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Tyrosine Phosphorylation in the SH3 Domain Disrupts Negative Regulatory Interactions within the c-Abl Kinase Core

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SUMMARY

Recent studies have shown that *trans*-phosphorylation of the Abl SH3 domain at Tyr89 by Src-family kinases is required for the full transforming activity of Bcr-Abl. Tyr89 localizes to a binding surface of the SH3 domain that engages the SH2-kinase linker in the crystal structure of the c-Abl core. Displacement of SH3 from the linker is an event likely to influence efficient downregulation of c-Abl. Hydrogen-deuterium exchange (HX) and mass spectrometry (MS) were used to investigate whether Tyr89 phosphorylation affects the ability of the SH3 domain to interact intramolecularly with the SH2-kinase linker in *cis* as well as other peptide ligands in *trans*. HX MS analysis of SH3 binding showed that when various Abl constructs were phosphorylated at Tyr89 by the Src-family kinase Hck, SH3 was unable to engage a high-affinity ligand in *trans* and that *cis* interaction with the linker was dramatically reduced in a construct containing the SH3 and SH2 domains plus the linker. Phosphorylation of the Abl SH3 domain on Tyr89 also interfered with binding to the negative regulatory protein Abi-1 in *trans*. Site-directed mutagenesis of Tyr89 and Tyr245, another tyrosine phosphorylation site located in the linker that may also influence SH3 binding, implicated Tyr89 as the key residue necessary for disrupting regulation after phosphorylation. These results imply that phosphorylation at Tyr89 by Src-family kinases prevents engagement of the Abl SH3 domain with its intramolecular binding partner leading to enhanced Abl kinase activity and cellular signaling.

Keywords

Hydrogen exchange; mass spectrometry; phosphorylation; Src-family kinase; Bcr-Abl; Hck

The Abelson (*c-abl*) proto-oncogene encodes a non-receptor protein-tyrosine kinase (c-Abl) that is tightly downregulated in cells ¹. In contrast, the oncoprotein Bcr-Abl, which results from a chromosomal translocation that fuses Bcr sequences to the N-terminal region of c-Abl, is constitutively active ^{2; 3}. The enhanced tyrosine kinase activity of Bcr-Abl fusion proteins is linked to chronic myelogenous leukemia (CML) and other forms of leukemia ³. Interestingly, almost all of the c-Abl protein sequence is retained in the context of Bcr-Abl. However, the molecular mechanisms of Abl kinase upregulation in Bcr-Abl are not completely understood.

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The tyrosine kinase core of c-Abl consists of an N-terminal cap (NCap) region, an SH3 domain, an SH2 domain, and a kinase domain (see Figure 1A). Multiple intramolecular interactions involving these regions have been observed in the crystal structures of the downregulated c-Abl core^{4; 5}. The SH3 domain binds the SH2-kinase linker, an interaction necessary to suppress kinase activity⁶. The NCap region is immediately N-terminal to the SH3 domain and is also essential for c-Abl downregulation^{4; 5; 7; 8}. The glycine residue at position 2 in NCap is myristoylated and binds to a deep pocket in the C-lobe of the kinase domain, thereby latching SH2 and SH3 in their downregulatory positions at the back of the kinase domain and stabilizing the intramolecular interactions between SH3/SH2 and the kinase domain^{1; 4}.

Recent work has shown that the Src-family tyrosine kinases Hck, Lyn, and Fyn phosphorylate Bcr-Abl within the Abl-derived SH3 and SH2 domains⁹. Tyr89 (Abl 1b numbering) in the Abl SH3 domain was found to be the most prominent phosphorylation site *in vitro* and was also highly phosphorylated by Src-family kinases within Bcr-Abl in CML cells⁹. Phosphorylation of Tyr89 was shown to be necessary for the full biological activity of Bcr-Abl, as substitution of this tyrosine residue with phenylalanine reduced the transforming potential of Bcr-Abl in a cytokine-dependent myeloid cell line¹⁰. The crystal structure of the c-Abl core (Figure 1A) shows that Tyr89 localizes to the binding surface between the SH3 domain and the SH2-kinase linker, a region important for maintaining the inactive, down-regulated state¹. Phosphorylation of this site by Src-family kinases may disrupt the conformation of the downregulated form of Abl and thereby contribute to its transforming activity.

In the present study, hydrogen exchange (HX) mass spectrometry (MS) was used to investigate whether phosphorylation at Tyr89 affects SH3 interactions with binding partners both in *cis* and in *trans*. We show that phosphorylation at Tyr89 by the Src-family kinase Hck inhibits SH3 binding both in *trans* to a peptide ligand and protein binding partner and in *cis* to the SH2-kinase linker, an interaction essential to negative regulation. Site-directed mutagenesis indicates that phosphorylation of Tyr245 in the SH2-kinase linker, which is also strongly phosphorylated by Hck, has little impact on the ability of SH3 to interact with the SH2-kinase linker. Overall our results provide direct biophysical evidence that phosphorylation of Abl SH3 domain Tyr89 disrupts SH3:linker interaction and efficient downregulation of kinase activity. Phosphorylation of this site in the context of both c-Abl and Bcr-Abl may contribute to Abl kinase activation *in vivo*.

RESULTS

Tyrosine phosphorylation of Abl by Hck

To characterize the structural consequences of Abl phosphorylation by Hck, we expressed and purified a number of different recombinant Abl proteins, many of which have been described in detail elsewhere^{11; 12}. These constructs contained the Abl SH3 domain either alone or together with the SH2 domain, the NCap and various lengths of the SH2-kinase linker (Figure 1B). Some of the proteins contained one site of known and heavy phosphorylation (SH3 Tyr89), others contained two sites (Tyr89 and linker Tyr245), and some contained more than two. Phosphorylation reactions were conducted by incubating the Abl proteins with purified Hck kinase in the presence of ATP/Mg²⁺ for 45 min at 30 °C. Previous work by Meyn *et al.*⁹ demonstrated that these conditions led to efficient phosphorylation of similar recombinant Abl SH3-SH2 proteins. Mass spectrometry data show that in the Abl SH3 domain there is the characteristic +80-Da increase in mass corresponding to the covalent addition of a single phosphate group by Hck (Figure 2A, top). Similar results were observed in other constructs that contained only Tyr89 (Abl NCap3, SH32, NCap32 and SH32L_{1/3}) using the same experimental conditions. Trypsin digestion experiments showed that Tyr89 was the sole residue phosphorylated in these constructs (for example, see Figure 2B, top). The ratio of

phosphorylated to unphosphorylated species (phosphorylation ratio) was about 5–15% in these proteins (Figure 2C). In larger constructs (Abl SH32L_{3/4}, Abl SH32L_{4/5}, Abl SH32L, and NCap32L), however, there was double phosphorylation (Figure 2A, bottom) and the phosphorylation ratios were much higher (Figure 2C). In the case of the NCap32L protein, which encompasses the entire regulatory region of Abl, >95% of the molecules were found to be doubly phosphorylated. Trypsin digestion experiments indicated that both Tyr89 and Tyr245 were phosphorylated in Abl SH32L_{3/4}, SH32L_{4/5}, SH32L, and NCap32L (Figure 2C).

To test the influence of the kinase domain on SFK-mediated Abl phosphorylation, we measured Hck-induced phosphorylation in two different forms of the c-Abl core. One form, designated c-Abl(1) had only half of the NCap and no myristoylation of the N-terminal glycine residue (Figure 1A). Thus, c-Abl(1) lacks the key regulatory interaction of the myristoyl group binding to the C-lobe pocket of the kinase domain (see Introduction). The second form, called c-Abl(2), contained more of the NCap and based on MS analysis (data not shown) was stoichiometrically myristoylated at Gly2 as shown originally by Nagar *et al.*⁴. This form of Abl represents the fully-downregulated conformation. The sites and extent of phosphorylation of both of these Abl core constructs were determined by MS (data not shown). While c-Abl(1) became phosphorylated at Tyr412, Tyr89 and Tyr245 within 10 min, c-Abl(2) was not phosphorylated even after 24 h of incubation (Figure 2C). Taken together, and in light of the data for the smaller Abl constructs, our data support the hypothesis that access to the SH3 domain must first be provided in order for Hck to phosphorylate the Abl SH3 domain on Tyr89. In other words, the SH3 domain must first move from its downregulatory position bound to the SH2-kinase linker, a process that is presumably much easier in c-Abl(1) due to the lack of negative-regulatory myristoylation^{1; 4}. To prove that SH3 is less able to interact with the linker following Hck-induced phosphorylation, we next turned to HX MS.

Phosphorylation of Tyr89 disrupts intermolecular interaction with SH3 ligands

We previously used HX MS to show that the Abl SH3 domain undergoes partial cooperative unfolding that is sensitive to ligand binding^{11; 12}. Previous work from our group has established that this unfolding event is a property of most SH3 domains¹³. By monitoring the appearance of a partial unfolding event in SH3 during the deuterium labeling time-course, the half-life ($t_{1/2}$) of unfolding can be determined. In the HX MS assay, inter- or intra-molecular ligand binding that stabilizes the SH3 domain shifts the unfolding half-life to longer times. By monitoring both protein unfolding dynamics and the actual deuterium uptake levels at each exchange time point, changes in binding as a result of phosphorylation were ascertained for each of the recombinant Abl proteins.

First we determined if phosphorylation itself had any effect on SH3 domain dynamics by comparing HX MS results of phosphorylated Abl SH3 with those of unphosphorylated Abl SH3. The mass spectra for unphosphorylated SH3 during deuteriation showed the characteristic broadening of the isotope distribution seen previously^{11; 12}, gradually changing from a narrow isotopic distribution to a wider distribution characteristic of an EX1 unfolding event¹⁴ (Figure 3A). An EX1 unfolding event occurs when portions of a protein that cooperatively unfold have a rate constant of refolding that is much less than the rate of isotopic exchange. The result is that during the time the region is unfolded, all exchangeable amide hydrogens in the region become deuterated at the same time. In mass spectra, this creates a bimodal distribution where the lower mass represents the protein molecules before cooperative unfolding and the higher mass represent the protein molecules after unfolding. More details on EX1 unfolding can be found in Refs 11, 12 and 14. The spectra for phosphorylated SH3 showed essentially the same results as the unphosphorylated SH3 alone, as did the deuterium uptake curve and peak width plot (Figure 3B). We conclude therefore, that phosphorylation itself does not alter the unfolding dynamics of the Abl SH3 domain. Any changes in unfolding dynamics of the phosphorylated

form must therefore be attributable to other factors such as peptide ligand binding, as shown below.

Next we investigated whether phosphorylation changed the affinity of the Abl SH3 domain for peptide ligands bound in *trans*. We compared the binding of phosphorylated Abl SH3 and unphosphorylated Abl SH3 to the high-affinity peptide ligand, BP1¹⁵. Unphosphorylated Abl SH3 + BP1 did not exhibit peak broadening like the unbound form (Figure 3A), indicating that SH3 bound to BP1, as shown previously¹¹. However, there was peak broadening during HX MS that was characteristic of an EX1 unfolding event¹⁴ when phosphorylated SH3 was incubated with BP1 at the same molar ratio as the unphosphorylated form of Abl SH3 (Figure 3A). The deuterium uptake curve showed that phosphorylated SH3 was more deuterated in the presence of BP1 than unphosphorylated SH3 + BP1 (Figure 3C, left). As expected from previous investigations¹¹, BP1-bound unphosphorylated Abl SH3 had a peak-width plot that was almost flat (Figure 3C, right), indicating that SH3 unfolding was almost completely abolished because BP1 binding was so tight¹¹. In contrast, the centroid value of the peak-width plot of phosphorylated Abl SH3 + BP1 was about 60 min (Figure 3C, right). In order to compare SH3 binding affinity, we define the term slowdown factor as the unfolding half-life of a construct divided by the unfolding half-life for free, unadulterated SH3 domain. This is a very useful parameter for monitoring changes in unfolding dynamics of proteins by HX MS (see examples in Hochrein *et al.*¹⁶). Larger slowdown factor (SF) values indicate a more rigid, less-dynamic protein that is less able to incorporate deuterium. The slowdown factor of phosphorylated SH3 + BP1 was approximately 10-fold larger (SF=11) than phosphorylated SH3 in the absence of BP1 (Figure 4A), but much smaller than the SF of unphosphorylated SH3 + BP1 (defined as infinity when the unfolding is so slow it cannot be measured). These results imply that phosphorylation of SH3 substantially decreases the BP1 binding affinity. We attribute this effect to steric interference at the binding interface as Tyr89 is near the SH3 interaction surface for ligands such as BP1.

Phosphorylation of NCap32L disrupts intramolecular interactions

The HX MS results described so far have been for intact, undigested proteins. HX MS analyses of peptic fragments from each protein can also be performed and provided much better resolution for larger proteins where measurement of peak width for the intact protein can be compromised by salt adducts. The consistency of results from intact protein analysis vs. peptide analysis validates the use of either as a good measure of unfolding¹².

Previously we showed that the sequence at the N-terminal end of c-Abl (called the NCap) stabilizes SH3 unfolding in the NCap32L construct by 2-fold as indicated by a longer unfolding half-life of a reporter peptide in the SH3 domain¹². We used the same methodology here to examine whether phosphorylation disrupts intramolecular interactions in NCap32L. Raw HX MS data (Figure 5) showed that the relative deuterium uptake of the SH3 domain reporter peptide in phosphorylated NCap32L was significantly higher than the same peptide derived from the unphosphorylated NCap32L protein. The peak width plot showed that the unfolding half-life of the reporter peptide in phosphorylated NCap32L was about 10 minutes, a value significantly shorter than the unfolding half-life of the reporter peptide in unphosphorylated NCap32L (which is about 20 min, Figure 5C). Conversion of these results into slowdown factor aids in the interpretation. In the Abl SH32L construct, phosphorylation caused the slowdown factor to decrease from 1.9 to 1.0, about half of the unphosphorylated value (Figure 4B). This change was indicative of the ability of the phosphorylation to prevent the linker from interacting with the Abl SH3 domain. Because SH3 was not binding to a ligand (in this case the linker), the dynamics were faster, *i.e.* SH3 was more able to flex and breathe in solution and became deuterated more quickly. In unphosphorylated NCap32L, the slowdown factor was 3.9 (Figure 4B) which, as shown previously¹², indicates that NCap stabilizes SH3 unfolding by 2-fold

compared with the control SH32L construct (where SF=1.9). However, the slowdown factor was 1.9 when NCap32L was phosphorylated, or about half the unphosphorylated value indicating that phosphorylation altered SH3 domain dynamics within NCap32L and made SH3 twice as dynamic compared to unphosphorylated NCap32L. This result mirrors the effects of phosphorylation seen in the SH32L construct. We conclude that phosphorylation changed the ability of the linker to interact with the Abl SH3 domain in both SH32L and NCap32L. Based on these data alone, however, we could not distinguish which tyrosine phosphorylation site (Tyr89 or Tyr245) regulated linker displacement.

Tyr89 is the crucial residue involved in disrupting intermolecular interactions in NCap32L

Intact mass analysis showed that there were two primary Hck phosphorylation sites in NCap32L (Figure 2A, bottom). Trypsin digestion revealed that both Tyr89 and Tyr245 were phosphorylated (Figure 2B). Hydrogen exchange data showed that phosphorylation reduced the ability of SH3 to bind to the linker but did not indicate which tyrosine phosphorylation site (Tyr89 or Tyr245) was primarily responsible. To address this question, site-directed mutagenesis was used to create two additional constructs in which each of these sites was individually mutated to phenylalanine (NCap32L Y89F, NCap32L Y245F) and a third construct corresponding to the double mutant (NCap32L dYF).

Both intact mass analyses and trypsin digestion experiments verified that the expected tyrosine was phosphorylated in each construct, *i.e.* Y89 was the only site of phosphorylation in the Y245F mutant and Y245 was the only site of phosphorylation in the Y89F mutant; no significant phosphorylation was observed with the dYF construct (data not shown). HX MS analyses of phosphorylated and unphosphorylated NCap32L Y245F were similar to those of wild type NCap32L and indicated that there was more deuteration of the reporter peptide in phosphorylated NCap32L Y245F compared to that in unphosphorylated NCap32L Y245F (essentially the same results as shown in Figure 5B). Similarly, peak width plots indicated that the unfolding half-life of the reporter peptide in phosphorylated NCap32L Y245F was half that of unphosphorylated NCap32L Y245F. A summary of the slowdown factors (Figure 4B) showed that the NCap32L Y245F mutant was essentially the same as the wild-type NCap32L construct, indicating that mutation of Tyr245 had no effect on linker engagement. Phosphorylation of the NCap32L Y245F protein still caused the slowdown factor to decrease by half, indicating that the remaining phosphorylation was still able to disrupt SH3:linker binding. The only site for phosphorylation to occur in the Y245F mutant was Tyr89, implicating this site as a key regulator of linker association as a function of phosphorylation.

In contrast, the relative deuterium uptake curves and peak width plots of the phosphorylated and unphosphorylated forms of both NCap32L Y89F and NCap32L dYF were similar, implying that phosphorylation had no effect on the SH3 dynamics in these mutants. In the NCap32L Y89F construct, an unexpected result was observed. Mutation of Tyr89 to phenylalanine caused the unfolding dynamics of the Abl SH3 domain (SF=1.8) to resemble what was seen in the construct that did not contain the NCap (SH32L, SF=1.9). Perhaps removing the hydrogen bonding potential of the Tyr89 side chain altered the ability of the linker to associate with the SH3 domain. Upon phosphorylation of NCap32L Y89F by Hck, there was a very minor decrease in the slowdown factor which was not nearly as dramatic as that seen in the wild-type or the Y245F mutant. Similarly, the slowdown factor for the reporter peptide was not significantly changed when the double mutant NCap32L dYF was treated with Hck (Figure 4B). Taken together, these results suggested that phosphorylation of Tyr89 alone contributes significantly to the disruption of intramolecular SH3:linker interaction.

Phosphorylation of Abl SH3 Tyr89 blocks binding to the Abl regulatory protein, Abi-1

HX MS data presented in the preceding sections show that phosphorylation of Abl SH3 at Tyr89 disrupts engagement with the SH2-kinase linker. In the context of full-length Abl, this event would be predicted to release the SH3 domain from the linker and promote kinase activation. Several Abl-interacting proteins (Abi proteins) have been described that may serve as both regulators and effectors of c-Abl^{17; 18}. Abi-1 is particularly intriguing, because it has recently been reported to repress Abl kinase activity despite its ability to bind to the Abl SH3 domain¹⁹. This led us to question whether phosphorylation of the Abl SH3 domain on Tyr89 by Hck also had the potential to disrupt *trans*-regulation of Abl by Abi-1, perhaps in a similar way as phosphorylation prevents BP1 binding (described above). As Abi-1 is difficult to overexpress and purify in sufficient quantities for HX MS, we turned to a co-precipitation assay to address this question. We first expressed and purified Abl SH3 and SH32L as GST fusion proteins, and incubated them *in vitro* in the presence or absence of Hck and ATP. GST alone was used as a negative control. The proteins were then incubated with lysates from 293T cells transfected with an Abi-1 expression plasmid. Following incubation and washing, Abi-1 binding was assessed by immunoblotting. As shown in Figure 6, Abi-1 bound to the unphosphorylated SH3 domain, and to an even greater extent to the SH32L protein. However, phosphorylation of the Abl fusion proteins with Hck significantly reduced Abi-1 binding to SH3 and abolished binding to SH32L. Control blots with phosphospecific antibodies showed that both the SH3 and SH32L protein were phosphorylated on Tyr89, while only the SH32L protein was phosphorylated on Tyr245 as expected. No phosphorylation or Abi-1 binding was observed with GST alone. These results show that Abl SH3 domain phosphorylation also impacts *trans* binding of the Abl regulatory protein, Abi-1, and may affect interaction with other SH3-binding proteins *in vivo*.

DISCUSSION and CONCLUSIONS

In the downregulated state of the c-Abl core, intramolecular interactions are crucial for maintaining an inactive conformation. The crystal structures show that the c-Abl SH3 domain engages the polyproline type II helix formed by the SH2-kinase linker^{4; 5}. In addition, the SH2 domain docks onto the back of the C-terminal lobe of the Abl kinase domain. This interaction is stabilized further by the NCap when the myristoyl group at Gly2 binds to a deep pocket in the C-lobe of the kinase domain and “latches” the SH2 domain against the back of the kinase domain^{1; 4}. Together, these unique interactions provide a regulatory clamp that allosterically holds the kinase domain in a tightly downregulated state.

Our experimental results suggest that in Abl constructs lacking the kinase domain, the preference of Hck for Abl SH3 phosphorylation is based on the length of the Abl SH2-kinase linker. Very little phosphorylation was detected in the SH32L_{1/3} protein but the addition of a few more residues (SH32L_{3/4}) caused a major change in the phosphorylation ratio (Figure 2C). Addition of additional C-terminal linker residues shifted the phosphorylation towards double phosphorylation (more Tyr245 was phosphorylated, data not shown) and addition of the NCap forced almost all phosphorylation to entirely double with >95% of the molecules modified at both positions. Thus, Hck phosphorylates both Tyr89 and Tyr245 in c-Abl proteins that lack the kinase domain.

A different situation occurs in versions of Abl that contain both the regulatory and kinase domains. Recombinant purified c-Abl (2) (identical in sequence to the downregulated crystal structure of Nagar, *et al.*⁴; see Figure 1) was not detectably phosphorylated by Hck even after 24 hours, suggesting that this form of c-Abl is tightly downregulated and thus resistant to Hck-mediated phosphorylation of the regulatory apparatus. However, another form of c-Abl tested here, c-Abl (1), which was not myristoylated at the N-terminus, was rapidly phosphorylated on three tyrosine residues (loop Tyr412, linker Tyr245 and SH3 Tyr89). These observations

are consistent with a previous report on phosphorylation of a form of recombinant Abl lacking N-terminal myristoylation²⁰ and suggest that the intramolecular restraints for c-Abl(1) may be disrupted as a result of the unmyristoylated NCap not being able to engage the pocket on the kinase domain. Such a form of c-Abl would be much better poised for phosphorylation by other kinases on sites that are normally buried in the downregulated core. In the Bcr-Abl fusion protein, the N-terminal myristoylation site is also removed; therefore Bcr-Abl may more closely resemble c-Abl(1) in solution than it does the tightly downregulated c-Abl(2). We speculate that this is one contributing factor that allows Bcr-Abl to be phosphorylated by Hck and other SFKs on the SH3 domain, SH2-kinase linker and other possible regulatory tyrosines in leukemia cell lines and *in vitro*⁹.

Based on biological data^{6; 9}, we hypothesized that phosphorylation physically disrupts downregulatory SH3:linker interactions in c-Abl, perhaps by a mechanism similar to that observed upon mutation of residues in the SH3:linker interface^{21–23}. To test this biophysically, we used HX MS to probe protein unfolding and dynamics in different constructs of Abl to determine whether phosphorylation destabilizes the unfolding of the SH3 domain in the absence of the kinase domain. Our data (summarized in Figure 4) showed that *trans* binding (to the exogenous ligand BP1) was significantly reduced upon Tyr89 phosphorylation as was intramolecular binding of SH3 to the SH2-kinase linker in SH32L and NCapSH32L constructs. In addition, *trans*-binding of the regulatory protein Abi-1 to the SH3 domain was also abolished by Hck-mediated phosphorylation of the SH3 domain (Figure 6). Site-directed mutagenesis and HX MS showed that Tyr89 is the key residue necessary for preventing regulation after phosphorylation. Our findings are consistent with the observation that mutations at SH3 Tyr89 result in Bcr-Abl imatinib resistance in four independent isolates from a random mutagenesis screen²⁴. Because imatinib prefers the inactive conformer of the Abl kinase domain, these mutations were presumed to destabilize the negative regulatory influence of SH3 on the linker in the context of Bcr-Abl. Our data imply that phosphorylation of Tyr89 by Hck may also favor the active conformation of Bcr-Abl and contribute to sustained kinase activity and imatinib resistance. The inhibition of binding, both in *trans* and in *cis*, likely only occurs in forms of the kinase that are not in the most downregulated state, such as Bcr-Abl.

Although Bcr-Abl exhibits constitutive tyrosine kinase activity, Hantschel and Superti-Furga have proposed that Bcr-Abl may retain some of the regulatory features observed in the c-Abl core¹. Compared to c-Abl core, Bcr-Abl lacks the regulatory impact of myristoylation, but the interaction between SH3 domain and the linker are suggested to remain. Smith *et al.*²⁵ showed that an important requirement for Bcr-Abl activity is oligomerization mediated by the N-terminal coiled-coil region of Bcr-Abl, and that mutations in the N-terminal coiled-coil that block transformation are overcome by mutations that disrupt SH3 domain interaction with the linker. This observation supports the idea that the SH3 domain still exerts some negative regulatory influence over Bcr-Abl tyrosine kinase activity. Work presented here provides new biophysical evidence that phosphorylation of the SH3 domain at Tyr89 by Hck, and perhaps other kinases as shown by Meyn *et al.*⁹, has a similar destabilizing effect. It appears that in the Bcr-Abl fusion protein, partial relaxation of the downregulated core conformation due to loss of the myristoylated NCap may allow other kinases to be recruited that phosphorylate Tyr89, an action that blocks the negative regulatory influence of SH3:linker interaction as well as interaction with *trans*-regulatory factors such as Abi-1. Selective inhibition of this phosphorylation activity, in combination with Abl-targeted drugs such as imatinib, may prove useful in CML therapy. Indeed, the dual Src/Abl inhibitor dasatinib is much more potent against CML cells than imatinib, and is active against most forms of imatinib-resistant CML²⁶.

MATERIALS and METHODS

DNA Constructs and protein purification

The human c-Abl SH3, SH32, SH32L, NCap3, NCap32, and NCap32L proteins were overexpressed and purified as described previously¹². The c-Abl(2) protein was purified from Sf9 insect cells upon co-expression with YopH, as described previously¹². The c-Abl(1) form, which was purified from *E. coli* and contains residues 65–534 (Abl 1b numbering), was a gift from Nathanael Gray at DFCI/HMS. The BP1 peptide was synthesized as described¹¹. All other chemicals and solvents were obtained from Sigma and used without further purification.

Phosphorylation reactions

Phosphorylation reactions of the Abl constructs were conducted in 50 mM Hepes pH 7.4, containing 10 mM MgCl₂ and 500 μM ATP for 45 min at 30 °C, and initiated by adding Hck kinase at a 1:50 Hck:protein ratio. The area under the isotope distribution for the unphosphorylated and phosphorylated form(s) of each intact protein was determined and used to calculate the ratio of phosphorylation, or the percent of protein molecules phosphorylated (see also Steen *et al.*²⁷). In order to identify phosphorylation sites, phosphorylated proteins were digested overnight with trypsin at a 1:25 trypsin:protein ratio. The digested peptides were separated using a Waters nanoACQUITY UPLC connected to a Waters Synapt HDMS mass spectrometer. Peaks for phosphopeptides and their unphosphorylated counterparts were identified in mass spectra based on their m/z ratios and the sequence of each peptide was verified by MS/MS analyses.

Deuterium exchange and MS analysis of deuterium incorporation

For SH3 incubation with BP1 in *trans*, the percent unphosphorylated SH3 bound was estimated using a K_d of 2 μM for BP1¹⁵. BP1 was added such that more than 90% of unphosphorylated SH3 molecules were calculated to be bound to peptide ligand in the labeling solution. For BP1 bound to phosphorylated SH3 where the K_d was unknown, BP1 was added in a 50-fold molar excess. All mixtures were incubated at room temperature for 30 min before labeling began. As a negative control, unphosphorylated SH3 and phosphorylated SH3 were incubated with 20 mM of the non-binding peptide angiotensin I (Sigma); therefore, all data listed as free are actually the constructs in the presence of angiotensin I.

Proteins were incubated in 50 mM sodium phosphate (pD 8.3), D₂O at 25 °C for various amount of time. The reaction was quenched by adjusting the pH to 2.5 with 0.5 M HCl at each time point. Quenched samples were immediately frozen on dry ice and stored at –80 °C until analysis. Intact protein analysis of deuterium incorporation was described as previously¹¹. For peptic analysis, each 50 μl sample (100–150 pmol) was injected onto a 50 mm×200 mm stainless steel column packed with pepsin immobilized on POROS-20AL beads (PerSeptive Biosystems) at 30–40 mg/ml. The resulting peptides were trapped on a C18 trap column. The total digestion time was ~20 seconds. The trapped peptides were eluted from the C18 trap onto a Magic C-18 column (Michrom BioResources, Inc.) and directed into the mass spectrometer with a six minute gradient of 2–45% acetonitrile in H₂O. The injector, column and tubing were all cooled with an ice-bath²⁸. The pepsin column was located above the ice-bath at an approximate temperature of 18 °C. Because all analyses were based on comparisons of data obtained at nearly the same time, no corrections were made for back-exchange (as described in detail in Wales & Engen²⁹). The average amount of back-exchange for peptides in this instrumental system was 15–18%, as measured with totally deuterated angiotensin and several other totally deuterated peptides. Intact protein analyses were performed with a Waters LCT premier mass spectrometer while peptide analyses were done with a Waters QToF Ultima instrument calibrated after each run with an infusion of myoglobin or glu-fibrinogen peptide.

The maximum error of determining the deuterium level for peptides was ± 2.0 Da for proteins and ± 0.5 Da for peptides.

Data processing

The deuterium uptake curves and peak width plots were made using HX-Express³⁰. The peak width was measured at full-width half maximum (FWHM) for each time point and plotted against the deuterium labeling time. Linear regression was performed on each side of the peak in each peak-width plot. The intersection point of the two linear equations was determined and used as the half life of unfolding¹¹. Slowdown factor is defined as the unfolding half-life of each construct divided by the unfolding half-life of free, unadulterated SH3 (described in more detail in Hochrein *et al.*¹⁶).

Abi-1 binding assay

The coding regions of the human Abl SH3 domain and SH3-SH2-Linker (32L) regions were amplified by PCR and subcloned into the bacterial expression plasmid pGEX-2T via the BamHI and EcoRI sites. SH3 amplification employed the primer pairs SH3-F (gcgggatccggaccagtgaaatgacc) and SH3-R (gtcagacctctgtgaggactcttaagggg); amplification of the SH3-SH2-Linker DNA sequence used the same forward primer plus the reverse primer 32L-R (5-gttgatg ctgttcaccctcactcttaagctc). The resulting GST-SH3 and GST-32L proteins as well as GST alone were expressed in *E. coli* and purified to homogeneity using glutathione-agarose beads according to manufacturers instructions (Sigma). The purified proteins were then phosphorylated *in vitro* as described in detail elsewhere⁹. Briefly, each GST-Abl protein or GST alone (10 μ M) was incubated in phosphorylation buffer (10 mM HEPES, pH 7.5, 10 mM MgCl₂, 500 μ M ATP) in the presence or absence of recombinant purified HckYEEI (1 μ M) for 30 min at 30 °C. The reaction was then quenched on ice. Human 293T cells were transfected with a mouse Abi-1 cDNA (PubMed accession number **BC004657**) in the expression vector pCMV-SPORT6 (Open Biosystems) and lysed in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 2 mM NaF, 2 mM sodium orthovanadate, protease inhibitor cocktail (Calbiochem), and 50 U Benzonase (Novagen). The cell lysates were clarified by centrifugation, and 1 mg lysate protein was combined with each GST-Abl protein or the GST control and rotated for 2 h at 4 °C. Glutathione-agarose beads were then added (20 μ l of a 50% w/v slurry) and the reactions were incubated for an additional 2 h. The protein complexes were then washed three times with RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate). The precipitated proteins were eluted in SDS-PAGE loading buffer, separated on 10% SDS-polyacrylamide gels, transferred to replicate PVDF membranes and probed with antibodies for Abi-1 (anti-SSH3BP1; Abcam), phosphospecific antibodies to Abl pTyr89 and pTyr245 (kind gift of Dr. Jiong Wu, Cell Signaling Technology) and GST (Santa Cruz Biotechnology).

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Figure 1.

Structural locations and details of the constructs used in this study. **(A).** Crystal structure of the Abl kinase core [PDB:2FO0, Nagar *et al.* ⁴]. Tyr89, Tyr245, and Tyr412 (Abl 1b numbering) are shown as sticks in color. The crystal structure begins at Ala65 as indicated. The unstructured part of the NCap is shown as dots in pink. Gly2 in Abl core is myristoylated *in vivo*. The color code is: pink = NCap, dark yellow = SH3 domain, green = SH2 domain, red = SH2-kinase linker, light blue = kinase domain. **(B).** Schematic diagram and sequence information for the constructs used in this study. The sequence of each different length NCap32L linker construct is shown on the top ¹¹. Tyr89, Tyr245, and Tyr412 are shown in the schematic diagram of the Abl core protein, the protein shown in Figure 1A. The sequence

for the NCap region is shown for c-Abl (1) and c-Abl (2). c-Abl(2) was expressed in Sf9 insect cells and was myristoylated at Gly2. c-Abl (1) was expressed in *E. coli* and was not myristoylated (see also Materials and Methods). The color code is the same as in part A.

Figure 2.

Phosphorylation analysis of different constructs. (A). Transformed ESI mass spectra of phosphorylated Abl SH3 (top) and NCap32L (bottom). (B). Representative electrospray mass spectra of the phosphorylated tryptic peptides from phosphorylated NCap32L (spectra similar for NCap3 and NCap32). The top spectra is of the tryptic phosphopeptide containing Tyr89 [position of the unphosphorylated ion (a) of m/z= 1175.9, phosphorylated ion (b) of m/z= 1202.6, +3 charge state, representing residues ⁷¹ENLLAGPSENDPNLFVALYDFVASGDNTLSITK¹⁰³]. The bottom spectra is of the tryptic phosphopeptide containing Tyr245 [position of the unphosphorylated ion (c) of m/z=948.9, phosphorylated ion (d) of m/z=988.9, +2 charge state, representing

residues ²⁴⁰NKPTVYGVSPNYDKWE²⁵⁵]. Unphosphorylated tryptic peptides (a, c) eluted at slightly different retention times and are not shown in this Figure. (C). Phosphorylation ratio (% protein molecules phosphorylated) of different intact protein constructs (see Materials and Methods for details of the calculations).

Figure 3.

Intact HX MS analyses of phosphorylated Abl SH3. (A). Transformed electrospray mass spectra of deuterium labeled phosphorylated Abl SH3 alone, unphosphorylated Abl SH3 alone (used as control), phosphorylated Abl SH3 + the high-affinity peptide ligand BP1¹⁵, and unphosphorylated Abl SH3 + BP1 (control). The dotted lines are provided for optical guidance and are centered on the widest peak observed in each case. For pSH3 and SH3, the time-points shown are not the same because SH3+BP1 spectra are vastly different than pSH3+BP1: even after 120 minutes in D₂O, the deuterium level has not reached the dotted line as it has after 60 minutes for pSH3+BP1 (see panel C for processed data). Relative deuterium uptake and peak width change during the time-course of deuterium labeling are shown for (B) Abl SH3 alone

and (C) Abl SH3 + BP1. BP1 binding to SH3 is used as a positive control for tight binding, as described previously¹¹. Back exchange has not been considered in these experiments (see Materials and Methods) and the deuterium level is therefore reported as relative. Peak width measurement was at FWHM. In panel B, the SH3 data (solid circles) are directly on top of the pSH3 data (open circles) in the deuterium uptake plot, left.

Figure 4. Summary of the slowdown factor in Abl SH3 unfolding for various constructs. **(A).** Comparison of slowdown factor of free SH3 and SH3 incubated with BP1. **(B).** Comparison of slowdown factor of phosphorylation in the constructs indicated. The -/+ symbols correspond to when the construct was unphosphorylated or phosphorylated with Hck.

Figure 5.

Analyses of the reporter peptide from HX labeling of phosphorylated NCap32L and unphosphorylated NCap32L. The reporter peptide is monitored with the ion of $m/z = 809.9$, +2 charge state, representing residues $^{119}\text{CEAQTKNQGQWVPSN}^{133}$; this peptide is directly related to SH3 unfolding, as described in Chen *et al.* ¹². (A). Electrospray mass spectra of deuterium labeled reporter peptide in phosphorylated NCap32L and unphosphorylated NCap32L. The (B) relative deuterium uptake and (C) peak width change of the reporter peptide from each construct during the time-course of deuterium labeling. Back exchange adjustment and peak width measurement were as reported in Figure 3.

Figure 6.

Phosphorylation of the Abl SH3 domain blocks binding to the Abl interacting protein, Abi-1. Recombinant GST and GST fusion proteins with the Abl SH3 domain alone (SH3) or the SH3-SH2-Linker (32L) were incubated in the presence or absence of recombinant Hck as indicated. Each protein was then incubated with an aliquot of lysate from 293T cells transfected with Abi-1. Following incubation, protein complexes were precipitated with glutathione-agarose beads, washed, and bound proteins were eluted in SDS-PAGE sample buffer and associated Abi-1 was visualized by immunoblotting (Top). Aliquots of the precipitates were also immunoblotted with phosphospecific antibodies to Abl pTyr245 (pY245), pTyr89 (pY89), as well as GST. The positions of GST, GST-SH3, and GST-32L are indicated by the arrows.