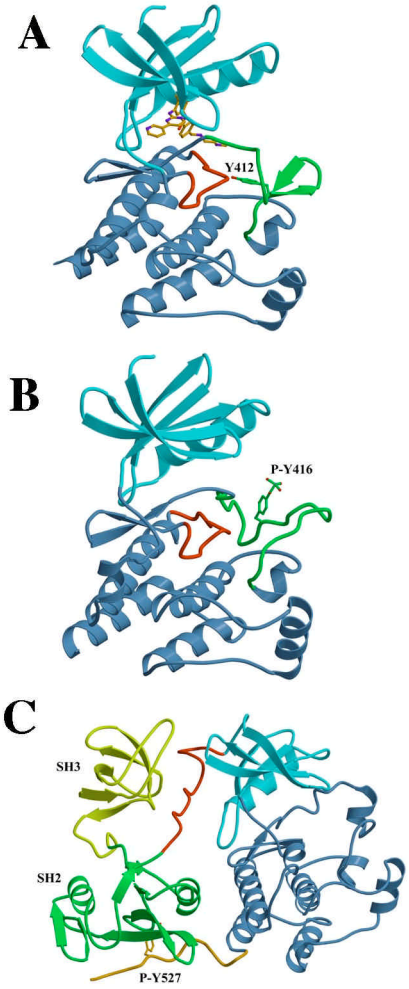

Figure 2. Three-dimensional structure of Abl and related tyrosine kinases. Structures are shown as ribbon diagrams; in all cases the orientation of the catalytic domain is the same, with the N-terminal lobe colored cyan and the C-terminal lobe blue. A. The catalytic domain of Abl in the inhibited, closed conformation, complexed with the small-molecule inhibitor STI-571 (29). The activation segment is depicted in green, and the sidechain of Y412, the major autophosphorylation site, is indicated. The catalytic loop is depicted in red and the inhibitor (bound in the ATP-binding site) is depicted in yellow. B. The catalytic domain of the Src family kinase Lck in the active, autophosphorylated conformation (86). Again the activation segment is depicted in green and the catalytic loop in red. Note that the phosphorylated activation segment tyrosine (P-Y416) has swung away from the catalytic site. C. The structure of Src in the inhibited, closed conformation, including the SH2 and SH2 domains (87). Src is phosphorylated on the regulatory C-terminal site (P-Y527), which binds to the SH2 domain (depicted in green). The SH3 domain (depicted in yellow) interacts with the SH2-linker segment, shown in red.

it does not include other domains believed to be involved in regulating activity and therefore reveals only part of the story. In contrast, the regulation of the Src family of kinases is very well understood on a molecular level due to extensive biochemical and structural studies. Because the domain structure of Src is very similar to that of the N-terminus of Abl, this is a worthwhile starting point in considering Abl regulation. As will be seen, there are both important similarities and fundamental differences when these two related kinases are compared.

The activity of Src family kinases is regulated by a series of intramolecular interactions involving its SH2 and SH3 domains (Figure 2c) (reviewed in (31)). Most critical is an interaction between the SH2 domain and a phosphorylated tyrosine residue in the C-terminus (Tyr-527). This interaction is further stabilized by a second intramolecular interaction, between the SH3 domain and a region between the SH2 and kinase domains (the SH2-linker). These two interactions are responsible for maintaining the closed, inactive conformation of the catalytic domain. Based on this structural information, three distinct mechanisms to activate Src can be envisioned: (1) dephosphorylating the Tyr-527 residue; (2) competing away the interaction between the SH2 and Tyr-527 with exogenous tyrosine-phosphorylated proteins or SH2 domains; or (3) competing away the interaction between the SH3 and SH2-linker with exogenous proline-rich peptides or SH3 domains. A recent study also suggests that the SH2-mediated and SH3-mediated interactions are not totally independent, but are in fact intertwined (32). Molecular dynamics simulations based on Src family crystal structures showed that these two domains are "dynamically coupled" by a relatively rigid linker; in other words, each of the individual interactions helps to stabilize the other interaction, and likewise disruption of one will destabilize the other.

Despite the similarities between Abl and Src in domain structure, one glaring difference is that Abl does not contain a recognizable C-terminal tyrosine residue that corresponds to Tyr-527 in Src. In fact there is no detectable tyrosine phosphorylation of c-Abl under normal conditions (33), and unlike the Src family kinases, deletion of the entire C-terminus or the mutation of the SH2 domain does not activate c-Abl (25,34). Therefore, Abl regulation cannot be identical to Src, and in fact two of the three proposed mechanisms for activating Src cannot apply to Abl.

5. ABL SH3 DOMAIN

There is a wealth of evidence that the Abl SH3 domain plays a critical regulatory role, though the extent to which it is analogous to the intramolecular interaction seen in the Src family kinases remains an area of some contention. The importance of the Abl SH3 domain was first highlighted by comparison of the sequence of the v-Abl oncoprotein with its cellular homolog. The recombination that created v-Abl replaced the entire N-terminus of c-Abl, up to and including the SH3 domain, with a portion of the retroviral Gag protein (16,35). The SH3 was more directly implicated in regulation when c-Abl mutants in which the SH3 domain was either completely deleted or made non-functional by point mutation were

constructed. In each case, the Abl mutant exhibited constitutive activity *in vivo* and was highly transforming (24,25,36). Other mutagenesis studies demonstrated that even when the SH3 is intact, relatively subtle changes in its location relative to other functional domains of the protein were sufficient to relieve inhibition (34).

Consistent with a role for the SH3 in repressing c-Abl activity, proline-rich ligands that engage the SH3 domain have been shown to mediate Abl activation. Examples include c-Cbl (37,38), RFX1 (39), ST5 (40), and c-Jun (41). In the case of c-Cbl, overexpression studies have shown that this substrate is efficiently phosphorylated by wt c-Abl, and that this phosphorylation is dependent on the proline-rich domains of Cbl; a Cbl mutant lacking the proline-rich region cannot be phosphorylated by c-Abl, yet can be efficiently phosphorylated by activated Abl mutants (38). This suggests that the proline-rich region of Cbl can engage the SH3 domain of c-Abl, thus relieving inhibition and leading to kinase activation. The larger implication is that certain substrates, when present in relatively high local concentrations, can play an active role not only in recruiting the kinase but also in activating it--in a sense the substrate can induce its own phosphorylation. Thus it is important to keep in mind that in some situations *in vivo*, "activation" of Abl may be the result of simply relocalizing it to a site with high concentrations of potential substrates containing proline-rich SH3-binding regions.

6. CIS VS. TRANS INHIBITION

In order to understand the inhibitory role of the Abl SH3 domain, a critical issue must first be addressed: Does this inhibition occur in cis or in trans? Does Abl regulate itself (either through an intramolecular interaction or by binding to other Abl molecules), or is there an exogenous inhibitor that binds to the SH3 domain and which regulates catalytic activity? While this question may seem fairly straightforward, the answer remains elusive as different groups (and sometimes the same group!) have generated conflicting evidence.

6.1. Inhibition in trans

The following evidence is often cited in support of a trans-acting inhibitor. First, overexpression of Abl at many times the endogenous level results in Abl activation (42,43), suggesting an inhibitor is titrated away at sufficiently high levels of Abl expression. However, the physiological validity of those observations is open to question, and alternative explanations are possible; for example, activation could be due to increased transphosphorylation of Abl molecules at very high expression levels. Second, there is generally little if any difference in *in vitro* kinase activity between wild-type c-Abl and constitutively active forms, either immunoprecipitated from mammalian cells or purified from insect cells (24,34,36). This is consistent with repression *in vivo* by a putative inhibitor, which dissociates and is washed away during purification or immunoprecipitation. Third, wild-type and SH3-deleted Abl have similar activity *in vivo* when expressed in the fission yeast *Schizosaccharomyces pombe* (which lacks an Abl homolog or any other identifiable tyrosine kinases) (44). In contrast c-Abl is much less active than oncogenic forms when expressed in *Xenopus* embryos (45), suggesting that *S. pombe* does not encode some endogenous inhibitor normally present in vertebrate cells.

6.2. Putative trans inhibitors

Based on these studies, a number of groups have attempted to isolate an Abl inhibitor by the screening of libraries for proteins that specifically bind to Abl. These include the following: 3BP1, 3BP2, Pag, Abi-1, Abi-2, and Aap1.

3BP1 and 3BP2 were isolated by screening a phage expression library with the SH3 domain of Abl (46,47). 3BP1 has regions of homology to the C-terminal segment of Bcr and to Rho-GAP. It was later shown to have specific GAP activity toward Rac-related G proteins, although the relevance of the SH3 domain binding site remains to be addressed (48). 3BP2 shares no significant homology with 3BP1 with the exception of the region that binds to the Abl SH3 domain. 3BP2 is believed to act as an adaptor in T-cells by coupling activated Zap-70/Syk to an LAT-containing signaling complex (49), and dominant mutations of 3BP2 have very recently been implicated in human cherubism (50). On balance these data are more consistent with both 3BP1 and 3BP2 being downstream effectors of Abl rather than trans-acting inhibitors.

The Pag (proliferation associated gene) protein was identified in a two-hybrid screen using the SH3 domain of Abl as a bait (43). Also known as macrophage stress protein 23 kDa (MSP23) and heme-binding protein 23 kDa (HBP23), Pag specifically interacts with the Abl SH3 domain as well as the ATP binding lobe of its kinase domain. In fact, the interaction with the kinase domain is stronger than the SH3-mediated interaction in pulldown experiments. Pag has been shown to associate with Abl and inhibits its kinase activity when overexpressed *in vivo*. Pag is expressed at a relatively high level and a fraction of total Pag is localized to the nucleus, both consistent with the expected properties of a trans-inhibitor of Abl. Several studies have suggested a role for c-Abl following genotoxic and oxidative stress, which is consistent with the redox-sensitive activity of Pag (reviewed in (3,51)). However, a concern with Pag is that it has not yet been shown to efficiently inhibit Abl in the context of mixing purified proteins. This raises the question of whether Pag acts together with yet another cellular factor to inhibit Abl.

Abi-1 and Abi-2 are related members of a family with similarity to homeobox transcription factors, and were identified in separate two-hybrid screens (52,53). Both have an SH3 domain that can bind to the proline-rich region of Abl immediately C-terminal to its kinase domain, as well as proline-rich regions capable of interacting with the Abl SH3 domain. Abi-1 has been shown to suppress v-Abl mediated transformation (52), but since v-Abl does not contain an SH3 domain this is not germane to its potential role as a putative SH3-binding inhibitor. When Abi-2 is expressed in fibroblasts as a carboxy terminal fragment, it can activate c-Abl and render it transforming (53), perhaps

by a dominant negative mechanism. To date neither Abi-1 and Abi-2 has been shown directly to inhibit c-Abl kinase activity *in vitro*. As with 3BP1 and 3BP2, therefore, both are more likely to be effectors of Abl rather than inhibitors.

The AAP1 protein was isolated with the Abl SH3-SH2 domains as bait in a two-hybrid screen (54). Although purified AAP1 decreased the phosphorylation of GST-Crk by Abl *in vitro*, an *in vivo* role has yet to be described.

6.3. Inhibition in cis

While the search for the elusive trans-inhibitor continues, other studies suggest a very different mechanism of inhibition – that Abl, like Src, regulates itself via an intra-molecular interaction. Two independent groups have recently shown that, in contrast to previous studies, significant differences in kinase activity between c-Abl and constitutively active Abl can be seen *in vitro*. One group overexpressed Abl in mammalian cells in the presence of the inhibitor STI1571, purified it by virtue of its His tag, and assayed its activity using a peptide substrate (55); others used a modified *in vitro* kinase assay following immunoprecipitation (H. Pluk and G. Superti-Furga, personal communication). Although neither group can fully account for why they have been able to see a difference between the two forms where others have not, the controversy seems to center on specifics of the overexpression and/or assay conditions. In any case, the fact that there is a measurable difference in activity between the wild-type and constitutively active forms of Abl (in the apparent absence of any co-purifying proteins) indicates that SH3-mediated inhibition can occur in cis.

Superti-Furga and colleagues have proposed a mechanism of cis inhibition closely modeled on the interaction between the SH3 domain and the SH2-linker region first seen in Src family kinases (56). They found that mutation of Pro-242 in the SH2-linker region of Abl resulted in increased activity, suggesting that binding of the SH3 domain to this region was important for regulation. More telling, they found that introduction of a complementary mutation in the SH3 domain (designed to compensate for the mutation in the SH2-linker) was able to reestablish inhibition in the double mutant, providing very strong evidence for an inhibitory interaction between these two regions. Despite these elegant studies, however, the authors were unable to explain and account for all of the evidence suggesting a trans inhibitor, specifically the *in vitro* kinase data as well as the *S.pombe* data. And while these results imply that an SH3/SH2-linker interaction may be necessary for the closed conformation, they do not address whether it is sufficient.

One possible resolution of all the experimental data is that Abl inhibition is actually achieved via *both* cis and trans interactions. Putative trans-acting "inhibitors" may function by stabilizing a weak intramolecular inhibition mediated by interaction of the SH3 domain and the SH2-linker region. This is quite reasonable, considering that Abl cannot take advantage of the SH2-PTyr interaction which plays such an important role in stabilizing the SH3-mediated interaction in the Src family kinases. Of course if this model is accurate, then screens to identify proteins that bind to the traditional ligand-binding site of the Abl SH3 domain would never identify such stabilizing factors; this is because the stabilizing factor would be predicted to bind to the closed conformation of Abl, in which the SH3 ligand-binding site is already engaged by the SH2-linker segment.

7. AUTOPHOSPHORYLATION VS TRANS-PHOSPHORYLATION

The role of Abl autophosphorylation/ transphosphorylation as a component of its mechanism of activation is now being actively investigated. A recent study shows that several phosphorylation events are critical to fully activate wild-type c-Abl (55). This group found that in the presence of ATP, unphosphorylated Abl (purified in the presence of STI-571) underwent rapid autophosphorylation at Tyr412, in the activation segment of the kinase domain; autophosphorylated Abl had approximately 20-fold higher specific activity than the unphosphorylated form when assayed with a peptide substrate. Tyr412 had long been known to be important for Abl activation (57), but this was the first study to assess its effects in a quantitative fashion. Phosphorylation was also observed at a second site, Tyr-245, but the biological significance of this is less clear. As expected, mutation of these tyrosines to phenylalanine prevented activation of c-Abl upon incubation with ATP. An SH3-mutated, activated mutant was only modestly affected by these mutations, however, suggesting that for a deregulated kinase it was less important that the open conformation be stabilized by phosphorylation (presumably, these mutants preferentially adopt the open conformation even in the absence of phosphorylation).

It had been noted earlier that for purified Src, phosphorylation of the activation loop tyrosine was concentration-dependent, suggesting that autophosphorylation was intermolecular (if phosphorylation of this site were entirely *intra*molecular, it would be expected to be independent of concentration) (58,59). This was also found to be the case for purified Abl (55). Thus phosphorylation of the activation loop tyrosine can be performed by a second Abl molecule in trans, resulting in the stable activation of the kinase. This implies that one mechanism of inducing the activation of Abl *in vivo* would be to induce its aggregation (increasing its local concentration), as this would dramatically increase the likelihood of the activating trans-phosphorylation event.

The hypothesis that Abl molecules phosphorylate and activate each other is consistent with the now well-established paradigm that receptor tyrosine kinases are activated by dimerization and transphosphorylation (60). The two oncogenic forms of human Abl, Bcr-Abl and Tel-Abl provide further support. Both Bcr and Tel are fused to the N-terminus of c-Abl, although their respective Abl fusions retain the Abl SH3 domain. Yet both fusion proteins exhibit increased kinase activity and are known oncoproteins, suggesting that there is a region or regions in

Bcr and Tel that can lead to activation despite the presence of the SH3 domain. Several studies have now identified oligomerization domains as critical for transforming activity: mutations within the coiled-coil domain of Bcr or the helix-loop-helix domain of Tel resulted in Abl fusions with decreased kinase activity and loss of transformation (12,19). This suggests that Bcr and Tel force Abl oligomerization that results in transactivation. However, because these fusions still include other portions of Bcr and Tel, it is difficult to draw any definitive conclusions.

Two other studies have more directly addressed the role of dimerization.
Fusion of the estrogen receptor (ER) hormone-binding domain to the C-terminus of Abl produced a protein that, in the absence of hormone, exhibited low or absent phosphotyrosine levels and did not induce transformation. With the addition of estradiol, which is predicted to induce hormone-dependent dimerization, both increased phosphotyrosine levels and morphological changes associated with transformation were observed (61). Another group added either one or two copies of the binding motif (FKBP) for the synthetic homodimerizer AP1510 to the N-terminus of c-Abl; they then demonstrated dimerizer-dependent induction of Abl activation and transformation (62). As a key control, they also showed that upon removal of ligand, the cells reverted and arrested in G1. Thus several lines of evidence suggest that dimerization alone is sufficient to activate c-Abl.

These oligomerization/dimerization results are important to keep in mind when one considers the potential effects of certain lab procedures on Abl activity. It is possible that the increases in c-Abl activity seen under conditions of significant overexpression, or those conditions created during lab purification processes (immunoprecipitation, GST pull-downs, etc.) are due at least in part to increases in local concentration. One must also consider the possibility that cellular processes that relocalize Abl *in vivo*, for example to focal adhesions or to the membrane, may also activate Abl simply by increasing its local concentration.

One final area to consider with respect to transphosphorylation is the effect of heterologous kinases. It has been shown that c-Src can phosphorylate Abl on the critical activation loop site, Tyr-412 (63). Upon PDGF treatment Src is activated by association with the autophosphorylated receptor, and these authors propose that Abl is then phosphorylated by Src and thus activated in turn. Therefore transphosphorylation of Abl need not be an autophosphorylation, but can be performed by other kinases that are in close proximity to Abl. Others have shown that the ATM serine/threonine kinase (which is mutated in ataxia telangiectasia and is activated by genotoxic stress) can phosphorylate Abl at Ser-465 (6). This site is close to the activation loop and catalytic cleft on the large lobe of the kinase domain, and is well situated to effect conformational changes that might induce or stabilize the open conformation.

Thus it appears that several phosphorylation events, catalyzed either by other Abl molecules or by different kinases entirely, have the ability to activate Abl. Although phosphorylation clearly plays an important role in determining the specific activity of the kinase, it is also important to realize that this is not an all-or-nothing activation switch. Even "inactive," completely unphosphorylated c-Abl will occasionally assume the open conformation, and thus it has some basal kinase activity (55); because of this it also has the potential to activate other Abl molecules by trans-phosphorylation.

8. SH2 DOMAIN AND SUBSTRATE BINDING

While the SH3 domain may play a similar inhibitory role both in Abl and in the better-understood Src family kinases, there are clear distinctions in the role of the SH2 domain. In Src, the SH2 is essential for maintaining the inactive state through its interaction with the inhibitory phosphotyrosine in the C terminus, and as expected mutations to the SH2 can activate c-Src and render it transforming (64-66). This result necessarily implies that for Src, a functional SH2 is not required for kinase activity *in vivo* (or for transformation), although mutations in the SH2 can have effects on some aspects of the transformed phenotype. On the other hand, Abl has no regulatory phosphotyrosine (33), and therefore SH2 mutations do not activate c-Abl (34,67). Furthermore, an intact SH2 appears to be essential for the transforming activity of constitutively activated Abl, such as SH3-deleted mutants or Bcr-Abl (34,68,69), and also for the activity of Abl expressed in fission yeast (44). The essential role of the SH2 appears to involve phosphotyrosine binding, as mutants that specifically abrogate this activity are unable to transform fibroblasts (34,67,68). Thus in Abl the role of the SH2 is almost exclusively to enhance activity in vivo, while in Src the most important function of this domain is in negative regulation.

How might the SH2 play a positive role in Abl activity? It is formally possible that the SH2 binds to a stimulatory phosphorylation site on the catalytic domain (e.g. Tyr-412, or less likely Tyr-245), thereby stabilizing the active conformation. However this would require some conformational gymnastics relative to the position of the SH2 seen in the Src family kinases, and at least in the case of the Tyr-412 site it is likely that binding of the relatively bulky SH2 domain would interfere with substrate binding. Furthermore, the presence of the SH2 does not affect the activity of purified Abl toward nonspecific substrates *in vitro* (34).

A more plausible explanation is that the SH2 plays a role in the recruitment and processive phosphorylation of certain substrates. The Abl catalytic domain, like those of most tyrosine kinases, is a relatively inefficient enzyme; the maximum catalytic rate for activated Abl is roughly 10 moles of phosphate per second per mole Abl (55). In contrast, it is well known that the constitutive levels of tyrosine phosphatase activity in the cell are very high, so the enzyme would seem to be fighting an uphill battle. The SH2 domain, however, gives the nonreceptor kinases a subtle advantage: it allows the kinase to bind stably to tyrosine-phosphorylated proteins. This

means that in many cases a newly phosphorylated substrate is not released, but is instead held in close proximity to the catalytic domain where it can be repeatedly or processively phosphorylated on multiple sites. By remaining physically associated with the kinase, the substrate can be stably phosphorylated even in the face of high phosphatase activity.

Several lines of evidence support a role for the SH2 in processive phosphorylation. First, a comparison of the substrate specificities of the catalytic domains of nonreceptor kinases and the binding specificities of their associated SH2 domains reveals an excellent correlation, suggesting that the SH2 of a particular kinase co-evolved with its associated catalytic domain to bind particularly well to the products of that catalytic domain (70). Second, Abl mutants in which the SH2 domain is replaced with an ectopic SH2 domain (with a different binding specificity) phosphorylate different spectra of substrates *in vivo*, consistent with a role for the SH2 in selecting those substrates that will be tightly bound to the kinase and thus processively phosphorylated (34). Third, *in vitro* kinase experiments with substrates containing multiple potential phosphorylation sites demonstrated that Abl and other nonreceptor kinases phosphorylate multiple sites much more efficiently in the presence of a functional SH2 domain (71-73); the increase in efficiency is due to a decrease in the K_m for substrate as expected (73). For substrates containing a single phosphorylation site, or for "nonspecific" substrates such as poly Glu-Tyr, however, the presence of the SH2 is unimportant (34,71,72), again suggesting that the SH2 does not regulate catalytic activity per se but instead regulates the ability to stably associate with and processively phosphorylate selected substrates.

In addition to possessing its own SH2 domain, there is some evidence that Abl might recruit other SH2 domains which broaden the range of proteins that it can processively phosphorylate. A proline-rich region just C-terminal to the catalytic domain binds to the SH3 domains of SH2/SH3 adaptors Crk, Grb2, and Nck (4,5). *In vitro* experiments with purified Abl demonstrated that addition of the purified Crk adaptor greatly increased the ability of Abl to processively phosphorylate $p130^{Cas}$, a protein containing multiple potential phosphorylation sites that bind with particularly high affinity to the Crk SH2 (72). Thus in this system binding of Crk to Abl increases the processivity of Abl toward $p130^{Cas}$; binding of Nck, Grb2, and other SH2/SH3 adaptors might similarly influence processivity toward other substrates. Whether this actually occurs *in vivo* is still an open question.

The fact that Abl has many domains that can interact with other proteins, especially in the long unique C-terminal domain, suggests that protein interaction will be a general mechanism to regulate the phosphorylation of particular substrates. This is not surprising, but perhaps not fully appreciated—the high local concentration of binding partners can drive efficient phosphorylation even for poor enzymes, and indeed the relatively weak intrinsic catalytic activity of tyrosine kinases may have evolved as a means of ensuring that phosphorylation can be tightly controlled. For example, phosphorylation of Crk by Abl has been shown to be greatly facilitated by binding of its SH3 domains to the proline-rich region in the Abl C-terminus (74). Another example is phosphorylation of the C-terminal domain (CTD) of RNA polymerase II by Abl. Not only is the Abl SH2 required for efficient phosphorylation of CTD, but also a region close to the C-terminus; this region alone has measurable affinity for CTD and functions by lowering the apparent K_m of this substrate by 100-fold (10). Others have shown that the extreme C-terminus of Abl can restore transforming activity to a mutant bearing a crippled SH2 and lacking the rest of the C-terminus, consistent with a role in substrate recruitment (75). Thus the C-terminus appears to regulate not only subcellular localization, but also association with specific substrate proteins, and therefore plays an important role in the kinase activity of the enzyme in the cell.

9. LOCALIZATION – NUCLEAR VS. CYTOPLASM AND BEYOND

One final area that merits further study is the effect of localization on Abl activity. This is an important consideration because the localization patterns of Abl are quite complex, and are known to correlate with transforming ability. Furthermore, a number of factors that can affect the biological activity of Abl are strongly dependent on subcellular localization, including the local concentration of potential inhibitors, activating kinases such as Abl itself, and substrates.

Initial studies of Abl localization in fibroblasts demonstrated that while overexpressed Abl is predominantly localized to the nucleus, there is also a significant amount in the cytoplasm, much of it bound to filamentous actin and the plasma membrane (76). While the majority of cell types maintain this localization pattern, there are several notable exceptions. Abl is found predominantly in the cytoplasm instead of the nucleus in both hematopoietic cell lines (77) and neurons (78). Furthermore, both the *Drosophila* Abl homolog, D-Abl, and vertebrate Arg, which is closely related to Abl, are apparently entirely cytoplasmic (79,80). Because *Drosophila* has only a single Abl family member, and because Arg can largely compensate for the lack of Abl in knockout mice (78), the significance of any biological role for Abl in the nucleus is unclear.

The complex subcellular localization of Abl is controlled by several distinct targeting signals, most of which reside in the long C-terminus. First, there are three distinct nuclear-localization signals (NLSs); each is sufficient to localize Abl to the nucleus in fibroblasts (14), though they may function differentially depending on the cell type in which Abl is expressed. Second, nuclear targeting is opposed by a nuclear-export signal (NES) (15). This NES is dependent on the Crm1 nuclear-export pathway, as demonstrated by its sensitivity to leptomycin B treatment. Third, the C-terminus also contains binding sites for F-actin and G-actin that are believed to be responsible for the fraction of cytoplasmic Abl associated with the actin cytoskeleton (12,13). Finally, as previously mentioned,

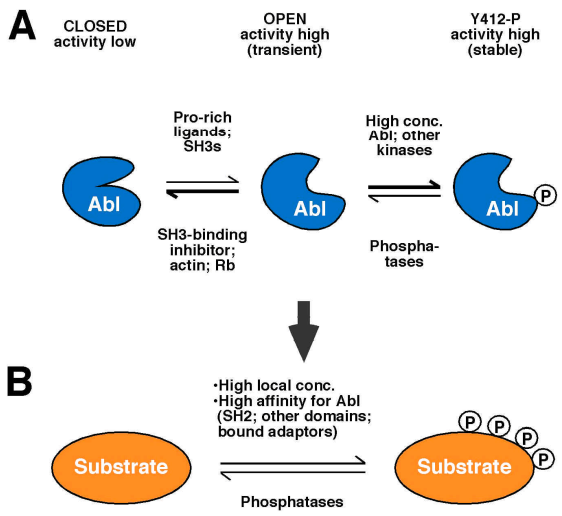

Figure 3. Regulation of Abl activity by intrinsic and extrinsic factors. A. The catalytic domain of Abl exists in a dynamic equilibrium between a closed, inactive conformation and an open, active conformation. The overall specific activity is determined by the percentage that is in the open conformation. Normally this equilibrium favors the closed form and specific activity is low. Phosphorylation of the Tyr-412 site stabilizes the open conformation and thus shifts the equilibrium toward the active form. These equilibria are affected by a variety of factors as indicated, such as proline-rich ligands that destabilize SH3-mediated inhibition, or other kinase molecules that can phosphorylate Tyr-412 in trans. B. Irrespective of the specific activity of the catalytic domain, other factors can affect the likelihood that a particular substrate will become highly phosphorylated. Factors that favor phosphorylation include high local concentration, high affinity for Abl, or interaction with adaptor proteins that can bind both Abl and potential substrates.

certain types of Abl contain a myristoylation signal at the N-terminus that directs association with the inner surface of the plasma membrane. Membrane localization is not obligatory, however, as both nuclear and actin-associated Abl appear not to be associated with membranes. As is the case for Src family kinases, membrane localization as well as the ability to transform fibroblasts are both lost when the myristoylation signal is removed from Abl (24,81).

There is evidence that the localization of Abl within the cell is quite dynamic. For instance, when fibroblasts are trypsinized and then re-plated on fibronectin, there is a detectable movement of c-Abl from the nucleus to focal adhesions (82). This is not a permanent relocalization, as one can observe the apparent return of Abl from focal adhesions to the nucleus within an hour of re-plating. This suggests that Abl may be constantly cycling between the nucleus and the cytosol, and that some of it can be temporarily trapped where high-affinity Abl binding sites are present (at focal adhesions, for example). A recent study has also shown that Abl relocalizes to mitochondria following oxidative stress (83). While it remains unclear if the increase in Abl associated with mitochondria is due to a relocation of Abl from either the nuclear or cytoplasmic pool, or if there is increased Abl synthesis, this study again suggests that Abl has the ability to move to a specific subcellular compartment under particular conditions.

A number of studies have suggested that two pools of cellular c-Abl, the cytoplasmic fraction vs. the nuclear fraction, have very distinct functions. It has long been appreciated that transforming Abl variants are always localized to the cytoplasm (76). In addition, fibroblast transformation absolutely requires the myristoylation signal and thus membrane localization (81). More recent studies in the areas of cell cycle control and the response to DNA damage suggest a critical role for the nuclear fraction of Abl for these activities, seemingly independent of the cytoplasmic pool of Abl (reviewed by (2,3)). The extent to which a particular molecule of Abl cycles between these different pools remains an open question, however.

Finally, it is worth noting that not only are potential substrates dependent on the localization of Abl, but so too is the composition of potential regulators (both activators and inhibitors). One example is binding of the retinoblastoma gene product, Rb, to nuclear Abl. This association is via the “C-pocket” of Rb and the ATP-binding loop of Abl, and has been proposed to inhibit the activity of nuclear Abl prior to S-phase (8). In another example, F-actin was recently shown to inhibit purified Abl protein, and this inhibition depended on the actin-binding site in the C-terminus (84). The authors propose that actin-mediated inhibition is responsible for inhibiting cytoplasmic Abl activity in fibroblasts that are detached from extracellular matrix. Finally, overexpressed c-Abl can be activated by overexpression of SH3 domains from the Nck adaptor; this activation is much stronger when the SH3 domains are directed to membranes via a myristoylation signal (85). The mechanism of activation by Nck is complex, but appears to involve multiple localization signals within the long C-terminus of Abl (J.M.S. and B.J.M., unpublished).

10. PERSPECTIVE

Abl is implicated in an ever-expanding list of cellular processes including cell cycle control, apoptosis, cytoskeletal regulation, growth factor signaling, cell growth and differentiation. Not surprisingly, it is difficult to reconcile all the known or suspected activities of Abl with all the known or suspected ways in which it can be regulated. One reason for the apparent complexity is that both intrinsic and extrinsic factors affect Abl activity, working together to ultimately determine which substrates get phosphorylated and to what extent (Figure 3). One level of regulation concerns the intramolecular interactions that keep Abl kinase activity low (the closed conformation), which is likely stabilized by interaction of the SH3 domain with the SH2-linker. These intrinsic factors control the specific activity of the catalytic domain

of Abl, irrespective of localization, presence of substrates, etc.; simply stated, Abl in the closed conformation is much less likely to phosphorylate a given substrate at a given concentration than is the activated enzyme in the open conformation. This intrinsic control can be perturbed by extrinsic factors, which may for example engage the SH3 domain or phosphorylate the activation segment. Such factors will shift the equilibrium toward the open, active conformation, effectively increasing the specific activity of Abl. Yet another level of regulation is provided by additional extrinsic factors such as local concentration of substrates, the presence of particular phosphorylation sites on substrates, or association with other proteins such as adaptors that can bind to substrates and confer processive phosphorylation. These latter factors do not affect the specific activity of the catalytic domain per se, but can have profound effects on the ability of Abl to phosphorylate a particular substrate.

Stepping back a bit, it may be that arguments about mechanisms of Abl regulation have been muddied by oversimplification. Perhaps it misguided to think about activation as a simple on-off switch; it is probably more appropriate to instead consider complex equilibria in which there are many different states of activity, dependent on binding partners, localization, phosphorylation state, concentration, and doubtless other parameters. Although the biological role of Abl and the mechanism of its regulation have served to fuel heated discussions for over a decade, it may be that in a sense everyone is right—Abl has evolved a level of subtlety and complexity in its activities that allow it to play a multitude of roles, to be regulated in a multitude of ways, depending on context and need.

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