Mitotic Phosphorylation Rescues Abl from F-actin-mediated Inhibition*

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We have previously shown that F-actin exerts a negative effect on Abl tyrosine kinase activity. This inhibition results from a direct association of F-actin with the C terminus of Abl and accounts, in part, for the loss of Abl activity in detached fibroblasts. We report here that Abl from mitotic cells or cells treated with the protein phosphatase inhibitor okadaic acid remains active when detached from the extracellular matrix. Aspartic acid substitution of Thr566, which is phosphorylated in mitotic or okadaic acid-treated cells, is sufficient to abolish F-actin-mediated inhibition and to maintain Abl activity despite cell detachment. A recent crystal structure of the Abl N-terminal region has revealed autoinhibitory interactions among the Src homology 3 (SH3), SH2, and kinase domains. We found that deletion of the SH2 domain also abolished the negative effect of F-actin on kinase activity. Immediately following the kinase domain in Abl is a proline-rich linker (PRL) that binds to several SH3 adaptor proteins. Interestingly, binding of the Crk N-terminal SH3 domain to the PRL also disrupted F-actin-mediated inhibition of Abl kinase. These results suggest that F-actin may reinforce the autoinhibitory interactions to regulate Abl kinase and that inhibition can be relieved through phosphorylation and/or protein interactions with the Abl PRL region.

The Abl tyrosine kinase transduces signals from cell surface receptors as well as DNA damaging agents to regulate actin dynamics, cell cycle, differentiation, and apoptosis (1–5). This wide range of biological activities depends on a number of modular functional Abl domains, which determine its subcellular localization, its interaction partners, and the regulation of its tyrosine kinase activity (1–4). Abl kinase is autoinhibited through intramolecular interactions involving its Src homology 3 (SH3),1 SH2, and kinase domains in the N-terminal region. These intradomain interactions are facilitated by three linkers, that of the CAP region preceding the SH3 domain at the N terminus, the SH3-SH2 linker, and the SH2-kinase linker (6). The Abl kinase is also negatively regulated in trans by several cellular inhibitors that directly bind to the Abl protein (7). Among the inhibitors of Abl kinase is F-actin, which binds to the Abl C terminus and contributes to the inactivation of Abl by cell detachment (8). Attachment of cells to fibronectin activates Abl kinase to stimulate the formation of F-actin microspikes (9, 10). Because activated Abl kinase can modulate the architecture of F-actin, which can in turn inhibit Abl kinase, Abl-dependent actin polymerization is self-limiting and may contribute to the dynamic membrane protrusive activity at the leading edge of spreading or migrating cells (2, 9, 11).

We have previously shown that F-actin binding to Abl reduces Abl tyrosine kinase activity in vitro and in detached cells (8, 9). F-actin-mediated inhibition results in reduced autophosphorylation as well as reduced substrate-phosphorylation by Abl (8). Disrupting Abl-F-actin association through either mutation of the Abl actin-binding domain (ABD) or treatment of fibroblasts with latrunculin A, which depolymerizes F-actin, increases Abl activity in detached fibroblasts and prevents the detachment-induced inactivation of Abl (8, 9). However, the ABD is not an autoinhibitory domain per se because its deletion does not affect Abl kinase activity in the absence of F-actin (8). To learn more about the mechanism of F-actin-mediated inhibition of Abl, we sought conditions under which the inhibitory effect of F-actin on Abl kinase could be suppressed.

The Abl protein is quantitatively hyperphosphorylated on serine and threonine residues during mitosis (12). Previous studies found that Abl kinase remains active in mitotic cells and concluded that mitotic phosphorylation does not affect Abl kinase activity (12). In those experiments, Abl from mitotic mouse fibroblasts that were detached from the extracellular matrix by mitotic shake-off was compared with Abl from interphase cells that were attached to the extracellular matrix (12). However, when a comparison was made between Abl from detached interphase cells and detached mitotic cells, it became clear that mitotic Abl retained kinase activity despite detachment. Here, we have examined the effect of one of the mitotic phosphorylation sites, Thr566, on Abl kinase activity. Substitution of Thr566 with Asp resulted in an Abl mutant that was resistant to F-actin-mediated inhibition in vitro and that retained kinase activity in detached interphase cells. Thr566 is in a proline-rich linker (PRL) immediately C-terminal of the kinase domain in Abl. The PRL contains several distinct binding sites for SH3 domains including those of Crk, Nck, and Abi1/2 Abl, threonine 566 of Abl mutated to alanine; T566D-Abl, threonine 566 of Abl mutated to aspartic acid; PRL, proline-rich linker; TRITC, tetramethylrhodamine isothiocyanate.

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1 The abbreviations used are: SH, Src homology; BSA, bovine serum albumin; CTD, C-terminal repeat domain of RNA polymerase II; mCTD, modified CTD; ABD, F-actin binding domain of Abl; GST, glutathione S-transferase; KD, kinase-defective; OA, okadaic acid; T566A-
Conformation-dependent Regulation of Abl Kinase by F-actin

EXPERIMENTAL PROCEDURES

Plasmids—Murine type IV Abl DNA was cloned 3′ of the long terminal repeat promoter in the pMSCV-hpl plasmid (17). KD and ΔF-actin Abl DNAAs were described previously (8). T566A-Abl and T566D-Abl DNA constructs were generated by PCR-based mutagenesis using Phu DNA polymerase (Stratagene) and confirmed by sequencing. All constructs used for transfection contained the eight-amino acid FLAG sequence, flanked by three glycine residues on each side inserted internally at the unique Sall site (amino acid 978). The FLAG tag did not affect adhesion regulation of Abl kinase as determined by side by side analysis with either endogenous Abl or transfected Abl without a FLAG sequence insert (8). The hybrid substrate, GST-crk-mCTD, was expressed from the pGEX plasmid. Oligonucleotides with the appropriate sticky ends and coding sequences for Crk amino acids 183–237 (encompassing the YAQP motif that is phosphorylated by Abl) fused to three modified repeats of the mammalian RNA polymerase II C-terminal repeated domain (CTD). The last three repeats (50–52) were modified to eliminate all but one serine and to maintain the secondary structure of the repeats. The modified CTD sequence is shown in Fig. 5.

Cell Culture, Transfection, and Phalloidin Staining—The stable mouse fibroblast cell line null for Abl activity was a gift from Dr. Rubio Ren (Brandeis University). Fibroblast cell lines were maintained in high glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, L-glutamine, and penicillin-streptomycin. Cell treatments with nocodazole (Sigma) or K12 antibodies (Santa Cruz Biotechnology). Detailed procedures for fibronectin stimulation, cell lysate preparation, immunoprecipitation, and kinase assays have been described previously (8). The procedures for treatment of cells with okadaic acid (OA) (Calbiochem) and nocodazole treatments with okadaic acid (OA) (Calbiochem) were modified to eliminate all but one serine and to maintain the secondary structure of the repeats. The modified CTD sequence is shown in Fig. 5.

Immunoprecipitation and Kinase Assay—Fibroblasts were lysed, and Abl was immunoprecipitated from cellular lysates using FLAGM2 monoclonal antibodies (Sigma) or K12 antibodies (Santa Cruz Biotechnology). Detailed procedures for fibronectin stimulation, cell lysate preparation, immunoprecipitation, and kinase assays have been described previously (8). The procedures for treatment of cells with okadaic acid and arresting cells in mitosis are described in the figure legends. Immunoblot analysis with the 8E9 (Pharmingen/BD Biosciences) or Ab-3 (Oncogene Sciences) Abl monoclonal antibodies was quoted and stored at 80 °C.

RESULTS

Mitotic Cells and Okadaic Acid-treated Cells Maintain Abl Kinase Activity in Detached Cells—Previous studies have shown Abl to be phosphorylated on at least 12 different Ser/Thr residues in mitotic cells (12). Hyperphosphorylation disrupts the DNA binding function of Abl (20) but does not appear to affect Abl catalytic activity (12). In those experiments, Abl activity in detached mitotic cells was compared with adherent interphase cells (12). However, when the comparison was made between detached nocodazole-arrested mitotic 10T1/2 mouse fibroblasts and detached interphase cells, it became clear that mitotic Abl activity was much higher than that of detached interphase cells (Fig. 1A). Mitotic hyperphosphorylation of Abl can be induced by treating interphase cells with OA (12). Therefore, Abl+/− mouse fibroblasts expressing either wild type or kinase-defective Abl (Abl-KD) were treated with 2 μM OA, and then Abl kinase activity was determined following immunoprecipitation of Abl from lysates of attached or detached cells (Fig. 1B). In immune complex kinase assays using GST-CTD as substrate and kinase-defective Abl (Fig. 1B, KD) as a negative control, we found that Abl activity from adherent fibroblasts was ~4-fold higher than Abl from cells in suspension (Fig. 1B, lanes 3 and 5 and histogram). Following OA treatment, however, Abl activity was maintained despite the loss of cell adhesion (Fig. 1B, lanes 4 and 6 and histogram). Because Abl is hyperphosphorylated in mitotic cells and OA-treated cells (12), these results suggest that hyperphosphorylation might contribute to the maintenance of Abl kinase activity in detached cells.

Phosphotyptic Mapping of Abl—To compare the sites of phosphorylation on Abl in OA-treated and mitotic cells, we performed phosphotyptic peptide mapping of Abl from 32P-labeled NIH3T3 mouse fibroblasts (Fig. 2). Abl from asynchronous fibroblasts contained three predominant phosphotyptic peptides (Fig. 2, spots a–c) and several minor phosphotyptic peptides (left panel). Phosphoamino acid analysis on each tryptic peptide (Fig. 2, spots a, b, and c) revealed only phosphoserine (not shown). The phosphoserine of peptide-a had previously been determined to be Ser568 (12). Peptide-b is likely to be phosphorylated at either Ser926 or Ser1029, and peptide-c, at Ser538 or Ser638 (21). Abl from mitotic cells contained 12 additional 32P-labeled peptides (Fig. 2, middle panel) (12). Abl from OA-treated cells contained some of the mitotic cell-specific phosphopeptides (Fig. 2, right panel) (12). One particular phosphopeptide (previously referred to as spot 1 (12), arrow) was prominent in Abl from both mitotic and OA-treated cells but was completely absent in Abl from asynchronously growing cells. This Abl phosphopeptide contained phosphothreone (not shown), and was previously determined to be Thr566 in the tryptic peptide DAPDPELLHTK of murine Abl (12). Thus, the Abl from mitotic or OA-treated cells is phosphorylated at Thr566.

Lewis et al. (22) have reported the transient appearance of multiple Abl bands, with a subset of the Abl protein migrating more slowly on SDS-PAGE, at early time points when detached fibroblasts were reattached to fibronectin. They suggested that Abl is activated by adhesion through a transient phosphorylation of the Abl protein based on the elimination of the slower peptides were purified using GST-agarose as described previously (19). Purified proteins were dialyzed against phosphate-buffered saline containing 1 mM dithiothreitol and 20% glycerol overnight and then aliquoted and stored at −80 °C.
migrating bands with in vitro serine and/or threonine phosphatase treatment. We examined the in vivo phosphorylation state of Abl in cells at 30 min following the replating of NIH3T3 cells on fibronectin by phosphotryptic mapping (not shown) but did not detect phosphorylation of Thr566. Thus, although Thr566 is phosphorylated in mitotic and OA-treated cells, its phosphorylation was not induced by cell adhesion to fibronectin.

**Mutation of Thr566 to Asp Prevents Detachment-induced Inactivation of Abl**—To determine whether phosphorylation of Thr566 may override the inhibitory effect of F-actin on Abl, we examined the consequences of substituting Thr566 with aspartic acid (T566D, TD), a phosphomimetic residue, or with alanine (T566A, TA), a nonphosphorylatable residue, on Abl activity. These mutant Abl proteins were tagged with the FLAG epitope, stably expressed in Abl−/− mouse fibroblasts, and purified using anti-FLAG agarose resin columns. Similarly, FLAG-tagged Abl, Abl-KD, and Abl-ΔABD were purified and used as positive and negative controls in the experiment. Although the in vitro kinase activity of Abl and TA-Abl toward GST-CTD was decreased in the presence of F-actin, the activity of TD-Abl was resistant to F-actin inhibition (Fig. 3A). Each purified Abl was tested for its ability to co-sediment with purified F-actin (Fig. 3B). Similar to Abl, we found that more than 90% of the Thr566 mutant Abl mutants (TD or TA) were recovered in the pellet (Fig. 3B, P) fraction after incubation with F-actin (Fig. 3B). In contrast, the ΔABD-Abl (Abl with the C-terminal three amino acids deleted resulting in a loss of F-actin binding) showed decreased co-sedimentation with F-actin. After correcting for nonspecific sedimentation of Abl (20 ± 10%, not shown) in the absence of F-actin, we calculated that 85% of the ΔABD-Abl remained in the supernatant fraction unbound to F-actin. Therefore, TD-Abl can bind to F-actin, but its kinase activity is resistant to inhibition by F-actin. We have previously shown that actin co-immunoprecipitates with Abl from detached cells and contributes to the reduction in kinase activity in immune complex kinase assays (9). We therefore compared the activity of transiently expressed TA- and TD-Abl immunoprecipitated from attached and detached cells (Fig. 3C). The autophosphorylation activity of Abl and TA- and TD-Abl was similar in attached cells (Fig. 3C). However, TD-Abl had higher activity than wild type Abl and TA-Abl in detached cells (Fig. 3C). Thus, TD-Abl is resistant to the inhibitory effect of F-actin, and its kinase activity is higher than Abl or TA-Abl in detached cells.
higher kinase activity than Abl or TA-Abl. The autophosphorylation of Abl before collection for immune complex kinase assays, the TD-Abl exhibited activities. When transfected cultures were detached by trypsinization first were left attached, the three Abl proteins exhibited similar autokinase (activity shown in the
tion experiments were performed for the measurement of Abl kinase with anti-FLAG antibodies coupled beads (Sigma). Independent transfec-
teins were each immunoprecipitated (IP) from attached or detached cells expressed by transient transfection in 293T cells. The indicated Abl pro-
sediment the F-actin. The quantity of Abl or F-actin in the supernatant
Asp; for activity with GST-CTD as the substrate and in the presence of 2 
Abl lacking the three amino acids at the extreme C terminus. B.
act-binding assay. The indicated purified Abl proteins were incubated with 2 μM F-actin, and reactions were centrifuged at 100,000 x g to sediment the F-actin. The quantity of Abl or F-actin in the supernatant (S) and pellet (P) fractions was determined by Western blotting with α-S6 or α-actin antibodies, respectively. C. Abl proteins (described in A) were expressed by transient transfection in 293T cells. The indicated Abl proteins were each immunoprecipitated (IP) from attached or detached cells with anti-FLAG antibodies coupled beads (Sigma). Independent transfection experiments were performed for the measurement of Abl kinase activity shown in the upper and lower panels. When transfected cultures were left attached, the three Abl proteins exhibited similar autokinase activities. When transfected cultures were detached by trypsinization first before collection for immune complex kinase assays, the TD-Abl exhibited more F-actin microspikes than those expressing Abl, T566A-Abl (T566A), or KD-Abl (KD).

FIG. 3. Mutation of Thr566 of Abl to aspartic acid abolges the F-actin-mediated inhibition of Abl. A, Abl protein was purified (αFLAGM2 resin) from Abl−/− fibroblasts stably infected with FLAG-tagged Abl type IV. Purified proteins were eluted off the resin and assayed for activity with GST-CTD as the substrate and in the presence of 2 μM BSA or 2 μM F-actin. Abl, wild type Abl; TD, Abl with Thr566 mutated to Asp; TA, Abl with Thr566 mutated to Ala; KD, kinase-defective Abl; ΔABD-Abl, Abl lacking the three amino acids at the extreme C terminus. B, actin-binding assay. The indicated purified Abl proteins were incubated with 2 μM F-actin, and reactions were centrifuged at 100,000 x g to sediment the F-actin. The quantity of Abl or F-actin in the supernatant (S) and pellet (P) fractions was determined by Western blotting with α-S6 or α-actin antibodies, respectively. C. Abl proteins (described in A) were expressed by transient transfection in 293T cells. The indicated Abl proteins were each immunoprecipitated (IP) from attached or detached cells with anti-FLAG antibodies coupled beads (Sigma). Independent transfection experiments were performed for the measurement of Abl kinase activity shown in the upper and lower panels. When transfected cultures were left attached, the three Abl proteins exhibited similar autokinase activities. When transfected cultures were detached by trypsinization first before collection for immune complex kinase assays, the TD-Abl exhibited higher kinase activity than Abl or TA-Abl. The autophosphorylation of Abl protein was measured by the incorporation of 32P (autoradiogram). WB, Western blot.

Crk N-SH3 but Not C-SH3 Disrupts F-actin-mediated Inhibition of Abl Kinase—Thr566 is located immediately C-terminal of the kinase domain within a PRL that contains binding sites for several SH3-adaptor proteins including Abi1/2, Crk, and Nck (13, 14, 23, 24). Previous studies have shown that the adaptor protein Crk binds to the PRL and is a substrate of the Abl kinase (14, 25). Interestingly, we found that Abl phosphorylation of GST-Crk in vitro was not inhibited by F-actin (Fig. 5, B and C). Abl autophosphorylation and phosphorylation of another substrate, the CTD of RNA polymerase II, by contrast, was inhibited by F-actin (Fig. 5, B and C). We have prepared a GST-Crk-mCTD fusion protein that contains just the phosphorylation site of Crk joined to three modified repeats of CTD (Fig. 5A), and the phosphorylation of this substrate by Abl was sensitive to inhibition by F-actin (Fig. 5, B and C). To examine directly the effect of Crk SH3 on Abl activity, we included either the N-terminal or the C-terminal SH3 domain of Crk in Abl autophosphorylation reactions (Fig. 5D). Purified Abl had detectable autokinase activity in vitro (Fig. 5D, lane 3), which was inhibited by F-actin (lane 4). Addition of the Crk N-terminal SH3, which binds to the Abl PRL, caused a 2.5-fold increase in autokinase activity (Fig. 5D, compare lanes 1 and 3). Addition of F-actin did not reduce the Abl autokinase activity when the Crk N-terminal SH3 was present (Fig. 5D, compare lanes 1 and 2 with lanes 3 and 4). By contrast, the Crk C-terminal SH3 did not significantly increase the Abl autokinase activity (Fig. 5D, compare lanes 3 and 5) and could not prevent F-actin from inhibiting Abl kinase (compare lanes 5 and 6 with lanes 3 and 4).

Abl-ΔSH2 Mutant Retains Kinase Activity in Detached Cells—The Abl SH2 domain has previously been shown to contribute to the processive tyrosine phosphorylation of Abl substrates such as the CTD of RNA polymerase II (26). Consistent with previous results, we found that ASH2-Abl from attached cells exhibited a 1.5–2-fold lower CTD-kinase activity than did Abl from attached cells (Fig. 6A). In contrast to Abl, the kinase activity of ASH2-Abl was not reduced in detached cells (Fig. 6A). This led us to test whether F-actin could inhibit the kinase activity of purified ASH2-Abl in vitro. The addition of F-actin lowered the purified Abl kinase activity but did not active in suspended fibroblasts and causes the formation of membrane protrusions rich in F-actin (9). The TD-Abl mutant, which also retains kinase activity in detached cells, exerted a similar effect (Fig. 4). The F-actin cytoskeleton of suspended Abl−/− cells stably expressing Abl, Abl-KD, or TA-Abl resembled those expressing only the retroviral vector (Fig. 4). In these suspended cells, F-actin was predominantly at the cell periphery as a ring underlying plasma membranes that appeared smooth. In contrast, cells expressing either of the two Abl mutants, T566D-Abl or ΔABD-Abl, contained numerous F-actin microspikes at the periphery (Fig. 4). This is consistent with our previous results showing that maintenance of active Abl kinase can produce F-actin protrusions in the absence of integrin-extracellular matrix engagement (9).
reduce the activity of ΔSH2-Abl (Fig. 6B). As a control, we showed that F-actin did not inhibit the kinase activity of ΔABD-Abl (Fig. 6B). We found that a point mutation that inactivates the phosphotyrosine-binding pocket of the SH2 domain (FLVR^{177}ES → FLVK^{177}ES) did not rescue Abl kinase from F-actin-mediated inhibition (data not shown). We have
also found that substitution of Abl SH2 with Src does not disrupt F-actin-mediated inhibition of Abl kinase (see Ref. 8). It thus appears that the complete removal of SH2 domain is required to prevent F-actin from inhibiting Abl. To further examine the basis for why ΔSH2-Abl kinase is resistant to F-actin, we performed sedimentation binding assays and found ΔSH2-Abl to be defective in co-sedimenting with F-actin (Fig. 6C). Previous work has shown that an ABD fragment of Abl (amino acids 979–1142), when purified as a GST fusion protein from bacteria, co-sedimented with F-actin (27). We have found that the ABL-SH2 domain alone did not co-sediment with F-actin (not shown). These results suggest that the SH2 domain may be required to maintain Abl in a conformation that allows the ABD to associate with F-actin. It is possible that the SH2 domain may be required in conjunction with the ABD to facilitate binding of full-length Abl to F-actin. Alternatively, deletion of the SH2 domain may affect the overall folding of the Abl protein resulting in the masking of the ABD.

**DISCUSSION**

**Maintaining Abl Kinase Activity in Detached Mitotic Cells**—The Abl non-receptor tyrosine kinase contributes to the cytoskeletal remodeling in fibroblasts and neurons (2, 28–30). Because Abl modifies actin structures through phosphorylating substrates (10, 31–34), the inhibitory effects of F-actin on Abl kinase must be repressed or relieved for Abl to exert its cytoskeletal effects. Our data suggest that the hyperphosphorylation of Abl during mitosis is one mechanism through which Abl escapes the F-actin mediated inhibition. We propose that mitosis-specific phosphorylation of Abl is needed to maintain its kinase activity despite the weakened interactions with the extracellular matrix in mitotic cells. We have shown that substitution of Thr\(^{566}\) with Asp is sufficient to disrupt F-actin-mediated inhibition and to maintain Abl kinase activity in detached cells. It should be noted that Thr\(^{566}\) is not conserved in the human ABL protein (human ABL contains Val at this position). However, other nearby mitotic Ser/Thr phosphorylation sites are conserved between mouse and human ABL. Moreover, we have found that human ABL as well as BCR-ABL are quantitatively hyperphosphorylated during mitosis (12). Although the T566D mutant abrogates F-actin-mediated inhibition of Abl kinase, T566A mutant remains sensitive to F-actin. However, the T566A mutant, similar to Abl, retained its kinase activity in detached mitotic cells (not shown). This suggests that other mitotic phosphorylation sites in Abl and/or additional regulatory mechanisms may exist to maintain Abl kinase activity in detached mitotic cells. At present, the reason why Abl activity is released from F-actin-dependent regulation during mitosis is unclear. Abl might play a role in the remodeling of F-actin cytoskeleton in mitotic cells or might contribute to initiation of post-mitotic cell spreading. However, because Abl-deficient cells do not exhibit mitotic defects in culture, the mitotic function of Abl tyrosine kinase may not be essential.

**Conformation-dependent Inhibition of Abl Kinase by F-actin**—The crystal structures of the N-terminal region of Abl have provided much insight toward an understanding of how intramolecular interactions can regulate its kinase activity (6). The Abl kinase domain is folded into an autoinhibited conformation that is stabilized by several interlocking interactions between the SH3 and SH2 domains, between the SH2 domain, the SH2-kinase linker, and the kinase N-lobe, and between the SH2 domain and the kinase C-lobe (6). The SH2/C-lobe interaction requires the binding of myristate to a lipid-binding pocket in the C-lobe (6) (Fig. 7). In one of the crystal structures (structure A), the myristate was added in trans, and it was bound into the kinase C-lobe. In another crystal structure containing murine Abl type IV amino acids 1–532 (structure B), the N-terminal myristoyl group bound into the same pocket (6). Folding of the N-terminal myristoyl group of human ABL-Ib (and murine Abl type IV) into the kinase C-lobe is likely to involve docking interactions of the N-terminal CAP with the SH3-SH2-kinase domains (6) (Fig. 7). Our finding that F-actin does not inhibit ΔSH2-Abl and only partially inhibits ΔSH3-Abl (8) suggests the autoinhibited conformation is required for F-actin to exert its inhibitory effect on Abl activity (Fig. 7).

The current crystal structure of Abl does not provide any insights into how the large C-terminal region of Abl (608 amino acids) may interact with the autoinhibited conformation because this region (murine type IV amino acids 535–1142) is not present in these structures. Data presented in this report suggest the C-terminal region can modulate the autoinhibited N-terminal structure and vice versa. We have found that deletion of the SH2 domain can disrupt the ABD/F-actin interaction. We therefore propose a model of mutual stabilizing interactions between the SH2/C-lobe assembly
and F-actin-ABD complex (Fig. 7). As a result, F-actin exerts a negative allosteric effect on Abl kinase by enforcing the autoinhibitory conformation.

We have also found that phosphorylation at Thr566 or binding of Crk N-terminal SH3 can disrupt the inhibition of Abl kinase by F-actin. Again, the current Abl crystal structures do not contain Thr566 or the PRL region, which begins with Pro545. The crystal structure has defined the myristate-binding site in the kinase C-lobe. The myristoyl group contacts 12 amino acids and induces the bending of helix a-I (6). The a-I helix is straight in crystal structures that do not contain myristate (6). When myristate is added to the Abl kinase domain, it engages several helices in the kinase C-lobe causing the a-I helix to adopt a bent conformation (6). Bending the a-I helix is required for interactions between the SH2 domain and residues in the C-lobe (6). Interestingly, the a-I helix extends to the C-terminal end of the crystal structure at Lys531. It is conceivable that a-I helix conformation may be subject to modulation by sequences that are immediately downstream but missing from the current crystal structure. We propose that Thr566 phosphorylation or binding of Crk N-terminal SH3 to the PRL is proposed to disrupt the SH2/C-lobe assembly because the PRL is immediately C-terminal to helix a-I, which controls the autoinhibitory interaction between the SH2 domain and the kinase C-lobe. By disrupting the SH2/C-lobe assembly, modulation of the PRL does not abolish F-actin binding but can abrogate the allosteric effect of F-actin on kinase activity.

![A hypothetical model for the conformation-dependent inhibition of Abl kinase by F-actin](image)

**Fig. 7. A hypothetical model for the conformation-dependent inhibition of Abl kinase by F-actin.** The N-terminal region of Abl is depicted according to the autoinhibited crystal structure, in which the myristoyl group at the N terminus is inserted into the kinase C-lobe (6). The CAP linker is depicted as a dashed line because it is not visible in the published crystal structure and is followed by the SH3 domain, which interacts with the SH2-kinase linker that scaffolds the SH3 and the kinase N-lobe. The SH3-SH2 linker is rigid in the crystal structure. The SH2 domain interfaces with the kinase C-lobe, and this interaction is dependent on myristate binding to induce a C-lobe conformational transition (6). We have found that the SH2 domain is required for the ABD to stably associate with F-actin and for F-actin to inhibit the kinase activity. We therefore propose that the SH2/C-lobe interaction revealed by the crystal structure to play a role in stabilizing the C-terminal ABD/F-actin interaction through as yet unknown mechanisms. Because the F-actin/ABD interaction leads to a decrease in kinase activity, F-actin may exert its negative effect by strengthening the SH2/C-lobe assembly in the autoinhibited conformation. Phosphorylation of Thr566 or binding of Crk N-terminal SH3 to the PRL is proposed to disrupt the SH2/C-lobe assembly because the PRL is immediately C-terminal to helix a-I, which controls the autoinhibitory interaction between the SH2 domain and the kinase C-lobe. By disrupting the SH2/C-lobe assembly, modulation of the PRL does not abolish F-actin binding but can abrogate the allosteric effect of F-actin on kinase activity.
gomerization, BCR-ABL lacks the myristoyl group and part of the CAP linker (6, 37); hence, its SH2/C-lobe interaction is likely to be weakened. Moreover, BCR-ABL stably associates with CrkL, a Crk-related protein, through the PRL region (38). Oligomerization, disruption of the SH2/C-lobe interaction, and CrkL binding to the PRL may each contribute to the release of BCR-ABL from F-actin-mediated inhibition.

In summary, the inhibitory effect of F-actin on Abl kinase can be overridden not only by mutating the F-actin binding consensus motif at the extreme C terminus of Abl but also through phosphorylation of Thr2565, binding of the Crk N-terminal SH3 domain, or deletion of the SH2 domain. At present, we cannot determine how the deletion of SH2 domain disrupts the F-actin binding function of Abl. Ultimately, co-crystallization of full-length Abl with F-actin bound to the C terminus is needed to fully elucidate how F-actin exerts its negative effects through the N-terminal autoinhibited conformation mediated by the interactions among the CAP-SH3-SH2-kinase domains. Unraveling the mechanisms that allow allosteric inhibitors such as F-actin to regulate Abl kinase will assist in the development of new strategies to inhibit deregulated BCR-ABL kinase of human chronic myelogenous leukemia.

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