Enhancement of ABL Kinase Catalytic Efficiency by a Direct Binding Regulator Is Independent of Other Regulatory Mechanisms*

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ABL family tyrosine kinases are tightly regulated by autoinhibition and phosphorylation mechanisms. These kinases maintain an inactive conformation through intramolecular interactions involving SH3 and SH2 domains. RIN1, a downstream effector of RAS, binds to the ABL SH3 and SH2 domains and stimulates ABL tyrosine kinase activity. RIN1 binding to the ABL2 kinase resulted in a large decrease in stimulations involving SH3 and SH2 domains. RIN1, a downstream effector of RAS, binds to the ABL SH3 and SH2 domains and stimulates ABL tyrosine kinase activity. RIN1 binding to the ABL2 kinase resulted in a large decrease in \( k_{\text{cat}} \) and a small increase in \( V_{\text{max}} \) toward an ABL consensus substrate peptide. The enzyme efficiency (\( k_{\text{cat}}/K_m \)) was increased more than 5-fold by RIN1. In addition, RIN1 strongly enhanced ABL-mediated phosphorylation of CRK, PSTPIP1, and DOK1, all established ABL substrates but with unique protein structures and distinct target sequences. Importantly RIN1-mediated stimulation of ABL kinase activity was independent of activation by SRC-mediated phosphorylation. RIN1 increased the kinase activity of both ABL1 and ABL2, and this occurred in the presence or absence of ABL regulatory domains outside the SH3-SH2-tyrosine kinase domain core. We further demonstrate that a catalytic site mutation associated with broad drug resistance, ABL1 T315I, remains responsive to stimulation by RIN1. These findings are consistent with an allosteric kinase activation mechanism by which RIN1 binding promotes a more accessible ABL catalytic site through relief of autoinhibition. Direct disruption of RIN1 binding may therefore be a useful strategy to suppress the activity of normal and oncogenic ABL, including inhibitor-resistant mutants that confound current therapeutic strategies. Stimulation through derepression may be applicable to many other tyrosine kinases autoinhibited by coupled SH3 and SH2 domains.

The ABL family non-receptor tyrosine kinases ABL1 (also known as c-Abi) and ABL2 (also known as Arg) function in the coordinated remodeling of actin cytoskeleton structures in response to external stimuli as occurs during cell attachment and motility (1–5). ABL kinases have been implicated in other actin reorganization functions including the formation of neuromuscular (6) and immune (7) synapses, the maturation and stability of neuronal synapses and dendrites (8, 9), and endocytic trafficking (10). The described ABL functions are mediated by the tyrosine phosphorylation of multiple actin remodeling regulator proteins including the adaptor proteins CRK and CRKL. These phosphorylation events are coordinated with the action of filamentous actin (F-actin) binding domains found at the carboxyl termini of both ABL proteins (for a review, see Ref. 11). In addition to its role in regulating cytoplasmic F-actin, ABL1 has a demonstrated nuclear function in the cellular response to DNA damage (for reviews, see Refs. 12 and 13).

The tyrosine kinase activity of ABL proteins is normally regulated at multiple levels. First an amino-terminal myristoylated peptide contributes to an inactive kinase domain conformation through multiple surface interactions (14, 15). Second the ABL SH3 and SH2 domains cradle the kinase domain and stabilize a low activity conformation (16). Finally tyrosine residues in the ABL kinase domain are substrates for auto- and trans-phosphorylation events that increase ABL kinase activity (17–20). In particular, SRC family kinase-mediated phosphorylation of a tyrosine in the ABL kinase domain activation loop stimulates activity severalfold. Recent work also suggests a role for regulatory serine phosphorylation of ABL proteins (21). Each type of ABL kinase regulation appears to be conserved between ABL1 and ABL2. Both proteins can be amino-terminal myristoylated, and they show 90% sequence identity throughout the SH3, SH2, and tyrosine kinase domains. Regulatory tyrosine residues subject to trans-phosphorylation are especially well conserved.

ABL kinase inhibitors such as ST1571 (imatinib or Gleevec) bind within the catalytic site of the enzyme and stabilize an inactive conformation (22). These compounds are effective in the treatment of chronic myeloid leukemia, which is characterized by expression of a translocation-derived BCR-ABL1 fusion protein. Unfortunately drug resistance often arises because of mutations within the kinase domain (23). These mutations block inhibitor binding while maintaining only slightly altered catalytic activity. Little is known, however, about how such catalytic site mutations affect regulation of kinase activity.

RIN1 is a RAS effector protein that enhances the activation of ABL kinases. Overexpression of RIN1 increased cellular phos-

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2 The abbreviations used are: SH, SRC homology; TK, tyrosine kinase; ERK, extracellular signal-regulated kinase.
phototyrosine and specifically elevated the level of tyrosine phosphorylated CRKL, and this was accompanied by alterations in the actin cytoskeleton (24). Conversely silencing of RIN1 by short hairpin RNA or deletion of RIN1 in a mouse model resulted in less basal tyrosine phosphorylation of endogenous CRKL (24). RIN1 silencing and deletion were associated with enhanced cell attachment to fibronectin and reduced cell migration, similar to the effects caused by treatment with the ABL kinase inhibitor STI571 (24). In addition, RIN1 enhanced the transforming potential of BCR-ABL1 (25). These findings suggest that RIN1 is a physiologically positive regulator of ABL kinase activity.

Signaling from RIN1 to ABL is initiated by the binding of a proline-rich sequence on RIN1 to the SH3 domain of ABL (26). This interaction leads to phosphorylation of RIN1 on tyrosine 36 and perhaps other amino-terminal tyrosines. RIN1 subsequently associates with the ABL SH2 domain (24). An appealing model links the resulting divalent interaction (RIN1 proline-rich motif and phospho-Tyr<sup>36</sup> bound to the ABL SH3 and SH2 domains, respectively) with ABL kinase domain activation. Deletion of the ABL SH3 domain blocks RIN1 binding (25), and mutation of RIN1 amino-terminal tyrosines severely diminishes both ABL binding and kinase activation (24, 27, 28), consistent with this model. Together these observations suggested that RIN1 directly activates the tyrosine kinase activity of ABL proteins, but the mechanism of enzyme activation is unclear. Here we demonstrate that RIN1 directly alters the kinetic properties of ABL in a manner consistent with derepression of ABL SH3- and SH2-imposed autoinhibition. Strong stimulation of ABL kinase activity by RIN1 required no other cellular proteins and was observed with three independent well characterized substrates. Furthermore stimulation through RIN1 binding was independent of trans-phosphorylation by other kinases or regulation by distal ABL domains. We also demonstrate that a clinically relevant ABL catalytic site mutant retains responsiveness to RIN1, suggesting that kinase inhibitor-resistant mutants may be susceptible to inhibitors that block RIN1 binding.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction and Mutagenesis**—The baculovirus vector construct encoding ABL1-(1–531) with a carboxyl-terminal cleavage site for the tobacco etch virus protease and a hexahistidine tag (14) was provided by Dr. John Kuriyan (University of California, Berkeley, CA). To create the threonine to isoleucine mutation at codon 334 of ABL1-(1–531) (equivalent to the BCR-ABL1<sup>T315I</sup> mutation), we first subcloned a fragment of ABL1-(1–531) from the amino-terminal BamHI site to the carboxyl-terminal EcoRI site into pFastBac1 digested with BamHI and HindIII using the linkers 5′-AATTCACTAGT-3′ and 5′-AGCTACTAGTG-3′ to connect the EcoRI and HindIII sites. A KpnI to AatII fragment from murine stem cell virus (equivalent to the BCR-ABL1<sup>T315I</sup> mutation) was subcloned into the pFastBac1 vector, and a full baculovirus construct was generated by recombination according to the manufacturer’s instructions (Invitrogen).

**Protein Expression and Purification**—The tagged ABL1-(1–531) and full-length RIN1 proteins were produced in S<sup>9</sup> insect cells following infection with the corresponding recombinant baculoviruses. Cells were harvested in Lysis Buffer (50 mM Tris, pH 8.0, 10% glycerol, and 15 mM β-mercaptoethanol with protease inhibitor mixture (Roche Applied Sciences) 48–72 h postinfection, then sonicated, and cleared at 17,000 × g for 30 min at 4 °C. The bacterial expression constructs His<sub>6</sub>-CRK, His<sub>6</sub>-PSTPIP1, and GST-DOK1 were kindly provided by Drs. Bruce Meyer, Stephen Goff, and Tony Hunter, respectively. The corresponding recombinant proteins were generated in transformed BL21 cells. Following induction with isopropyl 1-thio-β-D-galactopyranoside (1 mM for 3 h), cells expressing CRK or PSTPIP1 were harvested in Lysis Buffer (20 mM Tris, pH 8, 100 mM NaCl, and 5 mM imidazole), whereas cells expressing DOK1 were harvested in phosphate-buffered saline (at pH 7.4). Lysates were sonicated and cleared at 17,000 × g for 30 min at 4 °C. His-tagged recombinant proteins were captured by nickel-nitrilotriacetic acid-agarose (Qiagen), washed with Lysis Buffer, and eluted with Lysis Buffer containing 200 mM imidazole. Glutathione S-transferase-tagged proteins were captured on glutathione-Sepharose 4B (GE Healthcare), washed with phosphate-buffered saline, and eluted with phosphate-buffered saline containing 50 mM reduced glutathione. The expression and isolation of unphosphorylated ABL2 from S<sup>9</sup> cells has been described previously (20). Anti-ABL2 has been described previously (24). Purified protein concentrations were determined by a NanoDrop spectrophotometer (280 nm), and the approximate percent purity was evaluated by SDS-PAGE.

**In Vitro Kinase Assays**—All kinase reactions were performed under conditions in which total phosphorylation increased linearly with substrate concentration. A bixin-conjugated consensus ABL2 substrate peptide, bixin-AQDVYDVPAPKK (Bioanalytical, Ltd.; sequence was verified by mass spectrometry), was used to characterize the kinetic parameters of ABL2. The peptide was preincubated with purified ABL2 and RIN1 proteins at the indicated concentrations in the reaction buffer containing 100 mM NaCl, 10 mM Tris, pH 7.4, 1 mM dithiothreitol, 1 mM Na<sub>2</sub>VO<sub>4</sub>, and 10 mM MgCl<sub>2</sub> on ice. The reaction was initiated by adding an ATP mixture to a final concentration of 50 μM ATP and 20 μCi of [γ-<sup>32</sup>P]ATP with a final reaction volume of 50 μl. It was stopped by adding 50 μl of 0.5 mM EDTA, pH 8.0, to each sample after 15-min incubation in a 30 °C water bath. ABL2 substrate peptide was selected by incubating with 25 μl of 50% avidin-agarose (Pierce) at room temperature for 1 h. The beads were washed three times with 250 μl sodium phosphate buffer, pH 7.4, containing 50 μM ATP. The radioactivity bound to the beads was determined by liquid scintillation spectrometry. K<sub>m</sub> and V<sub>max</sub> values were determined using a

A baculovirus vector was used to produce human ABL2 with an amino-terminal hexahistidine tag attached to residues 74–1182 (natural carboxyl terminus) and has been described previously (20). Full-length human RIN1 cDNA with a carboxyl-terminal hexahistidine tag was subcloned into the pFastBac1 vector, and a full baculovirus construct was generated by recombination according to the manufacturer’s instructions (Invitrogen).
Lineweaver-Burk double reciprocal plot. The same data set was used to calculate $k_{\text{cat}}$ values based on an estimated molecular mass of 120 kDa for ABL2. For the other kinase assays, the reaction was initiated by adding ATP to a final concentration of 500 $\mu$M and was stopped by adding 10 $\mu$L of protein SDS loading buffer followed by boiling for 5 min. Protein phosphorylation was detected by immunoblotting with the phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology). A 10 mM stock of STI571 was prepared as described previously (24).

**Immunoprecipitation Analysis**—Purified proteins (RIN1, CRK, and ABL2) were mixed in 250 $\mu$L of buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl, and 1% Nonidet P-40 (RIN1 + ABL2 samples were first incubated in a 50-$\mu$L kinase reaction) and then precleared with 10 $\mu$L of 1:1 immobilized protein G-agarose (Pierce) at room temperature for 30 min. Supernatants were incubated with 1 $\mu$L of immunoprecipitation antibody and 25 $\mu$L of 1:1 immobilized protein G-agarose at room temperature for 1 h and then washed five times with buffer. Beads were resuspended and boiled in 25 $\mu$L of SDS protein loading buffer prior to immunoblot analysis.

**RESULTS**

**RIN1 Stimulates ABL Phosphorylation Kinetics**—To quantify the effect of RIN1 binding on the kinetics of substrate phosphorylation by ABL tyrosine kinases, we carried out kinase assays using ABL2 and RIN1 purified from an insect cell expression system. Working within the linear range of enzyme activity, we observed a substantial increase in the phosphorylation of a consensus substrate peptide by ABL2 when RIN1 was added to the assay (Fig. 1). Using an appropriate range of substrate concentrations, we evaluated the contribution of RIN1 to ABL enzyme kinetics. Analysis revealed that RIN1 decreased the $K_m$ for substrate by more than 4-fold while causing a modest increase in $V_{\text{max}}$ (Table 1). The net result was a greater than 5-fold elevation in ABL2 catalytic efficiency ($k_{\text{cat}}/K_m$). This is consistent with the model that ABL SH3 and SH2 domains function in part to inhibit the kinase domain and that engagement with RIN1 relieves this autoinhibition and increases ABL catalytic site accessibility to substrate.

**RIN1 Stimulates ABL Phosphorylation of Multiple Substrates**—The contribution of RIN1 to ABL-mediated phosphorylation of CRK, a well established substrate involved in actin remodeling, was examined next by *in vitro* kinase assay. RIN1 enhanced the kinase activity of ABL2 in a concentration-dependent manner up to an ~40-fold increase over a control sample without RIN1 (Fig. 2A). Parallel assays using bovine serum albumin in place of RIN1 showed no stimulation of ABL kinase activity (Fig. 2B), demonstrating that this was not a nonspecific effect of total protein concentration. The observed enhancement of ABL-mediated CRK phosphorylation following the addition of RIN1 was consistent with the large increase in phospho-CRK associated with overexpression of RIN1 in cultured cells (24) but much greater than previously measured *in vitro* effects using a bacterially expressed fragment of RIN1 (24). It should be noted.

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**TABLE 1**

Kinetic data for RIN1 stimulation of ABL2 kinase activity (from data presented in Fig. 1)

| Kinetic value     | RIN1 | + RIN1 |
|-------------------|------|--------|
| $K_m$ ($\mu$M)    | 23   | 5      |
| $V_{\text{max}}$ (pmol/min $\mu$g$^{-1}$) | 826  | 945    |
| $k_{\text{cat}}$ (min$^{-1}$) | 0.099 | 0.113  |
| $k_{\text{cat}}/K_m$ (min$^{-1}$ M$^{-1}$) | 4,200 | 23,000 |

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**FIGURE 1.** RIN1 enhances ABL2 enzyme efficiency. Kinase assays were performed using 10 nM ABL2 with varying concentrations of substrate peptide, and the results were analyzed by double reciprocal plot. Reactions were carried out in the absence (diamonds) or presence (squares) of 500 nM RIN1. Triplicate experiments were used to generate each data point and standard error (bars).

**FIGURE 2.** Multiple ABL2 substrates respond to RIN1-mediated stimulation. A, kinase assays performed with 0.2 nM ABL2, 2 $\mu$M CRK, and the indicated concentration of RIN1 were analyzed by immunoblot with anti-phosphotyrosine. - Fold induction was quantified by densitometry. B, kinase assays performed as in A but with the indicated concentration of bovine serum albumin (BSA) used in place of RIN1. C and D, kinase assays performed as in A but using 2 $\mu$M DOK1 or 2 $\mu$M PSTPIP1 in place of CRK. E, no detectable binding of RIN1 to substrate. Top, 50 nM RIN1 ± 50 nM CRK samples were preincubated and immunoprecipitated with anti-CRK and analyzed along with untreated samples by immunoblot as indicated. Bottom, 50 nM CRK ± 50 nM RIN1 samples were immunoprecipitated with anti-RIN1 and analyzed as for the top panel. F, RIN1 directly associates with ABL2. Following a kinase reaction, 50 nM RIN1 ± 50 nM ABL2 samples were immunoprecipitated with anti-ABL2 and analyzed along with untreated samples by immunoblot as indicated. IP, immunoprecipitation; pr, phosphotyrosine.
RIN1 Enhances ABL Tyrosine Kinase Efficiency

that the CRK phosphorylation assays were performed using 50-fold less ABL2 than was needed for the peptide phosphorylation assays. The reduced requirement for kinase in the protein substrate assays likely reflected the contribution of enzyme-substrate interactions involving sequences distal to the immediate target site in the protein but absent in the peptide. Such interactions have been shown previously for ABL1 and CRK (29). Even accounting for this factor, it appeared that RIN1 stimulated the phosphorylation of ABL2 more than of substrate peptide.

We noted that when using substantially higher concentrations of ABL2 (2 versus 0.2 nm) at the same concentration of CRK, the reaction proceeded much more rapidly, and the stimulatory effect of RIN1 was greatly diminished (data not shown). This is consistent with a positive regulatory role for RIN1 under limiting conditions as occur during cellular signaling.

To determine whether RIN1 enhancement of ABL kinase activity extends to targets other than CRK, we examined two additional cytoplasmic substrates. ABL kinases phosphorylate the adapter protein DOK1 during cell spreading-induced actin remodeling (5). DOK1 shows no similarity to CRK in overall sequence or domain structure, although the target phosphorylation sites in each substrate conform to an established consensus ((I/L/V)YYX_{1-5}(P/F) where Y is the phosphorylation site (30)). RIN1 enhanced the phosphorylation of DOK1 by ABL2, and this effect appeared to be similar in magnitude to that observed for the CRK substrate in a semiquantitative assay (Fig. 2C), suggesting that the kinase stimulatory effect of RIN1 is broad with respect to phosphorylation substrates. We continued this analysis by examining an established ABL substrate with a markedly different function. PSTPIP1 is an adaptor protein that recruits PEST motif tyrosine phosphatases to negatively regulate ABL (31). PSTPIP1 is structurally unrelated to CRK, except for an SH3 domain in each protein, or to DOK1. In addition, the phosphorylation site in PSTPIP1 deviates from the consensus ABL target found in both CRK and DOK1. Using kinase assay conditions identical to those used for CRK and DOK1, we observed a strong stimulatory effect of RIN1 on ABL2-mediated PSTPIP1 tyrosine phosphorylation (Fig. 2D), demonstrating that the RIN1 stimulatory effect is not limited to protein substrates with consensus target sites.

We considered whether RIN1 could bind directly to substrate proteins to facilitate access to the ABL kinase domain, but no evidence of this was observed (Fig. 2E). The previously documented interaction of RIN1 with ABL (24, 26) was clearly detected using these proteins, however (Fig. 2F).

These results indicate that RIN1 binding and derepression of the ABL kinase domain promote the phosphorylation of a wide spectrum of substrates. The magnitude of kinase enhancement provided by RIN1 appeared to be stronger for PSTPIP1 than for CRK and DOK1 (Fig. 2, A, C, and D) in this semiquantitative assay, suggesting the possibility that RIN1 may provide features that cooperate with ABL-encoded substrate binding surfaces and might participate in substrate selectivity.

The ABL1 and ABL2 proteins are extremely well conserved with greater than 90% amino acid identity throughout the SH3, SH2, and kinase domains. When assayed under the same conditions, RIN1 activated an ABL1 construct similarly to what was observed for ABL2 (Fig. 3, A and B). It should be noted that although both the ABL1 and ABL2 constructs include the full SH3-SH2-TK domain segment, they differ in the presence or absence of other regions common to both family members (Fig. 3C). The strong stimulation of both ABL constructs suggests that RIN1 function requires neither the ABL amino-terminal peptide implicated in autoinhibition (missing in the ABL2 construct) nor the large ABL carboxyl-terminal segment that includes F-actin-binding sites and several regulatory motifs (missing in the ABL1 construct).

Activation by RIN1 Is Independent of ABL Kinase Transphosphorylation—We next tested whether stimulation by RIN1 requires ABL kinase domain activation loop trans-phosphorylation by SRC family kinases. ABL2 prepared from Sf9 cells treated with a combination of kinase inhibitors showed no phosphorylation at Tyr^{399} in the activation loop, and more generally had no detectable tyrosine phosphorylation (Ref. 20 and Fig. 4A). When used in the CRK phosphorylation assay, we observed only a slight reduction in the level of ABL kinase stimulation by RIN1 (Fig. 4B). This result suggests that trans-phosphorylation of the kinase domain, a well characterized mecha-
introduced the T315I mutation into an ABL1 clone, creating kinase domain P-loop residue implicated in regulation (35). We CRK phosphorylation (Fig. 5) strongly resistance to the kinase inhibitor STI571 in an assay of this residue is altered in BCR-ABL1T315I, a mutant resistant to the ATP-binding site and a hydrophobic pocket (32). ABL kinase mutated in the “gatekeeper” residue that resides between the ATP-binding site and a hydrophobic pocket (32). This residue is altered in BCR-ABL1T315I, a mutant resistant to multiple kinase inhibitor drugs used in the treatment of chronic myeloid leukemia (23), and in EGFR T790M, a mutant resistant to epidermal growth factor kinase inhibitors used for lung cancer (33). BCR-ABL1T315I shows protein kinase activity that is comparable to BCR-ABL1 (less active with a peptide substrate) but had an altered cellular substrate profile (34, 35). BCR-ABL1T315I also has elevated phosphorylation of Tyr257, an ABL amino-terminal “cap” domain. This suggests either that RIN1 binding to the SH3 and SH2 domains is not, per se, relieve conformational autoinhibition. An ABI1-derived peptide that binds both the ABL SH3 and SH2 domains weakly stimulated tyrosine kinase activity at low concentrations but strongly inhibited activity at higher concentrations (38). Notably this inhibitory effect required peptide concentrations more than 1,000-fold above the concentration of full-length RIN1 protein used to stimulate kinase activity in our assays. Nonetheless it may well be that amino acids bordering the Tyr(P) and PXXP motifs could dramatically influence the magnitude and even the direction of kinase regulation.

The stimulation of ABL tyrosine kinase activity by RIN1 appears to be independent of the autoinhibition imposed by the amino-terminal “cap” domain. This suggests that RIN1 binding to the SH3 and SH2 domains overrides regulation by the cap peptide and myristoyl group or that these features are also engaged by the RIN1 protein through an uncharacterized manner (Fig. 5B). This result demonstrates that this kinase domain drug resistance mutation does not diminish the intrinsic requirement for RIN1 to achieve full ABL tyrosine kinase activity.

**DISCUSSION**

The activity of ABL family non-receptor tyrosine kinases is tightly controlled by structural elements that impose and stabilize a low activity kinase domain conformation. Our results demonstrate that RIN1 directly activates ABL kinases through an increase in enzyme efficiency. This likely occurs through an allosteric change in ABL SH3 and SH2 domains upon binding of PXXP and Tyr(P) motifs, respectively, found in the previously defined ABL binding domain of RIN1 (24). This model (Fig. 6) invokes a subsequent release of the kinase domain from a tight autoinhibitory fold imposed by its engagement with the SH3 and SH2 regulatory domains. Such a RIN1-induced conformational change may favor a more open and active ABL catalytic site with enhanced substrate access that is detected as a reduction in $K_m$ and an increase in catalytic efficiency ($k_{cat}/K_m$). Structural studies of the isolated ABL tyrosine kinase domain suggest that it may adopt distinct inactive structures that can interconvert (36), providing an additional level of regulatory complexity. An extended SH3-SH2-TK domain conformation, with critical SH2-TK domain interactions, has been implicated in the stable activation of ABL (16, 37). Determining the detailed structure of RIN1 bound to ABL would provide valuable insight into the conformation of the physiologically activated kinase.

Engagement of ABL SH3 and SH2 domains may not, per se, relieve conformational autoinhibition. An ABL1-derived peptide that binds both the ABL SH3 and SH2 domains weakly stimulated tyrosine kinase activity at low concentrations but strongly inhibited activity at higher concentrations (38). Notably this inhibitory effect required peptide concentrations more than 1,000-fold above the concentration of full-length RIN1 protein used to stimulate kinase activity in our assays. Nonetheless it may well be that amino acids bordering the Tyr(P) and PXXP motifs could dramatically influence the magnitude and even the direction of kinase regulation.

The stimulation of ABL tyrosine kinase activity by RIN1 appears to be independent of the autoinhibition imposed by the amino-terminal “cap” domain. This suggests that RIN1 binding to the SH3 and SH2 domains overrides regulation by the cap peptide and myristoyl group or that these features are also engaged by the RIN1 protein through an uncharacterized
RIN1 Enhances ABL Tyrosine Kinase Efficiency

interaction. In either case, the cap domain is clearly not required for RIN1-mediated stimulation. Transforming fusions of ABL, such as BCR-ABL1 and TEL-ABL2, have lost the cap domain, and this is believed to contribute to their elevated and constitutive kinase activity (39). Interestingly, however, these oncogenes retain the ABL SH3 and SH2 domains. This raises the possibility that these human leukemia oncogenes may still be responsive to RIN1.

Tyrosine phosphorylation by SRC family kinases leads to increased ABL kinase activity, and this signal transduction link contributes to stimulus-induced actin remodeling (18–20). The ABL binding partner ABI1 inhibits kinase activity in part through blocking trans-phosphorylation (38), consistent with a physiological role for ABL kinase domain phosphoregulation. Importantly, however, the activation of ABL via RIN1 binding appears to be independent of ABL trans-phosphorylation by other tyrosine kinases. This result suggests that conformational derepression and post-translational activation of the ABL kinase domain are uncoupled and implies that ABL kinases are responsive to distinct and non-overlapping regulatory signals. We cannot rule out, of course, the possible dependence of RIN1 on other ABL regulation mechanisms such as autophosphorylation, which was not examined here.

Although much effort has focused on mechanisms regulating the intensity of kinase activity, it is equally important to consider the potential influence of regulators on substrate specificity. For ABL tyrosine kinases, synthetic peptide selection initially identified preferred target sites with proline at position +3 or +4 (40) with subsequent studies leading to the broader consensus (I/L/V)YX_{1–5}(P/F) (30). Large scale proteomics analysis of BCR-ABL1-expressing cells also showed significant overrepresentation of YYX_{1–5}(P/F) sites (41). These and other (42) observations support a strong preference, although not a requirement, for a downstream proline. The fact that RIN1 enhanced ABL-mediated phosphorylation of substrates with a consensus site (CRK = yYAQLPSVEx where yP is phosphotyrosine) or a non-consensus site (PSTPIP1 = yYTAIAVQ) suggests that RIN1 increases ABL catalytic site accessibility for a wide range of substrates but does not rule out a potential contribution of RIN1 to substrate selectivity.

A large component of kinase substrate specificity can be provided by interactions peripheral to the target and catalytic sites. Mitogen-activated protein kinases, such as ERK1 and ERK2, refine their substrate selectivity by combining a low complexity target site with a distal docking site recognized by the enzyme (43–45). RIN1 might possibly contribute a port for an as yet uncharacterized substrate docking site. Such a mechanism could lead to both enhanced kinase activity and refined substrate recruitment. Further studies will be needed to determine the identity of any such docking sites.

The BCR-ABL1 T315I mutant has been found in chronic myeloid leukemia patients before exposure to kinase inhibitors (46) and is associated with relapse in patients treated with imatinib and related ABL kinase inhibitors (23). The fact that the ABL1 T315I mutant retains responsiveness to RIN1 is consistent with a model in which binding of RIN1 to the SH3 and SH2 domains promotes substrate access to the ABL catalytic site and suggests that BCR-ABL1 kinase domain mutants selected for drug resistance may still be vulnerable to inhibitors targeted outside the catalytic domain. Disruption of the interaction between RIN1 and ABL1 might also synergize with ABL catalytic site inhibition to reduce the rate of relapse in chronic myeloid leukemia patients.

The characterization of RIN1, a known RAS effector, as a potent direct positive regulator of ABL proteins suggests that RAS signaling can be channeled through a RAS-RIN1-ABL pathway. The majority of non-receptor tyrosine kinases have coupled SH3-SH2 domains as in ABL1 and ABL2. Our findings therefore raise the possibility that RIN1 may be representative of a larger class of tyrosine kinase conformational activators that cause derepression through direct binding of autoinhibitory SH3-SH2 domains to regulate cytoplasmic signaling. Interference with such activators could prove highly useful in efforts to redirect, dampen, or block tyrosine kinase function.

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REFERENCES

1. Kain, K. H., and Klemke, R. L. (2001) J. Biol. Chem. 276, 16185–16192
2. Miller, A. L., Wang, Y., Moooser, M. S., and Koleske, A. J. (2004) J. Cell Biol. 165, 407–419
3. Peacock, G. J., Miller, A. L., Bradley, W. D., Rodriguez, O. C., Webb, D. J., and Koleske, A. J. (2007) Mol. Cell. Biol. 18, 3860–3872
4. Woodring, P. J., Hunter, T., and Wang, J. Y. (2003) J. Cell. Sci. 116, 2613–2626
5. Woodring, P. J., Meisenhelder, J., Johnson, S. A., Zhou, G. L., Field, J., Shah, K., Bladt, F., Pawson, T., Niki, M., Pandolfi, P. P., Wang, J. Y., and Hunter, T. (2004) J. Cell Biol. 165, 493–503
6. Finn, A. I., Feng, G., and Pendergast, A. M. (2003) Nat. Neurosci. 6, 717–723
7. Huang, Y., Comiskey, E. O., Dupree, R. S., Li, S., Koleske, A. J., and Burkhart, J. K. (2008) Blood 112, 111–119
8. Moresco, E. M., Donaldson, S., Williamson, A., and Koleske, A. J. (2005) J. Neurosci. 25, 6105–6118
9. Sfakianos, M. K., Eismann, A., Gourley, S. L., Bradley, W. D., Scheetz, A. J., Settleman, J., Taylor, J. R., Greer, C. A., Williamson, A., and Koleske, A. J. (2007) J. Neurosci. 27, 10982–10992
10. Tanos, B., and Pendergast, A. M. (2006) J. Biol. Chem. 281, 32714–32723
11. Hernández, S. E., Krishnaswami, M., Miller, A. L., and Koleske, A. J. (2004) Trends Cell Biol. 14, 36–44
12. Shaul, Y., and Ben-Yehoyada, M. (2005) Cell Res. 15, 33–35
13. Wang, J. Y. (2005) Cell Res. 15, 43–48
14. Nagar, B., Hantschel, O., Young, M. A., Scheffzek, K., Veach, D., Bornmann, W., Clarkson, B., Superti-Furga, G., and Kuriyan, J. (2003) Cell 112, 859–871
15. Pluk, H., Dorey, K., and Superti-Furga, G. (2002) Cell 108, 247–259
16. Nagar, B., Hantschel, O., Seeliger, M., Davies, J. M., Weis, W. I., Superti-Furga, G., and Kuriyan, J. (2006) Mol. Cell 21, 787–798
17. Brasher, B. B., and Van Etten, R. A. (2000) J. Biol. Chem. 275, 35631–35637
18. Dorey, K., Engen, J. R., Kretzschmar, J., Wilm, M., Neubauer, G., Schindler, T., and Superti-Furga, G. (2001) Oncogene 20, 8075–8084
19. Plattner, R., Kadlec, L., DeMali, K. A., Kazlauskas, A., and Pendergast, A. M. (1999) Genes Dev. 13, 2400–2411
20. Tanis, K. Q., Veach, D., Duewel, H. S., Bornmann, W. G., and Koleske, A. J. (2003) Mol. Cell. Biol. 23, 3884–3896
21. Jung, J. H., Pendergast, A. M., Zipfel, P. A., and Traugh, J. A. (2008) Biochemistry 47, 1094–1104
22. Schindler, T., Bornmann, W., Pellicena, P., Miller, W. T., Clarkson, B., and
Kuriyan, J. (2000) *Science* (N. Y.) **289**, 1938–1942

23. Gorre, M. E., Mohammed, M., Ellwood, K., Hsu, N., Paquette, R., Rao, P. N., and Sawyers, C. L. (2001) *Science* (N. Y.) **293**, 876–880

24. Hu, H., Bliss, J. M., Wang, Y., and Colicelli, J. (2005) *Curr. Biol.* **15**, 815–823

25. Afar, D. E., Han, L., McLaughlin, J., Wong, S., Dhaka, A., Parmar, K., Rosenberg, N., Witte, O. N., and Colicelli, J. (1997) *Immunity* **6**, 773–782

26. Han, L., Wong, D., Dhaka, A., Afar, D., White, M., Xie, W., Herschman, H., Witte, O., and Colicelli, J. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 4954–4959

27. Hu, H., Milstein, M., Bliss, J. M., Thai, M., Malhotra, G., Huynh, L. C., and Colicelli, J. (2000) *Mol. Cell. Biol.* **20**, 1413–1423

28. Filippakopoulos, P., Kofler, M., Hantschel, O., Salah, E., Neudecker, P., Kay, L. E., Turk, B. E., Superti-Furga, G., Pawson, T., and Knapp, S. (2008) *Cell* **134**, 793–803

29. Levinson, N. M., Kuchment, O., Shen, K., Young, M. A., Koldobskiy, M., Karplus, M., Cole, P. A., and Kuriyan, J. (2006) *PLoS Biol.* **4**, e144

30. Gong, F., Spencer, S., Cote, J. F., Wu, Y., Tremblay, M. L., Lasky, L. A., and Goff, S. P. (2000) *Mol. Cell* **6**, 1413–1423

31. Noble, M. E., Endicott, J. A., and Johnson, L. N. (2004) *Science* (N. Y.) **303**, 1800–1805

32. Bell, D. W., Gore, I., Okimoto, R. A., Godin-Heymann, N., Sordella, R., Mullloy, R., Sharma, S. V., Brannigan, B. W., Mohapatra, G., Settleman, J., and Haber, D. A. (2005) *Nat. Genet.* **37**, 1315–1316

33. Griswold, I. J., MacPartlin, M., Bumm, T., Goss, V. L., O’Hare, T., Lee, K. A., Corbin, A. S., Stoffregen, E. P., Smith, C., Johnson, K., Mosennon, E. M., Wood, L. J., Polakiewicz, R. D., Druker, B. J., and Deininger, M. W. (2006) *Mol. Cell. Biol.* **26**, 6082–6093

34. Skaggs, B. J., Gorre, M. E., Ryvkin, A., Burgess, M. R., Xie, Y., Han, Y., Komisopoulou, E., Brown, L. M., Loo, J. A., Landaw, E. M., Sawyers, C. L., and Graeber, T. G. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103**, 19466–19471

35. Shah, N. P., Nicoll, J. M., Nagar, B., Gorre, M. E., Paquette, R. L., Kuriyan, J., and Sawyers, C. L. (2002) *Cancer Cell* **2**, 117–125