Allosteric inhibition of the nonMyristoylated c-Abl tyrosine kinase by phosphopeptides derived from Abi1/Hssh3bp1

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Received 30 July 2007; received in revised form 9 January 2008; accepted 28 January 2008
Available online 15 February 2008

Abstract

Here we report c-Abl kinase inhibition mediated by a phosphotyrosine located in trans in the c-Abl substrate, Abi1. The mechanism, which is pertinent to the nonmyristoylated c-Abl kinase, involves high affinity concurrent binding of the phosphotyrosine pY213 to the Abl SH2 domain and binding of a proximal PXXP motif to the Abl SH3 domain. Abi1 regulation of c-Abl in vivo appears to play a critical role, as demonstrated by inhibition of pY412 phosphorylation of the nonmyristoylated Abl by coexpression of Abi1. Pervanadate-induced c-Abl kinase activity was also reduced upon expression of the wild type Abi1 but not by expression of the Y213 to F213 mutant Abi1 in LNCaP cells, which are naturally deficient in the regulatory pY213. Our findings suggest a novel mechanism by which Abl kinase is regulated in cells.

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Keywords: c-Abl; Tyrosine kinase activity; Abi1; Hssh3bp1; Allosteric mechanism; SH3 and SH2 domain

1. Introduction

The ubiquitous nonreceptor tyrosine kinase, c-Abl kinase, plays an essential role in cell signal transduction, balancing events leading to apoptosis or to increased cell proliferation [1,2]. The critical role of c-Abl kinase in cell proliferation is illustrated by the dramatic manifestation of chronic myelogenous leukemia (CML) due to expression of BCR-Abl, a kinase-activated mutant form of c-Abl tyrosine kinase [3,4]. Rational approaches to curtail BCR-Abl kinase activity led to the development of STI-571 (imatinib mesylate, or Gleevec) [5] as the successful treatment of CML [6]. However, the appearance of Gleevec-resistance mutations [7,8], that becomes an issue in advanced stage CML [9] led to re-thinking of the mechanism of BCR-Abl regulation. This has brought attention back to c-Abl, since BCR-Abl and c-Abl share most regulatory domains [2,10].

Autoinhibition has emerged as the mechanism of regulation of c-Src and c-Abl [11,12]. These kinases share high structural homology conferred by the presence of highly conserved structural domains: SH3, SH2, and the catalytic domain. Crystal structures of c-Src [13,14] and c-Abl [15,16] revealed that SH3 and SH2 domains bind to the catalytic domain (CD) inducing an autoinhibitory conformation, which provides the basic mechanism of regulation of these kinases. This basic regulation is preserved in BCR-Abl, which retains the c-Abl SH2 and SH3 domains.

c-Src and c-Abl differ from each other in two mechanisms that inhibit activities of these kinases. In c-Src, inhibition is achieved by intramolecular interaction of the SH2 domain with the phosphorylated tyrosine 527 located in the C-terminal region of the same molecule [17]. In c-Abl there is no internal phosphotyrosine-SH2 domain interaction, precluding this inhibitory mechanism. Additional inhibitory constraints are imposed on c-Abl both by the myristoylated cap which binds directly to the C-terminal lobe of the kinase domain, and by the cap region phosphoserine 69 which binds to the SH2 domain [18]. These interactions further serve to lock the SH3-SH2 “clamp” onto the
catalytic domain. The myristoyl group [15,16], or small compounds mimicking its action [19], stabilize the position of the C-terminal helix of the catalytic domain, αI, resulting in the inhibited conformation of the kinase. The "molecular lock" imposed by the myristoylated cap, however, does not exist in the nonmyristoylated form of c-Abl, isoform 1a, which contains only a partial cap region, or in BCR-Abl, in which the cap region is replaced by BCR. Thus, the kinase activities of BCR-Abl, and c-Abl-1a, are not regulated by the myristoylated cap although the phosphoserine 69 is preserved in c-Abl-1a, where it may contribute to the autoinhibitory mechanism [18]. Intramolecular interactions of the cap region may also regulate accessibility, and therefore, may regulate binding of Abi SH3 or SH2 ligands including phosphotyrosine-containing peptides from growth factors, which may play a role in the myristoylated c-Abl kinase activation [15]. Considering the complexity of Abl regulation, activation of the kinase activity is likely to involve multiple steps leading to uncoupling of SH3 and SH2 domains from the catalytic domain thereby "freeing" the kinase from inhibition. Although not yet demonstrated with Abi proteins, peptides that combine both Abi SH3 and SH2 binding sites into a single consolidated ligand show enhanced binding affinities for the dual SH3–SH2 domain, pointing to the possibility that these domains may act as one functional unit in c-Abl [20].

Various proteins, including Abi1 and Abi2, that bind to c-Abl kinase have been proposed to be c-Abl co-inhibitors [12,21,22]. Abi1 and Abi2 have been thought to play a role in the regulation of cell growth [21–23] but the molecular mechanism is not clear. Abi1 and Abi2 were proposed to regulate c-Abl kinase activity by interaction with C-terminal PXXP sequences [21,22], and through interaction with the c-Abl SH3 domain [24]. No SH3- or SH2-based mechanism of c-Abl kinase regulation, however, has been demonstrated for Abi proteins. The LNCaP prostate tumor cell line contains a mutation in the Abi1 gene that results in deletion of exon 6 [23]. Exon 6 of Abi1 is within the SH3 domain-binding region [24] pointing to the possibility that this region might be critical for c-Abl kinase regulation.

We have further investigated regulation of c-Abl kinase by Abi1 and here report the discovery of a novel allosteric mechanism of inhibition of nonmyristoylated c-Abl kinase mediated by peptides derived from the Abi SH3- and SH2-binding regions of Abi1. Mutations in either binding site can abrogate the capacity to inhibit Abl kinase activity. These data are consistent with observed enhanced binding affinity of the consolidated Abi1 ligand for the dual Abi SH3–SH2 domain over single, i.e. SH3 or SH2, domain Abi1-derived ligands. We propose that Abi1 plays critical role in regulating Abi kinase activity in cells.

2. Materials and methods

2.1. Peptides and antibodies

See Fig. 1 for diagrams of peptides. All peptides were synthesized commercially. Anti-pY213 polyclonal antibody was produced to peptide pY213, and affinity purified using the phosphopeptide-specific column followed by absorption on the nonphosphopeptide (Y213) column. Polyclonal and monoclonal HA antibodies were from Covance (Berkeley, CA) and Roche Diagnostic Corporation (Indianapolis, IN). Antibodies to c-Abl were 8E9 (BD Biosciences, San Jose, CA), K12 (Santa Cruz Biotechnology, Santa Cruz, CA), and pY412 (Biosource International, Camarillo CA). Antibodies to Crk were from BD Biosciences, San Jose, CA (mouse monoclonal), Santa Cruz Biotechnology, Santa Cruz, CA (rabbit polyclonal), and Cell Signaling Technology (phospho-Crk pY221). Polyclonal antibody, Ab-2, to Abi1 was described previously [25]. Polyclonal antibody, Ab-5, to Abi1 was made to peptide TSPPPTIGVADSTTPPP. Monoclonal antibody, 7B6, to Abi1 was produced to recombinant Abi1. The epitope bound by this antibody is identical to that bound by Mab 4E2 [24]. Antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Ingemun Corporation (San Diego, CA). GST antibody was from Zymed (San Francisco, CA). GFP antibody was from Invitrogen (Carlsbad, CA). Generic antibody to phosphotyrosine, PY99, was from Santa Cruz Biotechnology, Santa Cruz, CA.

2.2. Abl kinase

His-tagged, partially capped, active (nonmyristoylated) c-Abl, E46 through C-terminus (isofrom 1b numbering) was produced in baculovirus from plasmid (a kind gift of Tony Koleske, Yale University, New Haven, CT) and purified as described [26] following treatment of insect cells with 30 μM STI-571 (Novartis Pharma AG, Basel, Switzerland) for 48 hrs prior to cell lysis. The expressed protein was affinity purified on nickel-nitriloacetic acid agarose, washed to remove inhibitor, and subsequently purified by ion-exchange chromatography using a Mono S column (Amersham Biosciences, Piscataway, NJ) GST fusions of c-Abl SH3 and SH2 domains and the SH2 variant containing an R171K mutation were obtained from Bruce Mayer (University of Connecticut Health Center, Farmington, CT). For use in fluorescence quenching experiments the dual domain SH3–SH2 polypeptide of c-Abl was expressed from plasmid pTXB1 (New England Biolabs, New England, CT) in E. coli BL21 cells. The recombinant fusion protein was purified through chitin affinity binding (New England Biolabs, New England, CT). After DTT cleavage the SH3–SH2 domain was further purified by SP Sepharose (GE Healthcare, Piscatway, NJ) cation exchange.

2.3. Expression plasmids

Wild type or mutant Abi1 (GenBank Accession No. NM 005470 and U87166) isofrom 2, residue numbering according to [24] were expressed from plasmids. The mutant Abi1-F213 contains a Y213F replacement. At residues 181–185 the mutant Abi1-Pro replaces the sequence AESEA with PPSP, which results in the loss of a PXXP SH3 binding motif. All Abi1 cDNAs were subcloned into the pEGFP-N2 plasmid (Clontech, Mountain View, CA) following removal of GFP-encoding sequences and introduction of an HA tag at the C-terminus. Untagged wild type isofrom 2 of Abi1 was also used for transfections. In vitro translation of the N-terminus of Abi1 was performed as described [23]. The C-terminal GFP fusion of the nonmyristoylated c-Abl (isofrom 1α) was obtained from Bruce Mayer.

2.4. Kinase assay

Measurement of kinase activity was essentially as described in [26], using biotinylated model substrate peptide GGEAIYAAPFKK, [27,28] and 32P-γ-ATP. SAM2 streptavidin-coated membrane (Promega Corporation, Madison WI) was used to capture the substrate. Kinase assays were carried out in kinase buffer (50 mM Tris–HCl pH 7.5, 10 mM MgCl₂, 1 mM EGTA, 2 mM dithiothreitol, 0.01% Brij 35, 100 μM ATP) along with 2N M Ab kinase, substrate peptides, and Abi1 ligand peptides as indicated. Reactions were carried out for 5 min. at 30°C. To evaluate c-Abl kinase activity in LNCaP cell lines, cells were treated with 0.1 mM sodium pervanadate for 10 min. prior to cell lysis; and the kinase was immunoprecipitated from lysed cells, c-Abl kinase activity was evaluated by measuring: 1) phosphorylation levels of activation loop tyrosine 412, 2) total tyrosine phosphorylation, and 3) phosphorylation of endogenous Abl substrate Crk.

2.5. Mass spectrometry

Mass spectrometric analyses of GST-Abi1 peptides were performed using an Applied Biosystems (Foster City, CA) Voyager DE MALDI mass spectrometer. Spectra were calibrated against an external or internal standard as needed.
2.6. Cell culture and transfections

LNCaP and Cos7 cells (ATCC, Rockville, MD) were maintained according to ATCC protocols. Co-transfections of Abi1 with c-Abl in Cos7 cells were performed with the isoform 1a of c-Abl (nonmyristoylated) and isoform 2 of Abi1 using Lipofectamine Plus Reagent (Invitrogen, Carlsbad, CA). At 22 h post-transfection, cells were processed for immunoprecipitation as described [25] following treatment with 10 µM Gleevec for 30 min. LNCaP cell lines stably expressing either wild type, clone Abi1(+), HA-tagged Abi1 isoform 2, or HA-tagged mutants of Abi1 isoform 2 were obtained using G418 selection (Invitrogen, Carlsbad, CA).

2.7. Immunoprecipitation and Western blotting

c-Abl tyrosine kinase was activated by treatment of LNCaP cells for 10 min with 0.1 mM sodium pervanadate (freshly prepared from 100 mM activated sodium orthovanadate and 100 mM H₂O₂), [29] prior to lysis. Immunoprecipitation was performed as described [25]. Western blotting and overlay binding assay to quantify Abi SH3 domain binding were performed as described [24]. All blots were developed using Supersignal West Pico Chemiluminescence Substrate (Pierce Biotechnology, Rockford, IL). Images were acquired using a Kodak GL 440 Imaging System and quantified using Kodak 1D Image Analysis Software (Version 3.6.4).

2.8. Surface plasmon resonance

Surface plasmon resonance was performed using a Biacore 3000 instrument (BIAcore Inc., Piscataway, NJ). Biotinylated 14-residue peptides, pY213 or Y213, were coupled to the surface of a streptavidin-coated (SA) biosensor chip (BIAcore Inc., Piscataway, NJ). Binding reactions were done in HBS-EP buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.05% (v/v) surfactant P20). The surface was regenerated before each new injection using 50 mM
NaOH and 1M NaCl. The Biacore instrument was programmed to perform a series of binding assays with increasing concentrations of GST Abl SH2 or GST Abl SH3–SH2 polypeptides over the same regenerated surface. Derived sensograms (plots of changes in response unit on the surface as a function of time) were analyzed using the software BIAeval 3.0. Affinity constants were estimated by curve fitting using a 1:1 binding model.

2.9. Intrinsic fluorescence measurements

Protein and peptide binding affinities were measured by intrinsic fluorescence quenching using a Fluorolog-3 fluorimeter (Horiba Jobin Yvon Inc., Edison, NJ), with excitation at 287 nm and emission detection at 345 nm as described previously [20]. Fluorescence intensity change was monitored as fluorescence quenching using a Fluorolog-3 fluorimeter (Horiba Jobin Yvon, Northampton, MA) to fit a modified Stern–Volmer equation [30].

2.10. Statistical Analysis

For Table 2 (evaluation of $K_m$ and $V_{max}$), and Fig. 4B (Abl kinase activity in LNCaP clones) two-tailed paired $t$-test was used.

3. Results

3.1. c-Abl phosphorylates Y213 of Abi1

Postulating that phosphotyrosines would be components of the active motif, we searched for tyrosine phosphorylation sites of Abi1. We determined that candidate tyrosine residues, Y198 and Y213, are located within the proline-rich region of Abi1 previously demonstrated to bind the Abl SH3 domain [24]. Using in vitro-translated polypeptides encoding the N-terminal half of the protein (Fig. 1A) we determined that Y213 is the preferred site in the N-terminus of Abi1 of phosphorylation by Abl kinase in vitro (Fig. 1B). The phosphorylation site at Y213 was confirmed by mass spectrometry of tryptic peptides following kinase reactions containing the recombinant Abi1 and active c-Abl (Table 1).

3.2. Identification of the minimal Abl SH3 and SH2 domain binding sites of Abi1

Using filter overlay assays we have previously localized binding of the c-Abl SH3 domain to residues 144–260 of Abi1 [24]. This region of Abi1 contains a PXXP SH3 binding motif located upstream of tyrosine 213. Deletion of residues 173 through 187 of Abi1 containing the PXXP sequence, 181PPSP185, almost completely abolished binding to the c-Abl SH3 domain (Fig. 2A).

The proximity of the Abl SH3 binding site to tyrosine 213 led us to hypothesize that phosphorylated tyrosine 213 would interact with the Abl SH2 domain. This putative interaction was analyzed using a biotinylated 14-residue peptide containing phosphorylated tyrosine 213 (pY213) and a GST-Abl SH2 domain fusion protein (Fig. 2B–D). First, we established that the Abl SH2 domain interacted with the phosphopeptide pY213 but not with the non-phosphorylated peptide Y213 (Fig. 2B). Then, using varying concentrations of Abl SH2 we determined that pY213 binds to the SH2 domain with high affinity (dissociation constant, $K_D = 3.32 \times 10^{-8}$ M, dissociation rate, $K_{off} = 3.76 \times 10^{-3}$ M$^{-1}$ s$^{-1}$) (Fig. 2C). Binding of pY213 with similar high affinity to the dual GST Abl SH3–SH2 domain was observed (dissociation constant, $K_D = 3.55 \times 10^{-8}$ M; dissociation rate, $K_{off} = 2.27 \times 10^{-3}$ M$^{-1}$ s$^{-1}$). However, control experiments showed that the Abl SH2 R171K mutant did not interact with pY213 (Fig. 2D) consistent with the finding that the R171 mutation renders SH2 domains incapable of interaction with phosphopeptides [31].

Table 1 Phosphorylation by Mass Spectrometry

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Samples were analyzed following trypsin digestion. Predicted and experimental masses for specific ions containing the indicated tyrosine 213 or phenylalanine replacement are listed. Sequence for wild type (WT) tryptic peptide: TLEPKPTVPNDpYMTSPAR; the phosphotyrosine 213 is in bold. *F213: same as WT but with Y213F replacement, in bold. TLEPKPTVPNDpFMTSPAR. T15WS contains residues 144–305 of Abi1 isoform 4 [24] and includes the same tryptic peptide as WT. 3-Met-O depicts oxidized methionine (underlined in tryptic fragments above) that was present in all samples; therefore two (nonphospho-sample) or four (phospho-sample) species of molecules were observed.

3.3. Consolidated Abi1 ligand exhibits enhanced binding affinity vs. single domain ligands for the dual domain Abl SH3–SH2 domain

High binding affinity measurements of the GST Abl-SH2 domain to pY213 using surface plasmon resonance (SPR) suggested the possibility of a dimerization effect of the GST fusion tag [32,33]. Therefore we employed an intrinsic fluorescence quenching method to determine binding affinities of Abi1 peptides to Abi1 SH3 and SH2 domains as previously demonstrated for optimized Abl ligands [20]. The binding affinities of Pro-Y198 (containing an SH3 domain-binding sequence), pY213 (containing an SH2 domain-binding sequence), and Pro-pY213 (containing the consolidated dual domain SH3–SH2-binding sequence) to the c-Abl SH3–SH2 dual domain were $2.3 \pm 0.4 \times 10^{-2}$ M, $2.4 \pm 0.4 \times 10^{-1}$ M, and $9.9 \pm 0.4 \times 10^{-1}$ M, respectively. These data, obtained via intrinsic fluorescence quenching, suggest that both pY213 and Pro-Y198 are relatively weak binders to their target domains (Fig. 2E). However, when two ligand binding sequences are conjoined as in Abi1, the binding of this natural consolidated ligand, i.e., the peptide Pro-pY213, is of much higher affinity.

3.4. pY213 phosphopeptide inhibits c-Abl kinase activity by a noncompetitive mechanism

Based on the crystal structure of c-Abl kinase, the SH2 domain-phosphopeptide interaction has the potential to regulate c-Abl kinase activity [15]. Therefore we performed experiments to test whether the sequences of Abi1 containing tyrosine 213
Fig. 2. Identification of the minimal binding region of c-Abl SH3 and SH2 domains in the N-terminus of Abi1. A. Binding to c-Abl SH3 domain. Recombinant GST fusion polypeptides containing the N-terminal terminal region of Abi1/Hssh3p1 were separated on SDS polyacrylamide gels and transferred onto the nitrocellulose membrane. The following Abi1 polypeptides were analyzed: N1-187, encoding residues 1–187; N1-172, encoding residues 1–172; N1-253, encoding residues 1–253; N1-253 ΔEx6, encoding residues 1–253 but lacking exon 6 sequences of Abi1 (Macoska et al., 2001); GST, glutathione S-transferase. The bottom panel shows results when the blots were probed with the anti-GST monoclonal antibody (Anti-GST) to indicate level of protein expression. The top panel was incubated with biotinylated GST-Abl-SH3 domain (GST-Abl-SH3-B). No binding to biotinylated GST (GST-B) was observed (middle panel). The histogram shows relative binding of polypeptides, as percent of N1-253 binding, quantified from three independent experiments (n=3, ±s.d.). B–D. Binding to c-Abl SH2 domain. Surface plasmon resonance was used to determine binding between biotinylated 14-residue peptides containing either phosphorylated tyrosine 213 (pY213) or nonphosphorylated tyrosine 213 (Y213) to GST-tagged Abl SH2 domain. B. Biosensor chips coupled with pY213 or Y213 peptides were injected with the Abl SH2 domain (1 μM). C. Biosensor chip coupled with pY213 was injected with different concentrations of Abl SH2 domain, as indicated. D. Biosensor chip coupled with pY213 was injected with 0.5 μM of either the Abl SH2 domain or with the Abl SH2 domain R171K mutant. No binding to the GST protein alone was observed (not shown). RU, response unit. E. Representative fluorescence titration data using purified Abl dual SH3-SH2 domain. Titration curves for the consolidated dual SH3-SH2 domain ligand, Pro-pY213, and single domain ligands, Pro-Y198 for Abl SH3 domain, and pY213 for Abl SH2 domain, are indicated.
affect c-Abl kinase activity. In these experiments we used active nonmyristoylated, uncapped, c-Abl [26], the model substrate peptide [27,34] and forms of the 14-residue peptide containing phosphorysine, pY213, or tyrosine to phenylalanine replacement at position 213, F213 (for peptide sequences see Fig. 1A). Addition of the pY213 phosphopeptide resulted in a 38% reduction of $V_{\text{max}}$ but no significant effect on $K_m$ of the substrate peptide, consistent with a noncompetitive mechanism of inhibition (Fig. 3A–B, and Table 2). No effect on the kinase activity was observed with the peptide F213.

3.5. Abi1 peptides derived from SH2 and SH3 domain binding sites regulate c-Abl kinase activity

To determine the role of SH3 domain binding in the regulation of c-Abl kinase activity we performed kinase assays using as inhibitors various peptides derived from residues 169–217 of Abi1 (Fig. 1A). As shown in Fig. 3C, peptide pY213 inhibited kinase activity in a concentration-dependent manner. Peptide F213, lacking the regulatory tyrosine, did not inhibit kinase activity. Peptide Pro-pY213, which spans the entire region, exhibited a more complex effect including slight enhancement

<table>
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<th>Peptide</th>
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<th>Fold change (PEP)</th>
<th>$K_m$ (µM)</th>
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<td>163.08±17.90*</td>
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<td>25.79±5.56</td>
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<tr>
<td>pY213</td>
<td>97.51±9.17**</td>
<td>0.62</td>
<td>24.90±5.63</td>
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</table>

Summary of $V_{\text{max}}$ and $K_m$ (mean ± s.d.; $n=3$) for Abi1 peptides at 1 mM for F213 and pY213. * $p=0.673$; ** $p=0.001$; PEP, substrate peptide. $K_m$ differences are not statistically significant. For description of peptides see Fig. 1A.

Fig. 3. Phosphopeptides from Abi1 regulate c-Abl kinase activity in vitro. A. Kinase activities (vertical bars represent s.d.; $n=3$) of c-Abl after addition of indicated peptides at 1 mM. Activity was measured with 5-min kinase assays in the presence of increasing substrate peptide concentrations. B. Double reciprocal Lineweaver–Burke plots of the data in A. Vertical bars represent s.d. ($n=3$). See Table 2 for $V_{\text{max}}$ and $K_m$ data from these experiments. C and D. Regulation of c-Abl kinase activity by Abi1 peptides. Concentration of Abi1 peptides is indicated on X axis; substrate peptide, PEP, was used at 100 µM (see Kinase assay, Materials and methods); vertical bars represent s.e.m. ($n=3$). See Fig. 1A for sequence information of peptides used here.
of kinase activity from 0.5 to 1 mM followed by profound inhibition of activity at higher concentrations. Further experiments demonstrated that Pro-pY213 is capable of up-regulating or down-regulating c-Abl activity in a concentration-dependent manner, and that the sequences \(^{181}\text{PPSP}^{185}\), Y198, and pY213 of Abi1 are critical for this regulation (Fig. 3D).

3.6. Abi1 pY213 and \(^{181}\text{PPSP}^{185}\) sequences regulate pervanadate-induced c-Abl tyrosine kinase activity

To determine if Abi1 regulates c-Abl tyrosine kinase activity in cells we used the LNCaP prostate cancer cell line, which, as a consequence of a heterozygous deletion of Abi1 exon 6, lacks the region containing Y213 on one allele. The mutated protein is not detected in LNCaP cells, and wild type Abi1 is expressed at a lower than expected level as compared to Abi1 expression in the primary prostate cell line, PrEC (data not shown). This result is consistent with haploinsufficiency and suggests the possibility that c-Abl kinase activity is dysregulated in LNCaP cells.

To test this possibility we first established that the endogenous c-Abl tyrosine kinase could be activated by pervanadate treatment [2] of LNCaP cells (Fig. 4A). Activation of c-Abl kinase was consistent with phosphorylation of the regulatory tyrosine pY412 [27] and with total tyrosine phosphorylation of c-Abl [34]. Therefore in this experimental setting we could examine whether expression of Abi1 inhibits Abi1 kinase activation. We stably transfected LNCaP cells with untagged wild type isoform 2 of Abi1 (Abi1(+)) or Ha-tagged (Wt.Ha), or with Ha-tagged Abi1 mutants Y213F (F213.Ha), and \(^{181}\text{PPSP}^{185}\) to \(^{181}\)AESEA\(^{185}\) (Pro.Ha). As shown in the immunoblots (Fig. 4B left panel) and the histograms (Fig. 4B, right panel) expression of intact Abi1 inhibited c-Abl kinase activity, whereas expression of Abi1 variants carrying a mutated SH2 domain-binding motif, Y213F, or a mutated SH3 domain-binding motif, \(^{181}\)AESEA\(^{185}\), did not.

Apparently, the introduction of the HA-tag at the C-terminus of Abi1 reduces the ability of the wild type Abi1 to inhibit Abl kinase activity (compare Abi1(+) to Wt.Ha cell lines, Fig. 4B). This would be consistent with a possible negative effect of the HA tag on the interaction between the SH3 domain at the Abi1 C-terminus and the PRL region of Abl as demonstrated in numerous studies [21,22].

3.7. Abi1 inhibits nonmyristoylated c-Abl kinase

The data from LNCaP cells suggested that Abi1 is capable of regulating c-Abl kinase activity. To determine if the observed mechanism of regulation is pertinent to the nonmyristoylated kinase as indicated by in vitro kinase data we co-expressed Abi1 and the nonmyristoylated Abi1 isoform 1a in Cos 7 cells. As shown in Fig. 4C Abi1 reduced levels of pY412 phosphorylation of the nonmyristoylated Abi1, albeit to a lower extent than treatment with STI-571. This treatment also inhibited phosphorylation of Abi1 pY213, and reduced the physical interaction with Abl (Fig. 4C). A pY213-dependent association of Abi1 with c-Abl was also observed in LNCaP cells (Fig. 4D).

4. Discussion

Based on these results we postulate that phosphorylation of Y213 of Abi1 by c-Abl kinase is followed by binding of Abi1 to the Abl SH2 domain with subsequent inhibition of c-Abl kinase activity. If verified, this would be the first demonstration of inhibition of c-Abl kinase by a phosphopeptide located in trans in another protein, in this case, Abi1.

We propose that Abi1 phosphopeptides inhibit c-Abl kinase through an allosteric mechanism. This mechanism involves binding of the phosphorylated Y213 to the Abl SH2 domain. An observed decrease of the \(V_{\text{max}}\), with no change of the \(K_{\text{m}}\), is consistent with a noncompetitive mechanism of inhibition of kinase activity by the phosphopeptide containing pY213. However, the effect of pY213 on Abl kinase activity is relatively weak (decrease of \(V_{\text{max}}\) of about 38% at peptide concentration of 1 mM). This is in contrast to high binding affinity data obtained from surface plasmon resonance studies using the GST tagged Abl SH2 domain. The binding data obtained from intrinsic fluorescence quenching experiments, obtained with the untagged protein, or from overlay binding assays [35] (K. Machida, B.J. Mayer and L. Kotula, unpublished results), indicate much lower binding affinity of pY213, i.e. in the micromolar range. These results suggest a strong effect of the GST tag, most likely due to its dimerization effect, on SH2 binding affinities obtained using SPR as previously suggested [33]. Our binding data for pY213-SH2, obtained with untagged protein, is consistent with the relatively weak effect on Abl kinase activity in vitro.

The in vitro kinase data demonstrated here pertains to the nonmyristoylated, partially capped, partially activated form of c-Abl kinase. However, there is no crystal structural information on the active kinase in the context of the SH3 and SH2 domains. Recent SAXS [18] studies indicate that the active kinase is likely to exist in the elongated form. Thus it is possible that pY213 decreases Abl kinase activity by increasing the rigidity of the kinase domain through the interactions of the SH2 domain and the N-lobe of the kinase domain, hence the non-competitive mechanism of inhibition. The noncompetitive nature of the inhibition is demonstrated by Lineweaver–Burke plot despite a relatively high concentration of the peptide used in kinase assay. Supporting the hypothesis that pY213 regulates c-Abl tyrosine kinase activity through interactions with the c-Abl SH2 domain, is the fact that pY213 regulates physical association of Abi1. This is demonstrated here by binding assays showing interaction of pY213 with the Abl SH2 domain (Fig. 2) as well as by immunoprecipitation results indicating that Abi1-pY213 interacts with the active Abl kinase in LNCaP cells (Fig. 4C and D). pY213 phosphorylation, and consequently the strength of Abi1-Abl kinase interaction, is STI-571-dependent as indicated by co-transfection experiments. Interestingly, treatment of K562 cells with STI-571 reduced pY213 levels as compared to untreated cells [36] suggesting the possibility that Abi1 is a substrate of, and functions downstream of, BCR-Abl.

The lack of a crystal structure of the nonmyristoylated kinase makes it difficult to interpret the effects of Pro-pY213 peptide...
on kinase activity. We base our interpretation on the following facts: 1) Pro-pY213 represents the region from Abi1 that regulates c-Abl kinase activity in vitro. At higher concentrations the peptide inhibits Abl kinase activity. 2) Mutated or truncated peptides either inhibit to a lesser extent than Pro-pY213 or do not inhibit at all. These data permit identification of three critical elements affecting activity: \textit{\textsuperscript{181}PPSPP\textsuperscript{185} (representing the core Abl SH3 domain binding PXXP motif); pY213 (representing the critical SH2 domain binding phosphotyrosine); and Y198, which works together with the \textit{\textsuperscript{181}PPSPP\textsuperscript{185} motif as demonstrated by Pro-Y198 peptide. 3) Multiple conformations of the autoinhibited (inactive) and active Abl kinase are possible based on recent findings on Abl [18] that include SAXS studies as well as Src kinase [37]. These studies indicate significant rearrangements of the SH3–SH2 dual domain around the catalytic domain of Src-like family kinases. Therefore, we hypothesize that different effects of the pY213 and Pro-pY213 and Abl kinase activity might be a result of different peptide affinities to transient Abl conformations. For example, the presence of residual autoinhibitory interactions of the SH3 and SH2 domain assembly with the catalytic domain of the kinase [16] in the nonmyristoylated Abl kinase might explain the lack of effect of autoinhibited (inactive) and active Abl kinase are possible based on recent findings on Abl [18] that include SAXS studies as well as Src kinase [37]. These studies indicate significant rearrangements of the SH3–SH2 dual domain around the catalytic domain of Src-like family kinases. Therefore, we hypothesize that different effects of the pY213 and Pro-pY213 and Abl kinase activity might be a result of different peptide affinities to transient Abl conformations. For example, the presence of residual autoinhibitory interactions of the SH3 and SH2 domain assembly with the catalytic domain of the kinase [16] in the nonmyristoylated Abl kinase might explain the lack of effect of
Fig. 3C and D). Tyrosine phosphorylation of Abl[10] due to autophosphorylation at or near Abi1 peptide binding regions may significantly influence their binding affinities.

The Pro-pY213 region of Abi1 represents an important element that regulates Abl kinase activity in vivo as demonstrated by nonphosphorylated, whereas kinase assays were performed with partially active, tyrosine phosphorylated kinase obtained from baculovirus. In this regard, a low level of pY412 and PY-99 immunoreactivity was confirmed in baculovirus-purified kinase (not shown). Importantly, these types of kinase preparations are extremely prone to activation due to autophosphorylation leading to observed differences in basal kinase activity (compare Fig. 3C and D). Tyrosine phosphorylation of Abl [10] due to autophosphorylation at or near Abi1 peptide binding regions may significantly influence their binding affinities.

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in LNCaP cells. Abi1 Y213F or 181AESEA185 mutants did not inhibit Abl kinase activation, which indicates that concurrent binding of Abi1 to both SH3 and SH2 domain of Abl is critical for regulation. This is consistent with in vitro binding data demonstrating significant enhancement of the binding affinity of the consolidated Abi1 ligand over single-site ligands. It is possible that the 181AESEA185 mutant, despite having a higher affinity binding site for Abl, is incapable of Abl inhibition in LNCaP cells because of lower expression of total Abi1 in comparison to the clone that expresses wild type Abi1-Ha. LNCaP cells express both isoforms of Abl i.e. myristoylated and nonmyristoylated as determined by mRNA analysis (data not shown). Thus, the effects of the recombinant Abi1 on both isoforms of kinase cannot be excluded in these cells.

The hypothesis that Abi1 acts on the nonmyristoylated isoform of Abl is suggested by inhibition of the kinase in co-transfection experiments in Cos7 cells. Apparently, the nonmyristoylated kinase is constitutively active upon transfection into cells, while Abl kinase must be activated with pervanadate in LNCaP cells in order to demonstrate regulation by Abi1. As pervanadate is considered a general tyrosine phosphatase inhibitor, the action of Abi1 on Abl may be through an allosteric effect, as we postulate, or through a “shielding” effect on the catalytic domain by Abi1 SH3 domain interacting with the proline-rich region of c-Abl [22]. Thus, steric hindrance caused by tagging of Abi1 at the C-terminal may decrease its inhibitory effect on Abl kinase as demonstrated here. We cannot exclude the possibility that Abi1 is also a competitive inhibitor in vivo in addition to its allosteric inhibition. Importantly, Abi1 must be phosphorylated at pY213, presumably by Abl, for the proposed regulation to occur. Consequently, in intact cells, Abi1 is both a candidate substrate and the candidate regulator of Abl kinase activity.

The proposed mechanism of regulation of c-Abl by Abi1 includes the possibility that Abi1 plays a role in the initial activation of c-Abl as proposed [38]. This hypothesis would most likely apply to the myristoylated, autoinhibited kinase. Structural studies of c-Abl [15,16] indicate that the phosphotyrosine binding site is partially occluded in the crystal structure of the myristoylated c-Abl fragment containing the SH3–SH2-catalytic domain assembly. Thus, Pro-pY213 could potentially activate the myristoylated kinase through the SH2 domain interaction as proposed [15,16]. It is also possible that Abi1 may downregulate c-Abl that has been activated by phosphopeptides [15]. This might occur, for example, by competing off the activating phosphopeptide by Abi1-pY213.

Conservation of the regulatory sequences suggests that other members of Abi/Hssh3bp1 family of proteins also regulate Abl. The region containing the regulatory tyrosines 213 is highly conserved between Abi1 and Abi2 from *Xenopus* through human, and is present in *Drosophila* Abi (Fig. 5). The conserved sequences also include the PXXP motif, 181PPSP185, which binds to the c-Abl SH3 domain, and tyrosine 198. All isoforms of Abi1 [22,24,39], or Abi2 [21] contain the regulatory sequence indicating the conservation of c-Abl regulation in all Abi isoforms. The conserved region of Abi1 apparently plays a role in the regulation of c-Abl kinase activity in cells; here we addressed the role of the regulatory sequences in the context of Abi1 isoform 2. The fact that multiple isoforms are expressed from the Abi1/Hssh3bp1 gene [24] suggests the possibility of differential effects on Abl kinase activity as well as multiple downstream effects on actin cytoskeleton and Wave complex regulation [40,41].

In summary, we identified a candidate molecular mechanism of regulation of nonmyristoylated Abl kinase. Nonmyristoylated, mutated forms of Abl, such as BCR-Abl, are implicated in chronic myelogenous leukemia and in some forms of acute lymphocytic leukemia [3,4]. Although the use of STI-571 has brought great promise for the treatment of these diseases, some patients have become resistant to the drug following long-term treatment. STI-571-resistance mutations are found in Abi SH3 and SH2 domains [42] suggesting the possibility that in these cases BCR-Abl escapes possible residual regulation by other interactions including that with Abi1. The studies described here may improve our understanding of the mechanisms of Abi regulation, and may directly impact studies of BCR-Abl.

Acknowledgments

We thank B Mayer and T Koleske for the plasmids and for their helpful discussions; J Farivar for the help in protein analysis; S Heck for the help with flow cytometry; R Kascak and J Chen for their assistance with Abi1/Hssh3bp1 monoclonal antibodies; J Xu for technical assistance; and E Luna, C Redman, and M Narla for helpful discussions. Supported by grants from Department of Defense Prostate Cancer Research Program (DAMD17-01-1-0096)(LK), NINDS (NS44968)(LK), NIGM (GM47021) (DC). S Hossain was supported by the FM Kirby Foundation, Inc., Morristown, NJ. The Voyager DE was purchased through funding from the Horace W. Goldsmith Foundation, (New York, NY) and the Hyde & Watson Foundation (Chatham Township, NJ). The Biacore 3000 was purchased through funding from the Abby R. Maize Charitable Trust (New York, NY).

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