ENZYME CATALYSIS AND REGULATION:
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J. Biol. Chem. 2000, 275:35631-35637.
doi: 10.1074/jbc.M005401200 originally published online August 29, 2000

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c-Abl Has High Intrinsic Tyrosine Kinase Activity That Is Stimulated by Mutation of the Src Homology 3 Domain and by Autophosphorylation at Two Distinct Regulatory Tyrosines*

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Using the specific Abl tyrosine kinase inhibitor STI 571, we purified unphosphorylated murine type IV c-Abl and measured the kinetic parameters of c-Abl tyrosine kinase activity in a solution with a peptide-based assay. Unphosphorylated c-Abl exhibited substantial peptide kinase activity with \( K_m \) of 204 \( \mu \text{M} \) and \( V_{\text{max}} \) of 33 pmol min\(^{-1}\). Contrary to previous observations using immune complex kinase assays, we found that a transforming c-Abl mutant with a Src homology 3 domain point mutant (P131L) had significantly (about 6-fold) higher intrinsic kinase activity than wild-type c-Abl (\( K_m = 91 \mu\text{M}, V_{\text{max}} = 112 \text{pmol min}^{-1} \)). Autophosphorylation stimulated the activity of wild-type c-Abl about 18-fold and c-Abl P131L about 3.6-fold, resulting in highly active kinases with similar catalytic rates. The autophosphorylation rate was dependent on Abl protein concentration consistent with an intramolecular reaction. A tyrosine to phenylalanine mutation (Y412F) at the c-Abl residue homologous to the c-Src catalytic domain autophosphorylation site impaired the activation of wild-type c-Abl by 90% but reduced activation of c-Abl P131L by only 45%. Mutation of a tyrosine (Tyr-245) in the linker region between the Src homology 2 and catalytic domains that is conserved among the Abl family inhibited the autophosphorylation-induced activation of wild-type c-Abl by 50%, whereas the c-Abl Y245F/Y412F double mutant was minimally activated by autophosphorylation. These results support a model where c-Abl is inhibited in part through an intramolecular Src homology 3-linker interaction and stimulated to full catalytic activity by sequential phosphorylation at Tyr-412 and Tyr-245.

c-Abl is a non-receptor tyrosine kinase of which the precise functions are not known, but roles for Abl in growth factor and integrin signaling, cell cycle regulation, neurogenesis, and responses to DNA damage and oxidative stress have been suggested (1). c-Abl kinase activity is increased in vivo by diverse physiological stimuli including ionizing radiation (2), entry into S phase (3), integrin activation (4), and platelet-derived growth factor stimulation (5). The mechanism of regulation of Abl tyrosine kinase activity by these processes is not well understood. Ionizing radiation may activate Abl kinase activity through phosphorylation of the Abl catalytic domain at Ser-465 by the Atm kinase (6), whereas platelet-derived growth factor stimulation is associated with tyrosine phosphorylation of c-Abl by c-Src (5). In contrast, the activation of nuclear c-Abl in S phase is through the detachment of the inhibitor Rb protein (3), whereas Abl may be activated by free radicals through dissociation of Pag/Msp23, an antioxidant protein that also inhibits Abl (7). Abl kinase activity can also be stimulated by the binding of several activator proteins, including the transcription factors c-Jun (8) and RFX1 (9), and the adapter protein Nck (10). These observations suggest complex regulation of c-Abl at multiple levels through binding or dissociation of activators and inhibitors and via direct phosphorylation.

Although the NH\(_2\)-terminal sequence of c-Abl is very similar to members of the Src family, biochemical and genetic studies suggest that the structural basis of regulation of c-Abl catalytic activity is significantly different from the catalytic activity of Src. When co-expressed with another kinase, such as Csk, that can phosphorylate the COOH-terminal regulatory tyrosine 527, c-Src (and the Src family member Hck) can be purified as an inactive monomer in which the phosphorylated Tyr-527 residue binds the SH2 domain in an intramolecular fashion. In this structure, the SH3 domain contacts the linker region between SH2 and the catalytic domain (the SH2-C2 linker) in an atypical interaction involving a single proline (Pro-250) (11, 12). Mutation or deletion of Tyr-527 (13, 14) or mutation of the SH2 or SH3 domains (15, 16) dysregulates and increases Src kinase activity both in vitro and in vivo. The precise mechanism of physiological activation of Src kinases is unknown, but in vitro studies demonstrate that activation may involve discrete steps that independently increase catalytic activity. In the presence of ATP and magnesium, Src or Hck that is monophosphorylated at the Tyr-527 homologue undergoes slow autophosphorylation at Tyr-412 that increases kinase activity about 10-fold (17–19). Dissociation of the SH2-Tyr-527 interaction by dephosphorylation or a competing SH2 ligand stimulates activity 2.5-fold, whereas the disruption of the SH3-linker interaction by an activating SH3 ligand, such as Nef (17), induces a further 3-fold increase in catalytic activity to a maximally activated state.

Unlike Src kinases, c-Abl lacks phosphorytrosine in its inactive state, and deletion of the C terminus or mutation of SH2 does not activate Abl (20, 21). However, deletion of the SH3 domain (20, 22) and SH3 point mutations that block PXXP ligand binding (23) do not stimulate Abl kinase activity in vivo as does the mutation of a proline residue (Pro-242) in the Abl

* This work was supported in part by a Howard Hughes predoctoral fellowship (to B. B. B.) and by National Institutes of Health Grant CA72465 (to R. A. V.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: SH2, Src homology 2; SH3, Src homology; GST, glutathione S-transferase.
SH2-CD linker region that is homologous to Src Pro-250 (24). Whereas the SH2-CD linker mutation implies an intramolecular role for the SH3 domain in Abl regulation, immunoprecipitated c-Abl and SH3-mutated Abl have similar high levels of kinase activity in vitro (22, 23, 25), suggesting that the regulatory function of the Abl SH3 domain is only apparent within the cell. An alternative model is that SH3 regulates Abl kinase activity through the binding of a cellular inhibitor (26). The expression of c-Abl in cells at up to 10-fold over endogenous levels does not result in Abl autophosphorylation (20, 27), but the expression at higher levels (20–50-fold) results in tyrosine phosphorylation of Abl and other cellular proteins (7, 26). Furthermore, c-Abl kinase activity is suppressed when expressed in Saccharomyces cerevisiae and Xenopus oocytes (28) but not in Schizosaccharomyces pombe (29). These observations suggest that wild-type and SH3-mutated c-Abl have similar intrinsic kinase activity, but the SH3-mutated c-Abl can no longer associate with a cellular inhibitor. By inference, the putative inhibitor is abundant but can be titrated upon the overexpression of Abl, does not efficiently immunoprecipitate with Abl, and is absent in fission yeast. Several Abl SH3-binding proteins have been identified as candidates for such an inhibitor, including Pag/Msp23 (7), AAP1 (30), Abi-1 (31), and Abi-2 (32). Of these inhibitors, Pag/Msp23 has been shown to inhibit c-Abl kinase activity upon co-expression in vivo (7).

To better understand the regulation of Abl tyrosine kinase activity, we purified c-Abl in a form that should correspond to its inactive state by using the specific Abl kinase inhibitor STI 571 to prevent activation and autophosphorylation upon overexpression in vitro. We found that unphosphorylated c-Abl had substantial intrinsic catalytic activity relative to inactive c-Src, and this basal activity was further stimulated by autophosphorylation at two distinct regulatory tyrosine residues. Surprisingly, we found that the mutation of the SH3 domain significantly increased the basal activity of c-Abl, supporting an intramolecular regulatory role for SH3. Together, these results suggest a model where c-Abl is activated in vivo by dissociation of an inhibitor followed by phosphorylation at tyrosine residues within the catalytic domain and the SH2-CD linker region.

EXPERIMENTAL PROCEDURES

Expression and Purification of Abl Proteins—The murine type IV c-abl cDNA in the vector pCDNA3 (Invitrogen) was modified to include six histidine codons at the 3’ end. The alteration changed the C-terminal amino acid sequence from . . . DIVRR to . . . DIVRRYPFRGNGG-GHHHHHH. Abl mutants were generated by inverse polymerase chain reaction and confirmed by DNA sequencing.

Abl proteins were expressed by transient transfection of 293T cells as described previously (33), except that medium was supplemented with 50 μM STI 571 (Novartis) where indicated. 48–60 h posttransfection, cells were collected and washed twice with phosphate-buffered saline supplemented with 5 mM EDTA, washed once with phosphate-buffered saline only, and then solubilized (1 ml/80-mm plate) in lysis buffer (0.5% Triton X-100, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5% glycerol, 5 mM β-glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamide, 0.7 μg/ml pepstatin, 10 μg/ml leupeptin, and 10 μg/ml aprotonin) and placed on ice for 15 min. Lysates were cleared by centrifugation at 13,000 × g for 20 min at 4 °C and then added to cobalt nitrilotriacetic acid-agarose (Talon resin, CLONTECH) at a ratio of 2 ml of lysate to 200 μl of packed volume agarose. Binding reactions were allowed to proceed at 4 °C for approximately 30 min with constant gentle shaking, and then the mixture was transferred to 5-ml disposable chromatography columns. Each column was washed with 1 ml of lysis buffer followed by 0.5 ml of wash buffer I (20 mM Tris, 10 mM imidazole, pH 8.0, 150 mM NaCl, 0.05% Brij35, 0.1 mM EDTA, and protease inhibitors), 0.5 ml of wash buffer II (same as wash buffer I but with 20 mM imidazole), and eluted with 0.5 ml of elution buffer (same as wash buffer I but with 100 mM imidazole). Eluted products were adjusted to 2 mM EDTA and 1 mM dithiothreitol and then dialyzed overnight against 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 0.01% Brij35, and 1 mM dithiothreitol. During dialysis, 15 μl of agarose-linked anti-phosphotyrosine antibodies (Oncogene Science, Inc.) was included to remove residual phosphotyrosine-containing proteins. Dialyzed samples were centrifuged for 30 min at 13,000 × g and then stored on ice for up to 3 days.

The concentration of purified Abl proteins was determined by SDS-polyacrylamide gel electrophoresis analysis and Coomassie Blue staining compared with purified bovine serum albumin standards (Pierce). These were quantitated by densitometry using a digital camera and NIH Image software. The typical yield from two transfected plates was 12–25 μg of total full-length Abl protein at 25–50 ng/ml.

Autophosphorylation Reactions—Abl phosphorylation reactions were carried out at various kinase concentrations at 30 °C in kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 2 mM dithiothreitol, 1 mM EGTA, and 0.01% Brij35) and in 500 μM ATP. For timed autophosphorylation reactions, all additions other than ATP were mixed and preheated for at least 5 min before the addition of ATP. Autophosphorylation reactions were terminated by the addition of Laemmli sample buffer and boiling. Proteins were then separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and detected with anti-phosphotyrosine (4G10, UBI), anti-Abl (3F12, a gift of R. Salgia, Dana-Farber Cancer Institute), monoclonal antibodies, and enhanced chemiluminescence (Amersham Pharmacia Biotech). Blots were digitized by scanning, and relative phosphorylation levels were determined using NIH Image and Origin 5.0 (Microcal) software.

Kinase Assays—Specific activity of unphosphorylated Abl kinases was determined using a peptide substrate containing the preferred Abl substrate sequence (34) and modified with an amino-terminal biotin, biotin-GEAIAAYAPFKK-amide. Kinase assays were carried out at 30 °C in kinase buffer plus 50 μM ATP, [γ-32P]ATP at 5000–7000 cpm/pmol, and peptide substrate. Assays were done in triplicate for each substrate concentration and were allowed to proceed for 5 min before termination by the addition of guanidine hydrochloride to 2.5 mM final concentration. After termination, portions of each reaction were spotted onto streptavidin-coated paper discs (Sigma-TECT, Promega) and then sequentially washed with 2 mM NaCl followed by 2 mM NaCl with 500 μM and kinase at 0.04 μM final concentrations. At indicated times, aliquots of autophosphorylation reactions were withdrawn and terminated for SDS-polyacrylamide gel electrophoresis-Western blot analysis or added to prewarmed mixtures of kinase buffer (γ-32P]ATP, and peptide substrate. Peptide phosphorylation reactions were done in duplicate or triplicate for 5 min as described above using a single concentration of peptide substrate (100 μM final concentration) to measure activity. Dilution of Abl kinases in the transfer from autophosphorylation reactions to peptide kinase reactions resulted in a final kinase concentration of 0.01 μM in the peptide kinase reactions.

Background binding to the discs was determined by omitting peptide substrate in a series of assays and was usually less than 0.03% total input counts. Incorporated counts for each kinase substrate combination were averaged, adjusted for background, and plotted on a double-reciprocal (1/V versus 1/[S]) graph using the Origin 5.0 program to calculate V and Kmax values. The most accepted model for the effects of autophosphorylation on kinase specific activity, the two assays detailed above were combined. Autophosphorylation reactions were carried out as described and typically contained unlabeled ATP at 500 μM and kinase at 0.04 μM final concentrations. At indicated times, aliquots of autophosphorylation reactions were withdrawn and terminated for SDS-polyacrylamide gel electrophoresis-Western blot analysis or added to prewarmed mixtures of kinase buffer, [γ-32P]ATP, and peptide substrate. Peptide phosphorylation reactions were done in duplicate or triplicate for 5 min as described above using a single concentration of peptide substrate (100 μM final concentration) to measure activity. Dilution of Abl kinases in the transfer from autophosphorylation reactions to peptide kinase reactions resulted in a final kinase concentration of 0.01 μM in the peptide kinase reactions.

RESULTS

Preparation of Non-tyrosine-phosphorylated c-Abl—Full-length myristoylated (type IV) murine c-Abl proteins containing a C-terminal hexahistidine tag were expressed in 293T cells and purified in a single step by affinity chromatography on Co2+-agarose. c-Abl normally lacks detectable levels of tyrosine phosphorylation in vitro in its inactive state (28) but high level expression of Abl in mammalian protein expression cells results in significant levels of Abl tyrosine phosphorylation (28). To isolate unphosphorylated c-Abl, transfected cells were grown in the presence of Novartis STI 571, an Abl-specific kinase inhibitor (35), and any residual tyrosine-phosphorylated proteins were removed from purified kinase preparations with agarose-conjugated anti-phosphotyrosine antibodies. c-Abl was the pre-

2 B. B. Brasher and R. A. Van Etten, unpublished observations.
Coomassie Blue staining (left) or Western blotting (B) with anti-Abl (left panel) and anti-phosphotyrosine (α-PTyr, right panel) antibody. The predominant protein was of the correct size for type IV c-Abl and was not present in CoCl₂-agarose-binding proteins that were isolated from non-transfected cells. A polypeptide of about 48 kDa was variably present in some c-Abl preparations but does not react with anti-Abl antibodies and is found in CoCl₂-agarose-binding fractions from non-transfected cells. No phosphotyrosine was detected on c-Abl using either 4G10 (shown) or Py20 anti-phosphotyrosine antibodies. C, peptide kinase activity of purified c-Abl in the presence of increasing amounts of Novartis STI 571 demonstrated a single activity with an IC₅₀ of 0.44 μM. The lower IC₅₀ value (0.025 μM), previously reported for inhibition of c-Abl by STI 571 (45), might reflect lower Abl concentrations or the use of a suboptimal substrate. D, purified unphosphorylated c-Abl (●) and c-Abl P131L (○) were incubated with [γ-³²P]ATP and increasing amounts of peptide substrate in triplicate 5-min reactions; peptide phosphorylation was measured and used to calculate specific activity for each concentration of the substrate. Kₘ and Vₘₐₓ were determined by fitting double-reciprocal plots (E).

Unphosphorylated c-Abl Has Significant Kinase Activity That Is Increased by SH3 Mutation—To study the in vitro catalytic activity of purified c-Abl, we employed a sensitive kinase assay using a biotinylated peptide with a sequence preferred by Abl kinases (34). Unphosphorylated wild-type c-Abl demonstrated substantial activity toward the peptide substrate with an average Vₘₐₓ of 33 pmol of phosphate min⁻¹ and Kₘ of 204 μM (Fig. 1D). Deletions (20, 22) and some point mutations (23) in the c-Abl SH3 domain dysregulate Abl kinase activity in vivo, inducing high levels of tyrosine phosphorylation of Abl and many other proteins and usually causing cellular transformation. However, the in vitro kinase activities of wild-type and SH3-mutated c-Abl are similar when measured after immunoprecipitation (22, 23). In contrast, we found that a transforming c-Abl protein containing a point mutation in the SH3 domain (P131L) that disrupts the binding of proline-rich ligands (23) exhibited significantly higher catalytic activity than did c-Abl when measured in solution with the peptide substrate. Unphosphorylated c-Abl P131L protein had a Vₘₐₓ approximately 3.5 times higher (112 pmol min⁻¹) and a reduced Kₘ (91 μM) relative to wild-type c-Abl (Fig. 1D). A CoCl₂-agarose affinity-purified preparation from non-transfected cells had only background levels of activity, again confirming that Abl was the sole kinase activity measured in our assay (data not shown). Therefore, the mutation or deletion of the SH3 domain appears to significantly increase the intrinsic tyrosine kinase activity of unphosphorylated c-Abl. We have also used a physiological protein substrate of c-Abl, GST-Crk (36), as a substrate in this assay and observed similar results (data not shown). However, Crk and many other polypeptide substrates of Abl can stably bind to c-Abl and may perturb Abl kinase activity. For this reason, the peptide substrate was employed exclusively in this report.

c-Abl Autophosphorylation Stimulates Kinase Activity—Autophosphorylation leads to increased catalytic activity for many kinases including c-Src (37), and we tested whether the same was true for c-Abl. Wild-type c-Abl and the P131L mutant were allowed to autophosphorylate, and intrinsic kinase activity was measured with the peptide assay. Both c-Abl and SH3-mutated c-Abl were rapidly tyrosine-phosphorylated when incubated in the presence of magnesium ion and ATP (Fig. 2A). Autophosphorylation of c-Src and Hck is concentration-dependent, suggesting an intermolecular reaction mechanism (17, 37, 38). When the Abl concentration was increased in the autophosphorylation reactions, the minimum time required to detect Abl phosphoryrosine decreased, and the rate of phosphoryrosine accumulation after initial detection increased (Fig. 2B). These results demonstrate that the c-Abl autophosphorylation rate is dependent on kinase concentration and suggest that autophosphorylation by c-Abl is also an intermolecular event. As autophosphorylation progressed, an increased ability of c-Abl to phosphorylate the peptide substrate was observed (Fig. 2C).
Although there was some variability in autophosphorylation-induced activation of c-Ab peptide kinase activity among different Abl preparations, increased peptide kinase activity closely matched the increase in Abl phosphotyrosine content in each experiment. c-Abl catalytic activity toward the peptide substrate continued to increase to as high as 22-fold over the basal level after 60 min of autophosphorylation (Fig. 2D).

The kinase activity of the SH3-mutated c-Abl was also stimulated by autophosphorylation (Fig. 2C), demonstrating that the SH3 mutation and autophosphorylation act independently to increase the catalytic activity of c-Abl. The activation of c-Abl P131L proceeded somewhat more rapidly than wild-type c-Abl, reaching a maximum after 10 min; however, the increase in activity of c-Abl P131L was only about 3.6-fold (Fig. 2D). The final activity of tyrosine-phosphorylated wild-type and SH3-mutated c-Abl was very similar (Fig. 2C) and equal to or greater than the specific activity of c-Abl that was purified from cells without treatment with STI 571 (data not shown). These results suggest that c-Abl is capable of maximal activation under these conditions and that autophosphorylation of c-Abl ultimately overcomes the intrinsic inhibitory effect of the SH3 domain.

Activation of c-Abl by Autophosphorylation Requires Catalytic Domain Tyrosine 412—Tyrosine 412 within the catalytic lobe of the c-Abl kinase domain is homologous to c-Src tyrosine 416, and autophosphorylation at tyrosine 416 has been shown to stimulate the kinase activity of Src kinases (19, 37). Tyrosine 412 is known to be a major site of tyrosine phosphorylation in transforming Abl proteins (39). A c-Abl Y412F mutant accumulated little phosphotyrosine upon high level expression in 293T cells in the absence of STI 571 (Fig. 3A), confirming that Tyr-412 is a major in vivo tyrosine phosphorylation site of c-Abl. Whereas mutation of the Tyr-416 homologue in Hck to alanine partially activates kinase activity (19), unphosphorylated c-Abl Y412F displayed similar peptide kinase kinetics as wild-type c-Abl (Fig. 3B), demonstrating that mutation of tyrosine 412 to phenylalanine did not significantly alter the basal catalytic activity of Abl. c-Abl Y412F was able to autophosphorylate upon incubation with magnesium and ATP (Fig. 3C), but the amount of phosphotyrosine was reduced in comparison with wild-type c-Abl, and phosphorylation was maximal by 20 min. Autophosphorylation of c-Abl Y412F was accompanied by increased peptide kinase activity, but the increase was very modest, peaking at a maximum of 4-fold activation 5 min after the addition of ATP (Fig. 3D). Peptide kinase activity then dropped slightly and remained steady for the next 60 min, very similar to the kinetics of autophosphorylation (Fig. 3C). These results demonstrate that Tyr-412 is required for most of the stimulatory effect of autophosphorylation on Abl catalytic activity and suggest that, as for c-Src, phosphorylation of this activation segment tyrosine increases c-Abl kinase activity. However, the fact that c-Abl Y412F can still autophosphorylate and exhibit some increased catalytic activity implies that additional kinase-activating autophosphorylation sites may exist within c-Abl.

It was previously demonstrated that tyrosine 412 is not required for the transformation of fibroblasts by SH3-deleted c-Abl (40). To determine the contribution of Tyr-412 autophosphorylation to the catalytic activity of SH3-mutated Abl, we also purified the c-Abl double mutant P131L/Y412F in the unphosphorylated state and assessed its autophosphorylation and kinase activity relative to c-Abl P131L. The c-Abl P131L/Y412F mutant rapidly autophosphorylated to about the same extent as c-Abl P131L (Fig. 3E). However, the catalytic activity of the double mutant was stimulated to only about 55% catalytic activity of c-Abl P131L (Fig. 3F). These results demonstrate that phosphorylation at tyrosine 412 also stimulates the activity of SH3-mutated c-Abl, but the relative contribution of Tyr-412 phosphorylation to overall Abl catalytic activity is lower in the presence of SH3 mutation. As noted above, these data also suggest the presence of additional tyrosines within Abl that can enhance in vitro kinase activity when phosphorylated.

Phosphorylation of Tyrosine 245 Independently Stimulates c-Abl Kinase Activity—Previous studies identified two major sites of tyrosine phosphorylation in the transforming p120 v-Abl protein (39). Although specific tyrosines were not identified, analysis of tryptic phosphopeptides revealed that one site was six residues and the other site was seven residues COOH-terminal to trypsin cleavage sites in v-Abl. c-Abl tyrosine 412 is seven residues away from a trypsin cleavage site and represents one of these phosphorylation sites. Because trypsin will not cleave at proline residues, there are two candidates for the second site in c-Abl, RNKPTY and KLGQQY (27). The latter site is not noticeably phosphorylated when c-Abl is overexpressed in 293T cells (41). However, the Tyr-245 site is of particular interest because it is in the SH2-CD linker region adjacent to Pro-242, of which the mutation to Ala (24) or Leu (38) stimulates c-Abl kinase activity in vivo and induces cell transformation.

To test the hypothesis that Tyr-245 may be a site of regulatory tyrosine phosphorylation in c-Abl, we mutated tyrosine 245 to phenylalanine and purified c-Abl Y245F in the unphosphorylated state. The c-Abl Y245F mutant also accumulated significantly less phosphotyrosine than wild-type Abl upon overexpression (Fig. 3A), confirming that Tyr-245 is phosphorylated in vivo under these conditions. Purified unphosphorylated c-Abl Y245F displayed very similar kinetics to wild-type c-Abl, demonstrating that this mutation does not significantly alter the intrinsic basal activity of c-Abl (Fig. 3B). However, c-Abl Y245F exhibited substantially less stimulation of kinase activity in response to autophosphorylation. The catalytic activity of c-Abl Y245F was rapidly stimulated about 7-fold within 5 min in the presence of magnesium and ATP but then increased only minimally over the next 60 min, reaching a final activity of only about half that of wild-type c-Abl (Fig. 3C). The two kinases accumulated similar levels of phosphotyrosine (Fig. 3D), but the ratio of phosphotyrosine to full-length Abl protein was slightly higher for wild-type c-Abl, particularly at later time points. We also produced a c-Abl Y245F/Y412F double mutant and observed greatly decreased autophosphorylation (Fig. 3C) and minimal (1.5-fold) stimulation of catalytic activity with autophosphorylation (Fig. 3D). These results suggest that autophosphorylation of Tyr-245 requires prior phosphorylation at Tyr-412 but has an independent stimulatory effect on c-Abl kinase activity, and phosphorylation at Tyr-412 and Tyr-245 together account for the majority of the positive regulatory action of autophosphorylation on c-Abl catalytic activity.

To determine if Tyr-245 contributes to the activation of the c-Abl P131L/Y412F mutant, we produced the c-Abl triple mutant P131L/Y245F/Y412F (denoted “LFF”). Peptide kinase kinetics for the unphosphorylated LFF protein were very similar to the peptide kinase kinetics of c-Abl P131L/Y412F (Fig. 3F) (and data not shown), again demonstrating that the Y245F mutation does not have an appreciable effect on intrinsic activity of unphosphorylated Abl kinases. The LFF kinase had only a slight reduction in the accumulation of tyrosine phosphorylation relative to c-Abl P131L and the c-Abl P131L/Y412F double mutant (Fig. 3E), suggesting that Abl can phosphorylize...
Y412F mutants accumulate less phosphotyrosine upon overexpression in vivo. Anti-phosphotyrosine (α-PTyr, top panels) and anti-Abl (bottom panels) blots of total lysates of 293T cells 48 h after transfection with equal amounts of expression vectors for c-Abl wild type (W.T.) or the Y412F, Y245F, and P131L mutants. Cells were grown without STI 571.

**DISCUSSION**

These studies represent the first reported enzymological analysis of c-Abl in solution and confirm that the structural basis of regulation of Abl kinase activity differs significantly from that of Src family members. We found that purified, unphosphorylated c-Abl had substantial intrinsic catalytic activity, transferring phosphate to a peptide substrate at up to 33 pmol min \(^{-1}\)pmol of Abl. By contrast, purified Hck that was monophosphorylated at tyrosine 527 phosphorylated peptide in a similar assay at about 4.9 pmol min \(^{-1}\)pmol of Hck (17). Although we cannot rule out the possibility that the non-tyrosine-phosphorylated c-Abl we have purified has been activated by in vivo overexpression through some other mechanism, it seems unlikely. The treatment of purified c-Abl with calf intestinal alkaline phosphatase did not alter the kinase activity (data not shown), suggesting that the Abl was not activated in vivo through serine/threonine phosphorylation. Furthermore, the degree of purity of the Abl excludes the possibility of co-purification of an activator protein. The substantial intrinsic catalytic activity of purified c-Abl might therefore be a consequence of purification away from a cellular inhibitor. In support of this theory, we have demonstrated that Pag/Msp23 protein can strongly inhibit the peptide kinase activity of purified c-Abl while completely suppressing autophosphorylation.\(^4\)

Many previous studies found the in vitro tyrosine kinase activity of wild-type and SH3-mutated c-Abl to be similar in immune complex kinase assays (22, 23) or when assayed as GST-Abl fusion proteins in solution (25). In contrast, we found that an SH3 point mutation led to a significant and reproducible increase in the intrinsic catalytic activity of purified, unphosphorylated c-Abl that was relative to wild-type kinase. The reason this difference was not detected in earlier studies is not clear. Because autophosphorylation tends to equalize the catalytic activity of wild-type and SH3-mutated Abl, the increased local concentration of Abl in an immune complex might stimulate rapid autophosphorylation of Abl and produce similar kinase activity when measured at times greater than 10 min. Similarly, autophosphorylation upon in vivo overexpression may account for the similar catalytic activity of wild-type and SH3-deleted c-Abl when purified as GST fusion proteins (25). A previous study demonstrated that the mutation of c-Abl Pro-242 in the SH2-CD linker or the destruction of a potential salt bridge between the SH3 and catalytic domains activated Abl kinase activity in vivo (24). This study suggested that the Abl SH3 domain interacts with the SH2-CD linker region and the catalytic domain to inhibit Abl kinase activity, similar to the mechanism elucidated for Src family members. Our results also

\(^4\) S.-T. Wen, B. B. Brasher, and R. A. Van Etten, unpublished data.

![Figure 3](http://www.jbc.org/)

**FIG. 3.** Autophosphorylation at tyrosine 412 and tyrosine 245 stimulates the catalytic activity of c-Abl. A, the c-Abl Y245F and Y412F mutants accumulate less phosphotyrosine upon overexpression in vivo. Anti-phosphotyrosine (α-PTyr, top panels) and anti-Abl (bottom panels) blots of total lysates of 293T cells 48 h after transfection with equal amounts of expression vectors for c-Abl wild type (W.T.) or the Y412F, Y245F, and P131L mutants. Cells were grown without STI 571. The relative amount of phosphotyrosine on the Abl proteins (arrowheads) was determined by densitometry, corrected for expression level, and expressed relative to c-Abl wild type (set at 1.0). B, Lineweaver-Burk double-reciprocal plot of kinetic parameters of purified unphosphorylated c-Abl wild type (■), c-Abl Y245F (▲), and c-Abl Y412F (○). C, anti-Abl (top panel) and anti-phosphotyrosine (bottom panel) blots of purified unphosphorylated c-Abl (W.T.), c-Abl Y412F, c-Abl Y245F, and c-Abl Y412F/Y245F incubated at 0.04 μM Abl with magnesium and ATP for increasing times. D, kinase activity (left panel) and -fold activation (right panel) for c-Abl (■), c-Abl Y245F (▲), c-Abl Y412F (○), and c-Abl Y412F/Y245F (●). Purified unphosphorylated Abl (0.04 μM) was incubated with magnesium and ATP, and aliquots were removed at the indicated times, diluted appropriately, and assayed for peptide kinase activity in triplicate 5-min reactions. Error bars represent standard deviation between triplicate samples from a representative experiment (left panel) or the average of multiple independent experiments (right panel). E, anti-Abl (top panel) and anti-phosphotyrosine (bottom panel) blots of purified unphosphorylated c-Abl P131L, P131L/Y412F, and the triple mutant LFF incubated at 0.04 μM Abl with magnesium and ATP for increasing times. F, kinase activity (left panel) and -fold activation (right panel) for c-Abl P131L (●), c-Abl P131L/Y412F (○), and c-Abl LFF (△), as described for D.
support a model where the principal negative regulatory effect of the Abl SH3 domain is through an intramolecular mechanism. If SH3 is bound intramolecularly to Pro-242 in purified unphosphorylated c-Abl, what could be the role of an SH3-associated Abl inhibitor? In Src proteins, the binding of phosphorylated Tyr-527 by SH2 helps stabilize the atypical SH3-linker interaction, whereas a critical Leu-255 in the SH2-CD domain with the linker region between the SH2 and catalytic domains. Upon purification away from the inhibitor, Abl acquires substantial catalytic activity that is further enhanced by primary phosphorylation at Tyr-412. This is rapidly followed by secondary phosphorylation at Tyr-412, which effectively disrupts the SH3-linker interaction. A similar level of activation is obtained by mutations in SH3 that disrupt ligand binding.

We demonstrate here that autophosphorylation of c-Abl is intermolecular and stimulates Abl catalytic activity. Phosphorylation of Tyr-412 increases c-Abl kinase activity about 9-fold, an effect that is similar to the stimulation observed upon phosphorylation of the homologous tyrosine in Src kinases and in the insulin receptor. The crystal structure of the isolated c-Abl catalytic domain complexed with STI 571 has recently been solved (43). In this structure, which probably represents the inactive configuration, Tyr-412 is positioned exactly as in a substrate peptide, but a critical Asp-Phe-Gly motif that is necessary for metal ion ligation is displaced, preventing efficient phosphate transfer. The orientation of this activation loop tyrosine in Abl is identical to the orientation of the insulin receptor (44) but distinct from that found in the inactive Src kinases (11, 12). It is likely that phosphorylation of Tyr-412 destabilizes the closed conformation of the activation loop because of electrostatic repulsion that leads to an open conformation characteristic of active protein kinases. In vivo, Abl might be physiologically activated by autophosphorylation or by phosphorylation of Tyr-412 by another tyrosine kinase, such as c-Src (5).

We also found that phosphorylation of Tyr-245 in c-Abl stimulates c-Abl kinase activity about 2.5-fold. However, the effects of phosphorylation of Tyr-245 and Tyr-412 are not entirely independent of one another. Because activation is greatly inhibited in the c-Abl Y412F mutant but only about 50% decreased in c-Abl Y245F, it supports a model where c-Abl might be activated in vivo in a stepwise fashion with phosphorylation at Tyr-412 followed by Tyr-245 (Fig. 4). It is interesting that Tyr-245 is three residues from Pro-242, which is postulated to bind the Abl SH3 domain. Tyrosine is found in this position in murine and human c-Abl and human Arg, but not in any Src family member. Src has a glutamine at this position whereas Hck has a proline, and the respective crystal structures (11, 12) show some degree of engagement of SH3 pocket 2 by this residue. It is plausible that phosphorylation of this tyrosine in Abl induces a negative charge or steric hindrance that disrupts the interaction of SH3 and Pro-242, mimicking the effect of SH3 mutation on the catalytic activity of c-Abl (Fig. 4). The Abl purification strategy and peptide kinase assay described here should be useful for furthering our understanding of the regulation of c-Abl and its oncogenic relatives.

Acknowledgments—We thank Drs. Brian Drucker (University of Oregon) for the generous gift of STI 571 and Bruce Mayer (University of Connecticut) for many helpful suggestions and for critically reading the manuscript.

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