UNIVERSITY OF CALIFORNIA, SAN DIEGO

The role of Abl tyrosine kinase in cell spreading

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Biology

by

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2007

The Dissertation of Hua Jin is approved, and it is acceptable in quality and form for publication on microfilm:

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2007

Dedication

This work is dedicated to my parents, Jianping Wu and Rongzhi Jin, and my other relatives. Thank you for your encouragement and support throughout my life. Without your loving and caring, I wouldn't have achieved these accomplishments.

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Acknowledgements

I thank my mentor Dr. Jean Wang for taking me as a graduate student from China. Thank you for guiding and helping me through graduate school. I thank my committee members, Dr. Michael David, Dr. Raffi Aroian, Dr. Richard Firtel, Dr. Shu Chien and Dr. Tony Hunter for giving me advice and guiding my research projects. I thank the Wang Lab members, especially Jonathan Zhu, for teaching and discussing experimental techniques. I also thank Marcus Smolka from Huilin Zhou's lab at UCSD for his contribution of the work in Chapter 3 of my dissertation.

Chapter 2, in full, is a reprint of the material as it appears in Molecular Biology of the Cell, 2007, Hua Jin; Jean Y. J. Wang, 2007. The author of the dissertation is the primary investigator and author of this paper.

Marcus Smolka is the co-author of Chapter 3. The author of the dissertation is the primary investigator and author of this paper.

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Jin, H. and Wang, J.Y.J. (2007) Abl Tyrosine Kinase Promotes Dorsal Ruffles but Restrains Lamellipodia Extension during Cell Spreading on Fibronectin. *Molecular Biology of the Cell.* 18, 4143~4154.

Lin, S., McLennan, AG., Ying, K., Wang, Z., Gu, S., **Jin, H.,** Wu, C., Liu, P., Yuan, Y., Tang, R., Xie, Y., and Mao, Y. (2001) Cloning, Expression, and Characterization of a Human Inosine Triphosphate Pyrophosphatase Encoded by the *ITPA* Gene. *Journal of Biological Chemistry.* 276, 18695~18701.

ABSTRACT OF THE DISSERTATION

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University of California, San Diego, 2007

Professor Jean Wang, Chair
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The non-receptor Abl tyrosine kinase regulates actin-dependent cellular processes. It stimulates F-actin microspikes and dorsal ruffles in response to adhesion and growth factor signals, respectively. Paradoxically, Abl inhibits cell migration, which also requires actin polymerization.

In the present study, we show that Abl tyrosine kinase inhibits cell spreading on fibronectin. Induced dimerization of an Abl-FKBP fusion protein, which activated Abl-FKBP kinase activity, interfered with cell spreading. The Abl kinase inhibitor,

imatinib, stimulated cell spreading and its effect was reversed by an imatinib-resistant AblT315I mutant. Re-introduction of Abl, but not its kinase-defective mutant, into *Abl/Arg* double knockout cells also inhibited cell spreading. Interestingly, Abl inhibits lamellipodia extension without affecting the overall levels of Rac-GTP. Furthermore, Abl kinase inhibited spreading of cells that expressed the constitutively active RacV12. Abl kinase caused the preferential localization of RacV12 to dorsal membrane protrusions, correlating with Abl kinase-dependent prolongation of dorsal ruffling during cell spreading. These results suggest that Abl tyrosine kinase may regulate the partitioning of actin polymerization machinery to favor dorsal ruffles at the expense of lamellipodia extension during cell spreading. This localized actin polymerization through Abl kinase may account for its positive role in dorsal ruffling and negative role in cell migration. But the Abl substrate(s) that is involved in dorsal ruffle formation remains unknown.

To identify Abl kinase substrates we generated a 293 cell line that inducibly expresses constitutively active Abl kinase, Abl PP. With this cell line, a mass spectrometry-based method was used to screen for Abl kinase substrates. We followed tyrosine phosphorylation changes of cellular proteins in the 293 cells with Abl PP induction in the presence or absence of Abl kinase inhibitor, imatinib. From two independent experiments, we found eighteen proteins have increased tyrosine phosphorylation with Abl PP induction in the absence of imatinib. Among the eighteen proteins, eight proteins were identified in both experiments. Six out of the eighteen proteins are established Abl substrates; the remaining twelve are novel Abl

substrates. Interestingly, among the eighteen proteins identified, eight of them are known Src substrates.

Chapter 1
Background

Identification of Abl tyrosine kinase

The *Abl* gene was first identified as the normal cellular counterpart to the Abelson murine leukemia virus oncogene *v-Abl* (Figure 1-1) [1]. Subsequently, the *Abl* gene was found to be involved in the generation of the *Bcr-Abl* oncogene through chromosomal translocation, which induces human myelogenous leukemia (Figure 1-1) [2-5].

The *Abl* gene is present in the genomes of multicellular eukaryotes from *C. elegans* to human and encodes a non-receptor tyrosine kinase [1, 6]. The mammalian *Abl* gene is expressed ubiquitously and has two alternative 5' exons with the usage of different promoters [7, 8]. This generates two Abl isoforms with different N-terminal sequences [7, 8]. The two Abl isoforms are denoted type Ia and Ib for human c-Abl, and type I and type IV for murine Abl [7, 8]. Both homozygous mutation and disruption of the *Abl* gene in mouse are neonatal lethal [9, 10], indicating its role during development. An *Abl*-related gene (*Arg*) is also present in the mammalian genome. Unlike the *Abl* knockout mice, *Arg* knockout mice are viable, but they exhibit behavioral abnormalities in walking, climbing, mating, and response to heat and acoustic stimuli [11]. However, *Abl* and *Arg* double knockout mice are embryonic lethal, they die at E9-11[11], suggesting that *Abl* and *Arg* may have overlapping functions during early and/or late embryonic development.

Functional domains of Abl protein

The N-terminus CAP region of type 1b/IV isoform of Abl contains 80 residues with an N-terminal myristoyl modification site (Figure 1-1). The N-terminus CAP region of type 1a/I isoform of Abl contains 60 residues without the myristoyl modification site due to the use of an alternative promoter [7, 8, 12]. Except for the difference in the N-terminal CAP region, both isoforms contain SH3, SH2 and kinase domains, followed by DNA-binding, and F-actin-binding domains at the C-terminus (Figure 1-1). Abl also contains three nuclear localization signals (NLS) and one nuclear export signal (NES) at the C-terminus, allowing it to shuttle between nucleus and cytoplasm [13]. Abl binds a variety of proteins mediated by one or more functional domains (Table 1-1).

Regulation of Abl tyrosine kinase

Like most kinases, Abl tyrosine kinase adopts a latent conformation in the absence of signals [14]. The inactive conformation is achieved by at least two mechanisms based on the crystal structure of Abl 1b (residues 2-531) with the inhibitor PD16632 or imatinib [15-17]. First, inactive Abl forms internal interactions among its SH3 and SH2 domains, and the two lobes of kinase domain (Figure 1-2), similar to that of Src family tyrosine kinases [16]. Disruption of these internal interactions, such as mutations in proline 242 and 249 to glutamic acids, or deletion of the SH3 domain, leads to Abl activation [18, 19]. Second, the N-terminal CAP (residues 1-80) plays an important role in maintaining the autoinhibitory conformation of Abl [15, 17]. The myristate from the N-terminal myristoyl modification of Abl 1b/IV binds to the kinase C-terminal lobe intramolecularly and induces

conformational changes that allow the SH2 domain to dock onto the kinase domain (Figure 1-2) [15, 17]. Furthermore, the N-terminal CAP segment of Abl1b/IV buttresses the SH3-SH2 substructure in the autoinhibited state and locks it onto the distal surface of the kinase domain [17]. Abrogation of the myristol-kinase domain interaction, such as mutation of the myristoylation site in the CAP region, or deletion of the CAP region, leads to Abl activation [12, 20]. Besides these two mechanisms, Abl is also kept inactive by binding to other proteins that function as Abl inhibitors. Examples of Abl inhibitor proteins include Rb, PAG and F-actin [21-23]. Cleavage or degradation of Rb activates Abl [22]; disruption of Abl and F-actin binding by mutating the F-actin binding domain of Abl also activates Abl [23].

Latent Abl can be activated by multiple signals such as DNA damage [24], growth factors (EGF and PDGF) [25], and adhesion (extracellular matrix proteins) signals [13]. In response to these signals, the auto-inhibitory conformation of Abl is disrupted by autophosphorylation, primarily at tyrosine 412 and 245 [18] by binding to its substrates, such as CrkII and Dok family proteins [26, 27] and by dissociation from Abl inhibitor proteins [22]. Taken together, the regulation of Abl kinase activity is complex and controlled by multiple mechanisms.

Biological functions of Abl tyrosine kinase

Nuclear Abl regulates DNA damage response

Nuclear Abl is activated by genotoxic agents or inflammatory cytokines, such as cisplatin and TNF, respectively [28, 29]. DNA damage signals induce caspase-

dependent cleavage/degradation of Rb [30, 31], which is a nuclear Abl kinase inhibitor [22]. Rb cleavage/degradation frees Abl from an inhibitory state and activation of Abl kinase leads to p73 protein induction [28]. Induction of p73 by Abl plays a role in DNA damage induced apoptosis. Cells lacking Abl kinase show defects in DNA damage induced apoptosis and the apoptosis defect is correlated with the failure to induce and activate p73 [28].

Nuclear Abl interacts with several proteins involved in DNA damage, including BRCA1 [32], ATM [33], Rad51, Rad52 [34-36], DNA-PK [37], and UV-DDB [38]. BRCA1, ATM, Rad51 and Rad52 are involved in homology recombination repair (HRR) of double-stranded breaks. DNA-PK plays a role in non-homologous end joining (NHEJ) repair of double-stranded breads. UV-DDB is a protein involved in UV damage. Despite the interaction between Abl and the DNA repair proteins, it is unclear whether Abl plays a role in regulating DNA damage repair.

Nuclear Abl phosphorylates RNA polymerase II [39-41]. Phosphorylation of RNA polymerase II by Abl enhances transcription from certain promoters (such as HIV-LTR) [40]. It is possible that Abl may enhance transcription from promoters of proteins that function in the DNA damage response. Abl phosphorylation of RNA polymerase II may also regulate mRNA splicing to generate specific transcripts that are important for the DNA damage response [42, 43].

Cytoplamic Abl regulates F-actin cytoskeleton

Cytoplasmic Abl stimulates membrane ruffling in response to PDGF or EGF [25]. Stimulation of fibroblasts with PDGF induces a transient activation of Abl kinase activity [25]. Abl activation by PDGF is dependent on Src family tyrosine kinases and phospholipase C-γ1 [25, 44]. The Src family tyrosine kinases can activate Abl by phosphorylating it at tyrosine 412 to disrupt its auto-inhibited conformation [25]. Phospholipase C-γ1 can activate Abl by removal of phospholipids (such as phosphatidylinositol-4,5-bisphosphate) that may inhibit Abl kinase [44]. PDGF induces remodeling of the F-actin cytoskeleton to form dorsal ruffles and dorsal ruffle formation is decreased in Abl knockout fibroblasts or fibroblasts with Abl kinase inhibition [25, 45]. Two Abl substrates, WAVE-2 and Cortactin, have been identified to mediate PDGF-induced dorsal ruffling. Abl phosphorylation of WAVE2 on tyrosine 150 increases WAVE2-driven actin polymerization [46]. WAVE2 knockout or Abl phosphorylation site mutant WAVE2 (WAVE2 Y150F) decreases dorsal ruffling of fibroblasts in response to PDGF [47]. Cortactin is a protein that stimulates actin polymerization and triggers F-actin ruffles in fibroblasts after PDGF treatment [48]. Abl phosphorylates Cortactin on tyrosine 421, 466 and 482 to promote dorsal ruffling and the simultaneous mutation of the three Abl phoshporylation sites of Cortactin abrogates its positive effect on dorsal ruffling [49].

Cytoplasmic Abl induces microspike formation in fibroblasts during cell spreading on fibronectin [50]. Integrin-mediated cell adhesion to fibronectin (an extracellular matrix protein) induces a transient activation of Abl kinase [13]. Activation of Abl correlates with the transient colocalization of Abl with integrins to

the early focal contacts [13]. Abl knockout fibroblasts spread like pancakes without microspikes and restoration of Abl kinase induces Abl knockout cells to form microspikes during cell spreading [50]. Dok-1 is the downstream effector of Abl that mediates microspike formation during cell spreading [51]. Dok-1 knockdown or the Abl phosphorylation site mutant Dok-1 (Dok-1 Y361F) abrogate the positive effect of Abl on microspike formation [51].

Cytoplasmic Abl kinase exerts a negative effect on cell migration. Inhibition of Abl kinase is correlated with increased chemotaxis response to HGF in thyroid carcinoma cells [52]. Abl knockout fibroblasts migrate faster than fibroblasts of their wild type counterpart [53]. Over-expression of Abl in Cos1 cells reduces migration [53]. CrkII has been shown to be one of the downstream effectors that mediate Abl induced reduction of cell migration. Phosphorylation of CrkII on tyrosine 221 by Abl disrupts the CrkII/p130Cas complex, which plays an important role in cell migration by activating the Rac small GTPase [53].

Cytoplasmic Abl can function downstream of Eph (ephrin) receptor activation. EphA-ephrin-A interaction at membrane contact sites triggers rapid loss of growth cone lamellipodia followed by axon retraction and cell-cell separation. Abl family kinases are major effectors of ephrin-A-induced retinal ganglion cell repulsion since the Abl inhibitor, STI571, prevents both loss of growth cone lamellipodia and axon retraction [54]. In breast cancer cells, EphB4 signaling inhibits cancer cell growth and invasion [55]. EphB4 activates an anti-oncogenic pathway involving Abl family

tyrosine kinases and the Crk adaptor protein. This Abl-Crk pathway inhibits breast cancer cell viability and proliferation in addition to motility and invasion, and also down regulates the pro-invasive matrix metalloprotease, MMP-2 [55].

Abl plays a role in neuronal development

Abl induces neurite outgrowth [56]. One of the mechanisms is through Abl phosphorylation of Cdk5, which is a protein involved in neuronal development [56]. In hippocampal neurons, Abl kinase promotes dendritogenesis by inhibiting RhoA activity [57]. It has been reported that Arg phosphorylates p190RhoGAP and results in down regulation of Rho activity [58, 59]. It is therefore possible that Abl can phsophorylate p190RhoGAP and regulate dendritogenesis through the same mechanism. In NGF-induced neuronal morphogenesis in PC12 cells, Abl associates with NGF receptor TrkA and is activated downstream of TrkA receptor activation [60, 61]. Upon Abl activation, phosphorylation of CrkII by Abl on tyrosine 221 is one of the signaling pathways that regulate NGF-induced morphogenesis [61].

Table 1-1: Abl interaction proteins

Proteins	Abl binding domain	Experimental system	References
14-3-3δ		Co-immunoprecipitation	[62]
Cable	SH3	Co-immunoprecipitation	[56]
Dok1		Affinity chromatography	[51]
Arg		Affinity chromatography	[63]
p85		Affinity chromatography	[63]
hNap1BP		Affinity chromatography	[64]
Sema6D		Co-immunoprecipitation	[65]
CRM1		Affinity chromatography	[66]
BCR		Affinity chromatography	[63]
Robo	SH3, SH2	Affinity chromatography	[67]
Abl	SH2	Affinity chromatography	[63]
ik3-2		Affinity chromatography	[68]
PLCγ1		Affinity chromatography	[63]
GAP		Affinity chromatography	[63]
RIN1		Co-immunoprecipitation	[69]
p47phox		Co-immunoprecipitation	[70]
v-Src		Affinity chromatography	[63]
WAVE1	SH3	Affinity chromatography	[71]
PDE4D4		Affinity chromatography	[72]
Rb	Kinase N-lobe	Affinity chromatography	[73]
EVL	SH3	Affinity chromatography	[74]
RPED-6	SH3	Affinity chromatography	[75]
Mena	SH3	Affinity chromatography	[76]
CrkI		Affinity chromatography	[77]
Dok-2	SH3	Co-immunoprecipitation	[27]

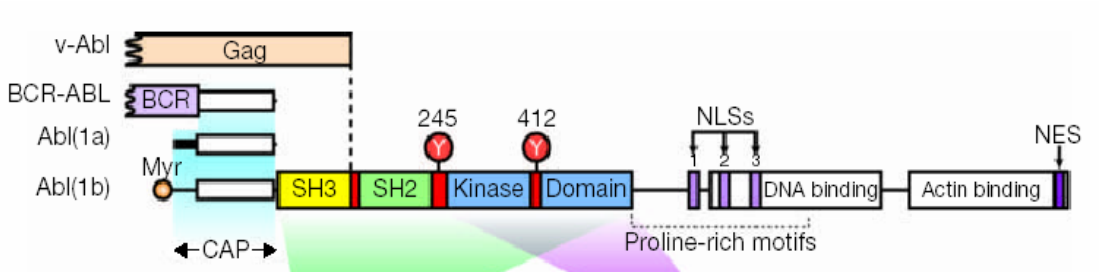
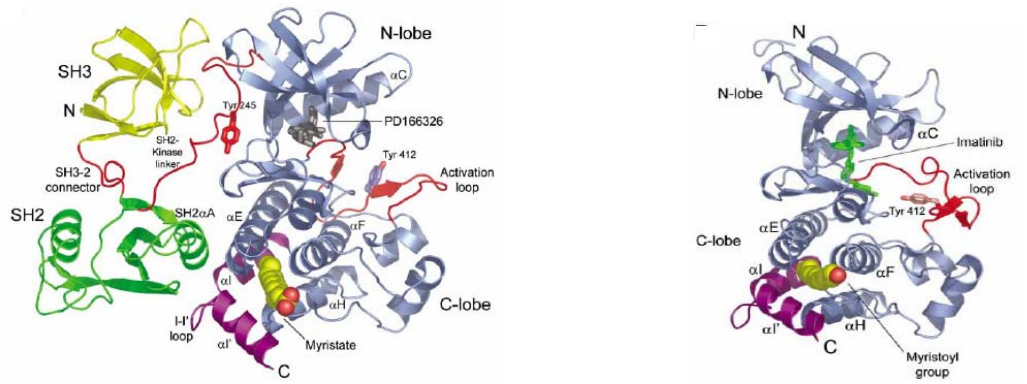


Figure 1-1: Functional domains of Abl [14]
Mammalian Abl has two alternative amino termini-1a (I in mouse) or 1b (IV in mouse) with different amino-acid sequences. Abl 1b, but not Abl 1a, is myristoylated (Myr). The CAP region contains the variable N terminus and common sequence (white box). Phosphorylation at Tyr 245 in the SH2 linker region, or Tyr 412 at the active site, can activate Abl kinase. NLS, nuclear localization signal; NES, nuclear export signal. Oncogenic v-Abl and BCR-Abl are N-terminal mutants of Abl. In v-Abl, viral GAG sequences are fused to Abl at the SH3-SH2 linker. In BCR-Abl, cellular BCR sequence is fused to Abl in the CAP region.



Structure A **Structure B**

Figure 1-2: Crytstal structure of c-Abl [15]
Structure A: structure of myristoylated c-Abl 1b (residues 2–531) in complex with the inhibitor PD166326. The N-terminal myristoyl moiety is sequestered in a hydrophobic pocket in the large lobe of the kinase domain. Only the myristoyl group is shown since the rest of the CAP region was disordered in the crystal. Structure B: structure of the kinase domain of c-Abl 1b bound to a myristoylated peptide corresponding to the N-terminal 16 residues of c-Abl 1b with the inhibitor imatinib. Only the myristoyl group is shown, since the rest of the peptide is disordered. Helices that change conformation upon myristoyl binding (αI and αI') are colored purple.

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Chapter 2

Abl Tyrosine Kinase Promotes Dorsal Ruffles but Restrains Lamellipodia Extension during Cell Spreading on Fibronectin

Abstract

The non-receptor Abl tyrosine kinase stimulates F-actin microspikes and membrane ruffles in response to adhesion and growth factor signals. We show here that induced dimerization of Abl-FKBP, but not the kinase-defective AblKD-FKBP, inhibits cell spreading on fibronectin. Conversely, knockdown of cellular Abl by shRNA stimulates cell spreading. The Abl kinase inhibitor, imatinib, also stimulates cell spreading and its effect is overridden by the imatinib-resistant AblT315I. Expression of Abl but not AbkKD in *Abl/Arg*-deficient cells again inhibits spreading. Furthermore, Abl inhibits spreading of cells that express the activated Rac, RacV12, correlating with RacV12 localization to dorsal membrane protrusions. Ectopic expression of CrkII, a Rac activator that is inactivated by Abl-mediated tyrosine phosphorylation, antagonizes Abl-mediated dorsal membrane localization of RacV12. Ectopic expression of a dynamin-2 mutant, previously shown to induce Rac-GTP localiazation to the dorsal membrane, abolishes the stimulatory effect of imatinib on cell spreading. These results suggest that Abl tyrosine kinase, through CrkII phosphorylation and in collaboration with dynamin-2 can regulate the partitioning of Rac-GTP to favor dorsal ruffles during cell spreading. The Abl-dependent dorsal membrane localization of activated Rac explains its positive role in ruffling and negative role in cell spreading and migration.

Introduction

The *Abl* gene is present in the genomes of multicellular eukaryotes from *C. elegans* to human and encodes a non-receptor tyrosine kinase with a conserved actin-binding domain [1-5]. The mammalian Abl tyrosine kinase is constitutively expressed and essential to the proper development as the knockout of mouse *Abl* gene causes neonatal lethality [6, 7]. An *Abl*-related gene (*Arg*) is also present in the mammalian genome; while the knockout of *Arg* did not cause developmental defects, the combined ablation of *Abl* and *Arg* leads to early embryonic lethality [8]. The Abl tyrosine kinase undergoes nucleo-cytoplasmic shuttling and plays an important role in the regulation of cell growth and cell death [9, 10]. The conserved interaction between Abl and actin suggests the regulation of actin dynamics to be a key function of this tyrosine kinase [3]. Indeed, the Abl tyrosine kinase is found to stimulate dorsal ruffles in response to growth factors such as PDGF and EGF [11, 12]. Abl kinase is also activated by cell adhesion to stimulate the formation of F-actin microspikes [13, 14].

Dorsal ruffles and microspikes are formed as a result of regulated actin polymerization at the plasma membrane. The ability of Abl kinase to promote actin polymerization is suggested by the findings that Abl phosphorylates components of the WAVE complex, including Abi-1/2 and WAVE2 [15-18]. The WAVE complex is composed of WAVE1/2, Abi1/2, NAP1, SRA and HSPC300 subunits [19, 20]. The WAVE complex can be activated by Rac-GTP binding the SRA, or NCK binding the NAP1 subunit [19, 21, 22]. Activated WAVE complex stimulates Arp2/3 to nucleate

actin-polymerization [23-25]. Abl-mediated tyrosine phosphorylation of WAVE2 is associated with an enhanced stimulation of the Arp2/3 complex and increased membrane ruffles [17, 18]. Activated Abl tyrosine kinase also phosphorylates the Dok-1 adaptor protein to recruit Nck-family of adaptors, leading to Rac-GTP-independent formation of F-actin microspikes [14, 26]. These results establish Abl as a positive effector in transducing growth factor and cell adhesion signals to the stimulation of actin polymerization.

Paradoxically, Abl tyrosine kinase inhibits cell migration, a process that is also dependent on actin polymerization [27]. Inhibition of Abl tyrosine kinase stimulates the migratory response of carcinoma cells to hepatocyte growth factor [28]. Ectopic expression of Abl tyrosine kinase in Cos1 or *Abl/Arg* double knockout cells inhibits cell migration [29]. During migration, cells form lamellipodia at the leading edge [30]. This process of lamellipodia extension also promotes cell spreading on extracellular matrix (ECM) proteins such as fibronectin [31]. The small GTPase Rac is essential for lamellipodia formation in migratory and spreading cells [32, 33]. Ectopic expression of a dominant negative Rac mutant, RacN17, inhibits lamellipodia extension and cell spreading [31, 34]. Fibroblasts derived from *Rac1* knockout mouse embryos spread slower than the *Rac1*-wild type cells due to the deficiency in lamellipodia formation [35].

Abl has been reported to exert positive and negative effects on Rac. The oncogenic v-Abl tyrosine kinase stimulates Rac and Rac-dependent pinocytosis to

promote cell proliferation [36]. The cellular Abl tyrosine kinase is activated by growth factors such as PDGF and EGF to increase the levels of Rac-GTP and stimulate membrane ruffling [12]. On the other hand, Abl-dependent tyrosine phosphorylation of Crk has also been shown to disrupt the Crk-Cas complex, correlating with a reduction of Rac-GTP level and the inhibition of cell migration [29].

Previous studies have established that Abl kinase is activated upon cell adhesion to ECM proteins such as fibronectin [13, 14, 37-39]. Abl activity peaks between 15-25 min after plating cells onto fibronectin-coated surface and returns to a basal level that is higher in attached than detached cells [13, 14, 37-39]. We show here that Abl tyrosine kinase exerts a negative role in cell spreading, consistent with its inhibitory effect on cell migration [28, 29]. Furthermore, we show that Abl kinase inhibits the spreading of cells that express the constitutively active RacV12 protein. The Abl kinase caused preferential localization of RacV12 to dorsal membrane protrusions. Ectopic expression of CrkII, antagonizes Abl-mediated dorsal membrane localization of RacV12 and reduced Abl-mediated inhibition of cell spreading. Ectopic expression of a dynamin mutant, previously shown to induce Rac-GTP localiazation to the dorsal membrane, abolishes the stimulatory effect of imatinib on cell spreading. Our findings suggest that Abl kinase-dependent dorsal membrane sequestration of Rac-GTP may reduce the pool of Rac-GTP for lamellipodia formation at the leading edge and thus account for Abl-dependent inhibition of cell spreading and migration.

Materials and Methods

DNA constructs, transfection and retroviral infection

Abl-FKBP, and AblKD-FKBP coding sequence was released from pC4M-Fv2E vector [40] by Xbal I and Bam HI digestion and blunt-end ligated into PMSCVhyg (clontech) at the Xho I site. The wild type Abl (murine type IV), AblT315I, and AblKD were ligated into PMSCVhyg at the Sal I site. The Phoenix™ retroviral expression system (Orbigen Inc.) was used to produce recombinant retrovirus. HA-RacV12 and GFP-Rac (gifts of Mark H. Ginsberg, UCSD, La Jolla, CA), GFP-RacV12 (a gift of Martin A. Schwartz, University of Virginia, Charlottesville, VA), myc-CrkII and myc-CrkI-Y221F (gifts of Kritiina Vuori, The Burnham Institute, La Jolla, CA), and HA-dynamin-2 and HA-dynamin-2-K44A (gifts of Sandra L. Schmid, The Scripps Research Institute, La Jolla, CA) were transfected using lipofectamine 2000 (Invitrogen). Stable polyclonal 3T3/Abl-FKBP, 3T3/AblKD-FKBP, 3T3/FKBP, *Abl/Arg* DKO/vector, *Abl/Arg* DKO/Abl and *Abl/Arg* DKO/AblKD were selected by hygromycin resistance following infection with the pMSCVhyg retroviruses.

shRNA Knockdown

Targeting sequences against LacZ and Abl were AACAGTTGCGCAGCCTGAATG and AACCTGTACACTTTCTGTGTG, respectively. Pairs of complementary oligonucleotides were annealed and ligated into HindIII/BglII-digested pRS shRNA expression retrovirus vector [41]. The phoenix™ retroviral expression system (Orbigen Inc,) was used to produce shRNA expression

virus. Stable polycolonal LacZ and Abl knockdown cell lines were selected by puromyocin resistence.

Cell culture and reagents

NIH3T3 fibroblasts and *Abl/Arg* double knockout fibroblasts were cultured in Dulbecco's modified Eagles medium (DMEM), supplemented with 10% fetal bovine serum (Hyclone), and antibiotics. Dimerizer, AP20187 (ARIAD Inc.) was used at 50nM. Imatinib was used at 5uM. Coverslides were coated with fibronectin at 10μg/ml at 4ºC overnight.

Cell spreading assays and immunofluorescence

Cells of ~80% confluence were trypsinized, resuspended in serum-free DMEM containing 0.5mg/ml soybean trypsin inhibitor, washed twice with serum free DMEM /0.1% BSA. Cells were held in suspension in DMEM/0.1% BSA for 45~75 min at room temperature and then plated on fibronectin-coated coverslides and allowed to spread at 37ºC. At various time points, cells were fixed in 4% paraformaldehyde, permeabilized in 0.3% Triton X-100/PBS, blocked in 10% goat serum/PBS and then stained with monoclonal anti-Abl 8E9 (BD Biosciences) followed by Alex 568-conjugated secondary antibody and/or Alex 546 or 488-conjugated phalloidin (Molecular Probe). Fluorescent images were captured with a CCD camera. The surface areas of cells were measured with Image-Pro software. At least 100 cells were analyzed per sample. Results were from three independent experiments.

Adhesion assay

Plates (96-well) were coated with fibronectin at concentrations of 0.1μg/ml to 20μg/ml. Suspension cells were prepared as in the spreading experiments and were seeded at $1x10^4$ cells per well and allowed to adhere for 5~120 min at 37ºC. Wells were washed twice with serum free DMEM/0.1%BSA and adherent cells were fixed with 5% glutaraldehyde, and then stained with crystal violet (0.1%). Following extensive washing to remove the free dye, the cell-bound crystal violet was extracted with 0.5% Triton X-100 and absorbance measured at 595nm.

Rac-GTP and Rho GTP pull down assay

Suspension cells were prepared as in spreading experiments and were allowed to spread on fibronectin-coated 6-well plates for the indicated time. Cells were lysed on plates in the presence of GST-PBD (Pak Rac-binding domain). Rac-GTP was pulled down with bacteria purified GST-PBD as described [42]. Rho-GTP was pulled down with bacteria purified GST-RBD (Rhotekin Rho binding domain) [43]. X-ray films of the immunoblotting experiments were scanned and digital signals quantitated with chemilmager 4400 software.

Immunoprecipitation, immunoblotting and antibodies

Cells were lysed in RIPA buffer (50mM Tris, pH 7.4, 150mM Nacl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, protease inhibitor cocktail (Roche), 1mM PMSF, 1mM EDTA, 10mM sodium vanadate, and 10mM β-glycerol-phosphate). Whole cell lysate (50μg) was fractionated on SDS-PAGE, transferred to nitrocellulose membrane, and blotted with primary antibodies followed by horse peroxidase-

conjugated secondary antibodies and visualized by chemiluminescence. For immunoprecipitation, 1mg of cell lysate was used. Antibodies: anti-Abl, 8E9 (BD Biosciences); anti-phosphotyrosine, 4G10 (Upstate); anti-tubulin (Santa Cruz); anti-HA (Convance); anti-GFP (Clontech); anti-Rac1 (stategene); anti-RhoA (Santa Cruz); anti-dok-1 (Santa Cruz); anti-actin (Sigma).

Microscopy

Fluorescent images were obtained with a fluorescence microscopy (Zeiss) or a delta vision deconvolution microscope system (Nikon TE-200 microscope). SEM images were obtained with a Hitachi S-2700 scanning electron microscope.

Quantitation of dorsal localization of RacV12 and F-actin

The localization of GFP-RacV12, GFP-Rac, and GFP was examined in fully spread cells by deconvolution microscopy. Dorsal membrane protrusions are mostly absent form fully spread 3T3/vector cells. With 3T3/Abl cells that overexpressed Abl, prominent dorsal membrane protrusions with GFP-signals were observed in fully spread cells. Therefore, we made a binary distinction between cells with GFP-signals in dorsal membrane protrusions (of various degrees) and cells without GFP-signals in dorsal membrane protrusions. A similar binary distinction was applied to the quantitation of dorsal F-actin ruffles. The percentage of cells with or without dorsal F-actin ruffles was determined by examining at least 50 cells for each sample from three independent experiments. For experiments in Figure 7E, 3T3 cells plated in the presence or absence of imatinib were allowed to spread for 20 or 40 min, fixed and

stained with Alex-546 conjugated phalloidin. Individual cells were analyzed for size and dorsal F-actin protrusions. Cells were grouped into 11 categories based on size, and in each category, at least 50 cells were counted to determine the percentage of cells with dorsal F-actin ruffles.

Results

Dimerization of Abl-FKBP inhibits cell spreading on fibronectin

We generated an Abl-FKBP, as well as an Abl kinase defective-FKBP (AblKD-FKBP) fusion protein and adopted the strategy of activating Abl kinase activity with a chemical inducer of dimerization [40, 44]. Previous studies have shown that in the presence of a bivalent FKBP ligand, FKBP-Abl or Abl-FKBP undergoes dimerization leading to its autophosphorylation and kinase activation [40, 44]. The Abl-FKBP and AblKD-FKBP used in this study was constructed by fusing two copies of FKBPv, a variant of FKBP that specifically interacts with a synthetic ligand, AP20187 [45], and the HA epitope to the C-terminus of Abl (murine type IV) (Figure 1A). The Abl-FKBP or AblKD-FKBP fusion cassette was then introduced into an MSCV-based retroviral vector for the production of recombinant retroviruses, which were used to infect mouse 3T3 fibroblasts and to establish polyclonal cell lines stably expressing Abl-FKBP, AblKD-FKBP or FKBP (3T3/Abl-FKBP, 3T3/AblKD-FKBP and 3T3/FKBP). The level of Abl-FKBP and AblKD-FKBP in 3T3/Abl-FKBP and 3T3/AblKD-FKBP cells was comparable to that of endogenous Abl (Figure 1B). The Abl-FKBP protein contained basal tyrosine phosphorylation in the absence of AP20187 (Figure 1B). Following the addition of dimerizer, we observed a further ~2 fold increase in Abl-FKBP tyrosine phosphorylation (Figure 1B), confirming that inducing Abl-FKBP dimerization elevated its kinase activity [40, 44]. As a control, the AblKD-FKBP protein did not contain detectable tyrosine phosphorylation in the absence or presence of dimerizer (Figure 1B).

To examine the effect of Abl-FKBP dimerization on cell spreading, exponentially growing cells were trypsinized and then treated with dimerizer (or vehicle control) while they were held in suspension in serum-free media. After a 30-min pre-incubation with dimerizer, cells were plated on fibronectin-coated coverslides, allowed to spread under serum-free condition and then collected for image analyses following staining with fluorescently labeled phalloidin. Dimerizer treatment did not change the spreading of control 3T3/FKBP cells (Figure 1C), as measured by either cell surface area (Figure 1D), or percentage of spread cells (Figure 1E). The spreading of 3T3/Abl-FKBP cells were reduced when compared to the 3T3/FKBP cells even in the absence of dimerizer (Figure 1, C-E). With dimerizer treatment, the spreading of 3T3/Abl-FKBP cells was further decreased (Figure 1, C-E). By contrast, the spreading of 3T3/AblKD-FKBP cells was increased relative to 3T3/FKBP cells in the absence of dimerizer (Figure 1, C-E). Furthermore, dimerizer treatment did not alter the spreading of 3T3/AblKD-FKBP cells (Figure1, C-E). Thus, dimerization-induced activation of Abl-FKBP kinase caused a reduction in cell spreading on fibronectin.

Inhibition of endogenous Abl kinase promotes cell spreading

Abl kinase is transiently activated upon cell adhesion to fibronectin [13, 14, 39]. To examine if endogenous Abl also inhibits cell spreading, we stably expressed *Abl-shRNA* (3T3/Abl-shRNA) to reduce its levels by about 2 fold (Figure 2B). We found the 3T3-*Abl-shRNA* cells to spread faster than 3T3/*LacZ-shRNA* cells (Figure 2, A and B), consistent with Abl being a negative effector in cell spreading. We also

performed spreading experiments with 3T3 cells in the presence or absence of an Abl kinase inhibitor, imatinib (Gleevec^{TM}) [46, 47]. Cells treated with imatinib consistently spread faster than those treated with vehicle control (Figure 2, A and B). To demonstrate the effect of imatinib was due to the inhibition of Abl kinase, we used an imatinib-resistant Abl kinase, AblT315I, which was constructed based on the mutation identified in a imatinib-resistant BCR-ABL kinase [48]. Wild type (wt) murine Abl and its 315I derivative (AblT315I) were expressed in 3T3 fibroblasts via retroviral-mediated gene transfer. Cell spreading and immunoblotting experiments were performed 48 hours after infection to ensure high levels of ectopic Abl and AblT315I expression (Figure 2C). By measuring the phosphotyrosine content of Dok-1, a known substrate of Abl kinase [14], we confirmed that AblT315I activity was resistant to imatinib (Figure 2C). Cells expressing ectopic wt-Abl (3T3/Abl) spread slower than those infected with retroviral vector (3T3/vector), but the negative effect of Abl was overridden by imatinib (Figure 2D). The ectopic expression of AblT315I inhibited spreading to a similar extent as that of Abl (Figure 2D, vehicle), however, imatinib did not reverse the negative effect of AblT315I (Figure 2D, imatinib). Thus, the stimulatory effect of imatinib on cell spreading is mediated through its inhibition of the Abl tyrosine kinase.

In time course experiments, we found that the stimulatory effect of imatinib was observed during the first 20 min of plating cells onto a fibronectin-coated surface (Figure 2E, upper panel), consistent with previously reported transient activation of Abl kinase activity upon cell adhesion to fibronectin [13, 39]. The overproduction of

wt-Abl significantly reduced the rate of cell spreading (Figure 2E, lower panel), however, the addition of imatinib accelerated spreading to a level comparable to that found with imatinib-treated 3T3/vector cells (Figure 2E, compare the two imatinib time courses). Therefore, the negative effect of Abl overproduction on cell spreading requires its kinase activity. We also examined the effect of Abl overproduction on cell adhesion as a function of time and fibronectin concentrations (Figure 2F). Abl overproduction did not affect cell adhesion at the fibronectin concentration tested (0.1μg/ml~20μg/ml); neither did it affect cell adhesion between 5 and 120 minutes after plating on fibronectin (Figure 2F). Thus, Abl kinase is unlikely to interfere with cell spreading by impairing cell adhesion.

Expression of Abl kinase in Abl/Arg double knockout mouse fibroblasts inhibits cell spreading

Previous studies have employed fibroblasts derived from *Abl/Arg* double knockout (DKO) mouse embryos to study the effect of Abl kinase on the F-actin cytoskeleton [12, 14, 17]. The *Abl/Arg* DKO cells are deficient in forming F-actin microspikes when spreading on fibronectin-coated surfaces, but microspikes are formed through Abl reconstitution [14]. We expressed murine wt-Abl (type IV) or its kinase-defective mutant (AblKD) in the *Abl/Arg* DKO cells via retroviral-mediated gene transfer, and generated polyclonal populations of stable expressers through hygromycin-selection (Figure 3A). As previously reported [14], Abl-reconstituted *Abl/Arg* DKO cells displayed F-actin microspikes when spreading on fibronectin,

which was not observed in *Abl/Arg* DKO cells expressing vector or AblKD (Figure 3B). In addition, we found that Abl-reconstituted *Abl/Arg* DKO cells spread slower than those reconstituted with vector or AblKD (Figure 3, B and C). These results further provide genetic evidence for the negative effect of Abl tyrosine kinase on cell spreading.

Imatinib does not affect the GTP-loading of Rac and Rho during cell spreading

It is well established that adhesion signals activate Rac-GTP to stimulate lamellipodia extension through actin polymerization [19, 20, 23]. Because imatinib stimulates cell spreading, we determined its effect on Rac-GTP during cell spreading using the Rac-GTP-binding domain of PAK (PBD) as an affinity ligand GST-pull down assay [42]. As expected, the Rac-GTP levels were low in detached 3T3 cells but increased rapidly following cell plating on fibronectin (Figure 4, A and B). The addition of imatinib did not affect the Rac-GTP levels during the time frame when it stimulated cell spreading (Figure 4, A and B). In other experiments, we found the GTP-loading of Rac and Cdc42 to be similar between 3T3/vector cells and 3T3/Abl cells that overproduce the Abl protein (not shown). Thus, we were unable to observe an effect of either the endogenous Abl or overproduced Abl on adhesion-induced GTP-loading of Rac.

The levels of Rho-GTP are regulated by cell adhesion as well, but in a different manner from that of Rac-GTP [49, 50]. Detached fibroblasts maintained Rho-GTP.

Upon cell adhesion, the level of Rho-GTP is initially reduced to allow the extension of lamellipodia [49, 50]. The Rho-GTP level then rises during cell spreading to promote the formation of actin stress fibers [49]. Using the Rho-GTP binding domain of Rhotekin as an affinity ligand [51], we were able to detect the transient reduction in Rho-GTP followed by a restoration of Rho-GTP levels during cell spreading on fibronectin (Figure 4, C and D). Again, we were unable to detect any significant effects of imatinib on the Rho-GTP levels (Figure 4, C and D). These results show that imatinib stimulates cell spreading without affecting the overall level of Rac-GTP or Rho-GTP.

Ectopic expression of RacV12 does not override the negative effect of Abl on cell spreading

To demonstrate that Abl can inhibit cell spreading despite the activation of Rac, we performed spreading experiments with constitutively active RacV12 [52]. We transfected 3T3/vector or 3T3/Abl cells with plasmids expressing HA-RacV12 and/or GFP. The levels of HA-RacV12 were determined by immunoblotting of whole cell lysate (Figure 5A, upper panel), and the levels of HA-RacV12-GTP by GST-PBD pull down (Figure 5A, middle panel). As expected, ectopic expression of RacV12 stimulated cell spreading (Figure 5B, compare bars 1 and 2; 4 and 5). However, RacV12-transfected 3T3/Abl cells still spread slower than the RacV12-transfected 3T3/vector cells (Figure 5B, compare bars 2 and 5). The overproduction of Abl did not affect the levels of RacV12 protein, nor did it affect the binding of RacV12 to GST-PBD (Figure 5A, compare lanes 5 and 2). The levels of RacV12 and its binding

to GST-PBD were also unaffected by imatinib treatment (Figure 5A, lanes 3 and 6). Nevertheless, imatinib treatment was able to stimulate the spreading of RacV12-transfected 3T3/vector or 3T3/Abl cells to a similar extent (Figure 5B, bars 3 and 6). In addition to co-transfection with GFP, we also examined a GFP-RacV12 fusion protein and observed similar results (Figure 5C). Therefore, ectopic expression of RacV12 does not override the negative effect of Abl on cell spreading.

Abl kinase-dependent RacV12 localization to dorsal membrane protrusions

Previous studies have shown that Abl tyrosine kinase stimulates dorsal ruffles in response to growth factor stimulation [11, 12]. Dorsal ruffles are membrane protrusions also stimulated by Rac-mediated actin polymerization [53]. Given the observations that Abl kinase did not affect the level of RacV12 (Figure 5A) but reduced cell spreading (Figure 5, B and C), we examined whether Abl altered the localization of RacV12 (Figure 6). Among fully spread 3T3/Abl cells, we found GFP-RacV12 localized to dorsal membrane protrusions in ~60% of the cells (Figure 6A, left panels). In the other 40% of fully spread 3T3/Abl cells, GFP-RacV12 was found at the peripheral membranes and cytosolic space (Figure 6A, middle panels), a distribution similar to that in fully spread 3T3/vector cells containing physiological levels of Abl (Figure 6B, left panel). Thus, overproduction of Abl causes dorsal membrane localization of GFP-RacV12 in fully spread cells. This effect of Abl overproduction was completely abrogated by treatment with imatinib in that GFP-RacV12 was distributed at the peripheral membranes and cytosolic space in ~96% of fully spread cells (Figure 6A, right panels). We also examined the distribution of

GFP-Rac in 3T3/Abl cells. Without imatnib, ~40% of fully spread 3T3/Abl cells showed GFP-Rac in dorsal membrane protrusions, which were less prominent than those found in 3T3 cells expressing GFP-RacV12 (Figure 6C, left panels). With imatinib, virtually none of the GFP-Rac was localized to dorsal membrane protrusions (Figure 6, right panels). As a control, GFP signal was found at the cell periphery and diffusely in the cytosolic space of, and we did not observe dorsal membrane localization of GFP with or without imatinib fully spread 3T3/Abl cells (Figure 6D). These results showed that Abl tyrosine kinase could sequester Rac at the dorsal membrane, correlating with the formation of dorsal protrusions.

Abl kinase prolongs dorsal ruffling during cell spreading

Activated Rac-GTP stimulates membrane protrusions including ruffles and lamellipodia [54]. If the Abl-mediated RacV12 localization to dorsal ruffles also applied to the endogenous Rac-GTP, we would expect 3T3/Abl cells to exhibit increased dorsal ruffling relative to their imatinib-treated counterparts. We therefore examined dorsal ruffles, which was scored as F-actin-rich wrinkles on the topside of spreading cells away from the fibronectin-coated surface by deconvolution microscopy (Figure 7, A and B). Immediately after plated on fibronectin-coated coverslides, 3T3/Abl cells displayed dorsal ruffles irrespective of imatinib treatment (small cells, 1000~1250 micron^2, Figure 7, A and C), indicating that adhesion-induced dorsal ruffles does not require Abl kinase activity. Among the larger and more spread-out 3T3/Abl cells, 70-80% retained dorsal ruffles in the absence of imatinib but only

20-30% retained dorsal ruffles in the presence of imatinib (large cells, 1750~2000 micron^2, Figure 7, B and C).

The observation that overproduction of Abl kinase promoted dorsal ruffles (Figure 7, A-C) prompted us to examine whether endogenous Abl also regulates dorsal ruffling during cell spreading. By scanning electron microscopy, we confirmed that fibronectin-induced dorsal ruffling occurred irrespective of imatinib treatment (Figure 7D), consistent with the result that imatinib did not affect fibronectin-induced GTP loading of Rac (Figure 4A), which stimulates membrane protrusions [54]. We then determined the percentage of cells with dorsal F-actin ruffles as a function of cell area, collecting data at 20 min and 40 min after plating cells on fibronectin (Figure 7E). We found an inverse correlation between cell area and dorsal ruffles. Interestingly, this negative correlation was significantly enhanced with imatinib-treated cells (Figure 7E), suggesting imatinib-treated cells lose their dorsal ruffles faster than their un-treated counterparts. This result is consistent with the notion that Abl tyrosine kinase maintains dorsal ruffling during cell spreading.

Effects of Crk and dynamin on Abl-dependent dorsal localization of RacV12

To explore the mechanism of Abl kinase-dependent dorsal membrane location of activated Rac, we examined the effect of CrkII. Previous studies have shown CrkII to stimulate cell spreading and migration by activating Rac [29, 55]. Previous studies have also established CrkII to be a substrate of Abl [56, 57], and that tyrosine phosphorylation of CrkII at Y221 leads to an intramolecular SH2-Ptyr interaction that

inactivates the CrkII SH2 adaptor function [58]. When CrkII was coexpressed with GFP-RacV12 in 3T3/Abl cells, we observed a reduction in the dorsal membrane localization of the GFP signal (Figure 8A). Correspondingly, expression of CrkII stimulated the spreading of 3T3/Abl cells (Figure 8B). The ectopic expression of CrkII did not completely aborgate the effects of imatinib on GFP-RacV12 localization and spreading (Figure 8, A and B). We also examined the effect of CrkII-Y221F mutant. We found that the Y221F mutation did not create a constitutively active CrkII in that it did not reduce Abl-dependent dorsal membrane localization of GFP-RacV12, neither did it stimulate cell spreading (Figure 8B). Furthermore, CrkII-Y221F maintained dorsal localization of GFP-RacV12 in imatinib-treated 3T3/Abl cells (Figure 8A), conrresponding to a slower rate of cell spreading (Figure 8B). These results suggest that the interplay between CrkII and Abl may regulate the localization of active Rac and cell spreading.

We also examined the role of dynamin-2 in Abl-dependent dorsal localization of RacV12 because a previous report has shown dynamin-2 to play a role in Rac translocation to the leading edge [59]. The ectopic expression of wild-type dynamin-2 did not affect the localization of GFP-RacV12 or the spreading of 3T3/Abl cells (Figure 8, C and D). Expression of a dominant negative dynamin-2-K44A, which has been shown to induce dorsal membrane localization of Rac-GTP and inhibit cell spreading [59], did not cause a significant further spreading inhibition of 3T3/Abl cells that already spread slowly due to the overproduction of Abl. However, in the presence of dynamin-2-K44A, the spreading stimulatory effect of imatinib was partially

abolished (Figure 8D), correlating with sustained dorsal localization of GFP-RacV12 (Figure 8C). These results suggest that the Abl kinase and the dominant negative dynamin mutant are likely to act through the same or an inter-dependent mechanism to regulate active Rac localization and cell spreading.

Discussion

Abl regulates cell spreading

We have established that Abl tyrosine kinase regulates cell spreading with several lines of evidences. Expression of Abl, but not its kinase-defective mutant (AblKD), in *Abl/Arg* double knockout cells inhibits cell spreading (Figure 3, B and C). Dimerization-induced activation of Abl-FKBP (but not AblKD-FKBP) or overproduction of Abl exerts a negative effect on 3T3 cell spreading (Figure 1, C-E; figure 2D). Conversely, shRNA-mediated Abl knockdown or inhibition of Abl kinase with imatinib stimulates cell spreading, and an imatinib-resistant AblT315I kinase overrides this effect (Figure 2, A and B; figure 2D). Cell spreading is a complex and dynamic process. Using total internal reflection fluorescence (TIRF) microscopy, a previous study has described two modes of spreading- anisotropic and isotropic [60]. The isotropic spreading, with a higher initial rate of area increase and 78% of the cell edge extending, is more prevalent with serum-starved cells [60]. The Abl kinase is activated by growth factors and by cell adhesion [11, 13]. We conducted the spreading experiments in serum-free condition that favors isotropic spreading. Although we did not apply TIRF in this study, our results suggest that inhibition of Abl tyrosine kinase further enhance isotropic spreading in sereum-starved cells. In other words, the Abl tyrosine kinase may antagonize the isotropic mode or stimulate the anisotropic mode of spreading as indicated by the slower rate of area increase and the prolonged dorsal ruffling during cell spreading with increased Abl activity.

Regulation of Rac-GTP localization

Rac is activated by cell adhesion to induce lamellipodia formation and thus promotes cell spreading [31, 34, 35]. Although Abl has been shown to enhance growth-factor-stimulated Rac-GTP levels [12], we have found that Abl kinase does not affect the GTP loading of Rac stimulated by cell adhesion to fibronectin (Figure 4, A and B). We also show that Abl kinase activity is not required for fibronectin to initiate dorsal ruffles (Figure 7, A, C and D); instead, Abl kinase prolongs dorsal ruffling during cell spreading (Figure 7, B, C and E). Furthermore, Abl inhibits the spreading of cells that express the activated Rac mutant, RacV12 (Figure 5, B and C), correlating with an Abl kinase-dependent localization of RacV12 to the dorsal membrane protrusions (Figure 6). These results suggest that Abl tyrosine kinase may regulate the partitioning of Rac-GTP to favor dorsal ruffles and thus reducing the pool of Rac-GTP for lamellipodia extension to inhibit lamelliodia extension.

The membrane localization of Rac-GTP is stimulated by cell adhesion signals [61-63]. Targeting of Rac-GTP to cholesterol-enriched membrane microdomains is required for Rac-GTP to activate its downstream effectors [63]. Results from this study suggest that the distribution of Rac-GTP within the plasma membrane may be further regulated through integrin-dependent activation of Abl tyrosine kinase. Shlunck *et al* have proposed that Rac-GTP is translocated through a dynamin-dependent endocytosis pathway to the basal membrane that contacts the ECM and thus fueling the continuous extension of the laemellipodia during cell spreading [59]. This notion is consistent with the findings that Rac-GTP is distributed in the plasama membrane and the cytoplasm [61].

We have replicated the result of Shlunck *et al* showing that the dominant negative dynamin-2-K44A mutant inhibits the spreading of 3T3 cells (not shown). With 3T3/Abl cells that already spread slower due to the overproduction of Abl, dynamin-2-K44A does not further reduce cell spreading (Figure 8D). However, the stimulatory effect of Abl kinase inhibitor imatinib is stunted in 3T3/Abl cells expressing dynamin-2-K44A (Figure 8D). This result suggests that Abl either acts upstream or in parallel with dynamin to regulate RacV12 localization to the dorsal membrane. A recent report has shown that the Abl tyrosine kinase can inhibit the endocytosis of activated EGF receptor [64]. It is conceivable that Abl tyrosine kinase may prevent the endocytosis of Rac-GTP-containing lipid domain and thus reducing the rate and/or frequency of lamellipodia extension on the fibronectin matrix. The precise mechanism for how Abl tyrosine kinase sequesters Rac-GTP at the dorsal location will await further investigation.

Role of Abl Substrates

We have established that the negative effect of Abl on cell spreading requires its tyrosine kinase activity. The Abl tyrosine kinase phosphorylates a number of proteins that have been implicated in the regulation of F-actin polymerization [3]. These include the Crk-family, the Dok-family, the p130Cas-family of adaptor proteins, and the components of the WAVE complex [15, 17, 26, 29, 65]. Tyrosine phosphorylation of Dok-1 by Abl has been shown to promote F-actin microspikes during cell spreading [26]. We also observed Abl-dependent formation of microspikes

in Abl-reconstituted *Abl/Arg* double knockout cells (Figure 3B). Despite increased Dok-1 phosphorylation, we did not observe F-actin microspikes in spreading 3T3 cells overexpressing Abl either in the presence or absence of imatinib (Figure 2, B and C). These observations suggest that microspike formation is not necessary for Abl to inhibit cell spreading; additionally, neither wild type Dok-1 or Dok-1 phosphorylation site mutant, Dok-1 Y361F [26] reverses Abl-mediated inhibition of cell spreading (data not shown), suggesting Dok-1 phosphorylation by Abl may not be sufficient to inhibit cell spreading. Nevertheless, Dok-1 and WAVE2 phosphorylation by Abl is likely to stimulate actin polymerization at the dorsal membrane [18, 26] which may contribe to the sequestration of Rac-GTP.

Our results suggest that Abl-dependent phosphorylation of Crk is involved in the regulation of Rac localization and cell spreading. During cell spreading on fibronectin, CrkII stimulates membrane localization of Rac-GTP without affecting the overall levels of Rac-GTP [55]. We have found that overproduction of CrkII reduces the dorsal membrane localization of RacV12 and partially reduces the spreading defect of 3T3/Abl cells (Figure 8, A and B). By contrast, we found that CrkII-Y221F, which is not phosphorylated by Abl [56], does not stimulate the spreading of 3T3/Abl cells (Figure 8B). Thus, mutation of Y221 does not create a constitutively active CrkII for cell spreading; rather, it abolishes the spreading-stimulatory activity of CrkII. Abassi and Vuori have shown that CrkII stimulates the membrane targeting of RacV12 and that CrkII-Y221F was defective in this activity [55]. We have found that expression of CrkII-Y221F maintains the dorsal membrane localization of Rac-GTP in 3T3/Abl

cells (Figure 8A), suggesting that this mutant is not defective in targeting Rac-GTP to the membrane, but rather, it preferentially target Rac-GTP to dorsal membrane ruffles. Treatment with imatinib did not reverse the dorsal sequestration of RacV12 in cells expressing CrkII-Y221F (Figure 8A). These results suggest that the interplay between Abl and CrkII is involved in the regulation of Rac-GTP localization and thus cell spreading. However, this interaction is likely to be modulated by other mechanisms to account for the net negative effect of Abl kinase on cell spreading.

Acknowledgements

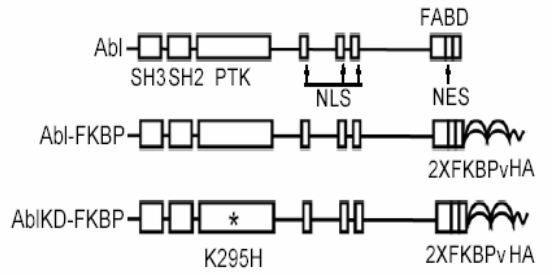
We thank Dr. Mark H. Ginsberg for the HA-RacV12 and GFP-Rac plasmids; Dr. Martin A. Schwartz for the GFP-RacV12 plasmid; Dr. Kritiina Vuori for the CrkII and the CrkII-Y221F plasmids; Dr. Sandra L. Schmid for the dynamin2 and dynamin-2-K44A plasmids; Kersi Pestonjamasp from the UCSD-Cancer Center microscopy service for delta vision deconvolution microscopy; and the SDSU electronic microscopy facility for scanning electronic microscopy. This work was supported by a grant from the National Institutes of Health, HL57900.

This Chapter is a reprint of the material as it appears in Molecular Biology of the Cell, 2007, Hua Jin; Jean Y. J. Wang, 2007. The author of the dissertation is the primary investigator and author of this paper.

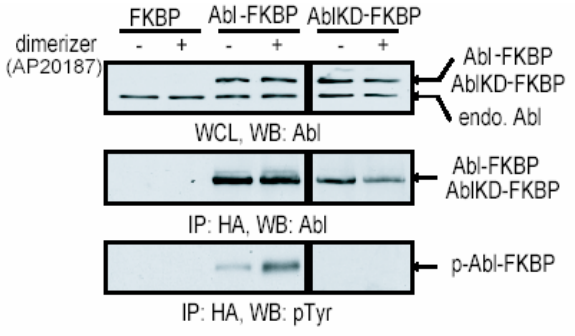
Figure 2-1: Dimerization of Abl-FKBP causes slower cell spreading on fibronectin

(A) Diagram of Abl (murine type IV) and Abl-FKBP fusion proteins. PTK, protein tyrosine kinase domain; NLS, nuclear localization signal; NES, nuclear export signal; FABD, F-actin binding domain; FKBPv, modified FK506 binding domain (Clackson *et al.*, 1998). The kinase defective mutant (KD) was created by substitution of Lys 295 with His (Welch and Wang, 1995). (B) The indicated cells were detached, held in suspension for 45 min in serum-free DMEM, and then treated with AP20187 (50 nM) or vehicle (0.1% ethanol) for 30 min. Cells were harvested for immunoprecipitation (IP) and immunoblotting (WB) with indicated antibodies as described in Materials and Methods. (C) The indicated cells were treated with AP20187 or vehicle as in (B). Cells were fixed 40 min after plated on FN (10μg/ml)-coated slides and stained with Alex 546-conjugated phalloidin. Scale bars equal 50 μm. (D) Quantitation of cell spreading in (C) by measuring cell surface area shown as box plots with the minimum, the median, the maximum, the 25th and the 75th percentile values. Values shown are from at least 100 cells for each sample. (E) Quantitation of cell spreading in (C) by counting the number of spread cells (marked by arrows in C) among at least 100 cells. The values and standard deviations were from three independent experiments.

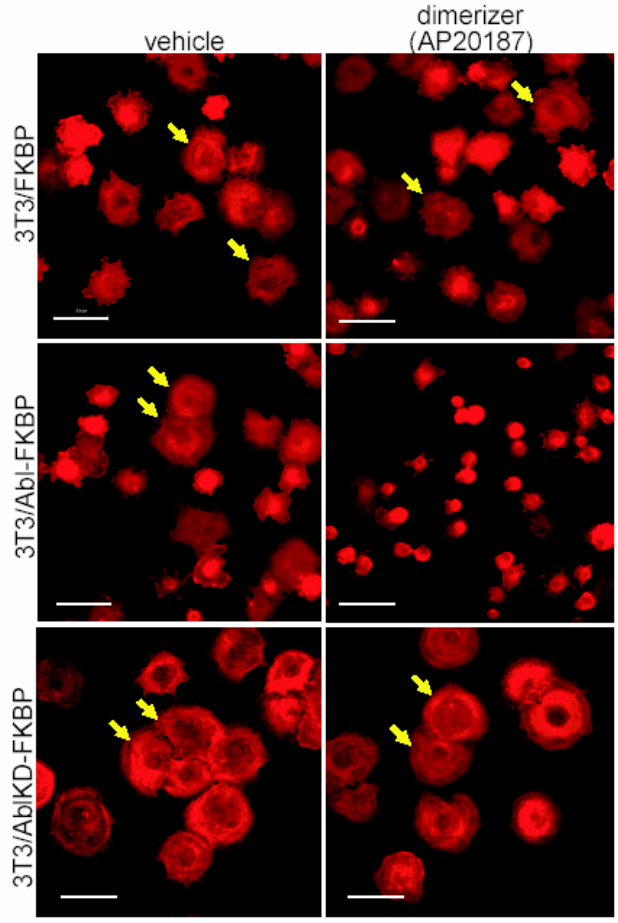
2-1A


FABD
Abl
SH3 SH2 PTK
NLS
NES
Abl-FKBP
2XFKBPvHA
AblKD-FKBP
K295H
2XFKBPv HA

2-1B


FKBP
Abl-FKBP
AblKD-FKBP
dimerizer
(AP20187)
- + - + - +
Abl-FKBP
AblKD-FKBP
endo. Abl
WCL, WB: Abl
Abl-FKBP
AblKD-FKBP
IP: HA, WB: Abl
p-Abl-FKBP
IP: HA, WB: pTyr

2-1C



2-1D

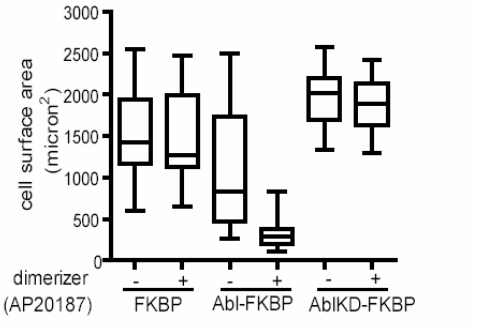


Figure 2-1: continued

2-1E

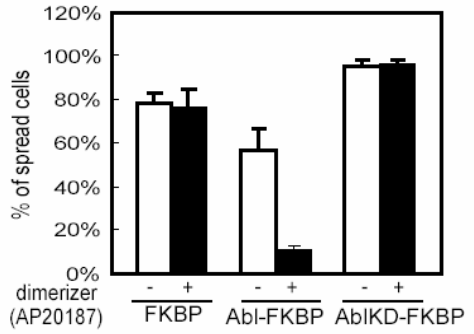
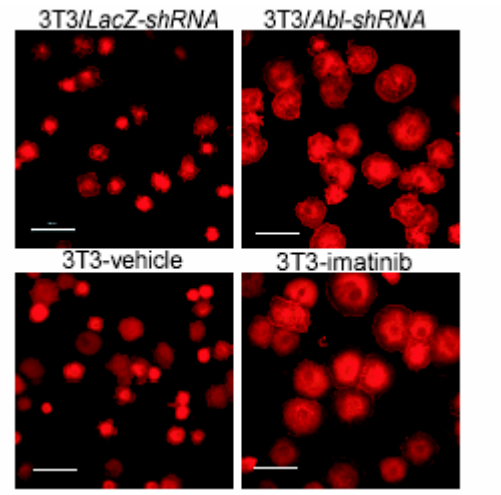


Figure 2-1: continued

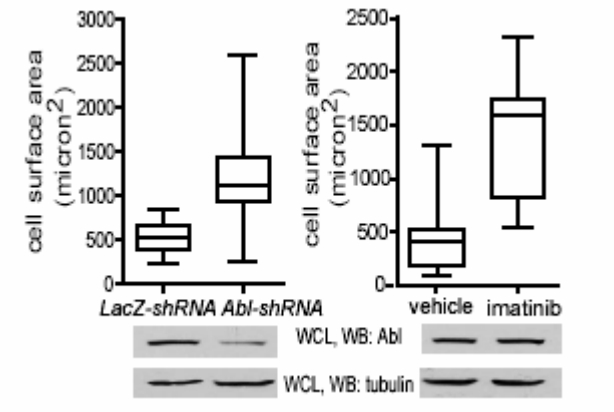
Figure 2-2: Knockdown of Abl or inhibition of Abl kinase by imatinib promotes cell spreading

(A) Top left panel: 3T3 cells stably expressing LacZ shRNA; top right panel: 3T3 cells stably expressing Abl shRNA. Bottom left panel, 3T3 cells treated with vehicle; bottom right panel, 3T3 cells treated with imatinib (5uM). The indicated cells were detached and held in suspension for 30 min in serum-free DMEM, and then treated with imatinib (5 μM) or vehicle (0.1% DMSO) for 45 min before plating on FN (10μg/ml)-coated coverslides. Cells were fixed 20 min later and stained with Alex 546-conjugated phalloidin. Scale bars equal 50 μm. (B) Quantitation of cell spreading in (A) by measuring cell surface area shown as box plots. Values shown are from at least 100 cells for each sample. (C) 3T3 cells were infected with retrovirus encoding vector, Abl, or Abl315I, treated with imatinib or vehicle as in (A) 48 hr post infection, and then harvested for immnoblotting (WB) and immunoprecipitation (IP) with the indicated antibodies. (D) Cells in (C) were fixed 20 min after plated on FN-coated coverslides and then stained with anti-Abl /Alex 568- conjugated secondary antibody (red) and Alex 488-conjugated phalloidin (green). Scale bars equal 25 μm. (E) The indicated cells treated with imatinib or vehicle as in (A) were plated on FN-coated coverslides, fixed at the indicated time, and the percentage of spread cells determined. In the 3T3/Abl sample, only cells with increased anti-Abl immunofluorescence signal were scored. At least 100 cells were counted in each sample from three independent experiments. (F) The 3T3/vector or 3T3/Abl cells were detached and replated on fibronectin. Cell adhesion was measured at the indicated time after plating on 10 μg/ml fibronectin, or at 1 hr after plating cells on different concentrations of FN (0, 0.1, 0.5, 1, 5, 10, 20 μg/ml) as described in Materials and Methods.

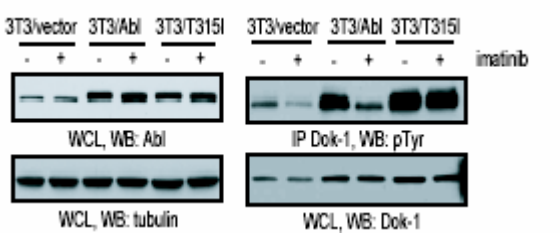
2-2A


3T3/LacZ-shRNA
3T3/Abl-shRNA
3T3-vehicle
3T3-imatinib

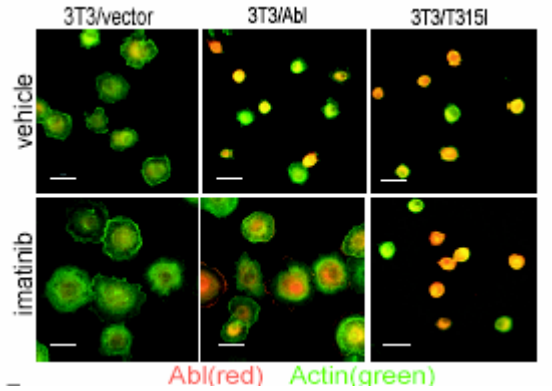
2-2B


cell surface area (micron2)
3000
2500
2000
1500
1000
500
0
LacZ-shRNA
Abl-shRNA
cell surface area (micron2)
2500
2000
1500
1000
500
0
vehicle
imatinib
WCL, WB: Abl
WCL, WB: tubulin

2-2C


3T3/vector
3T3/Abl
3T3/T315I
3T3/vector
3T3/Abl
3T3/T315I
imatinib
WCL, WB: Abl
IP Dok-1, WB: pTyr
WCL, WB: tubulin
WCL, WB: Dok-1

2-2D



2-2E

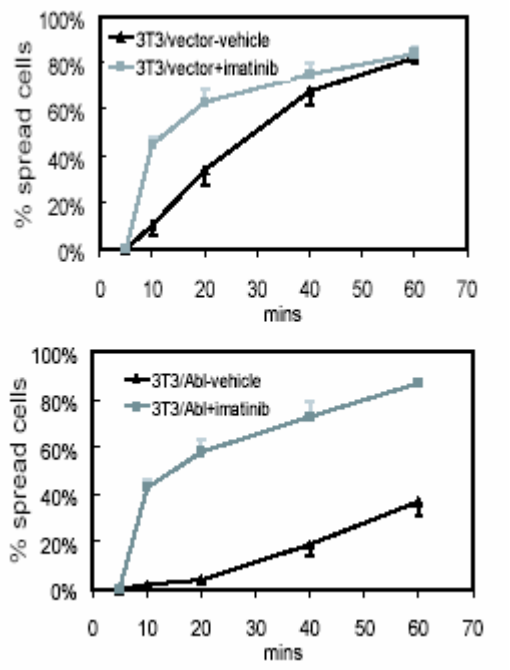


Figure 2-2: continued

2-2F

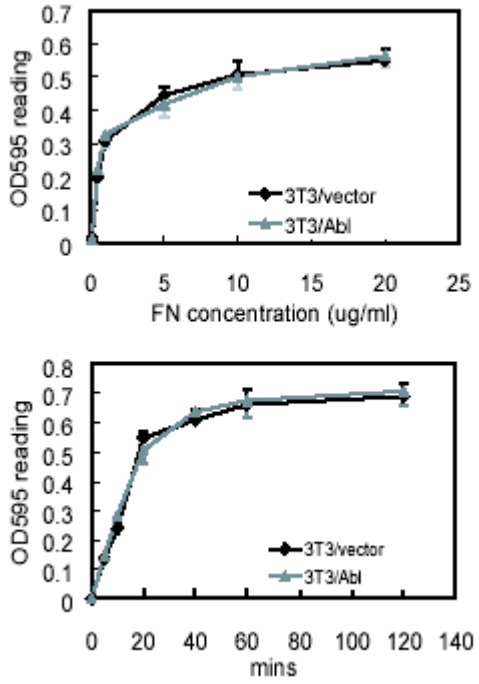
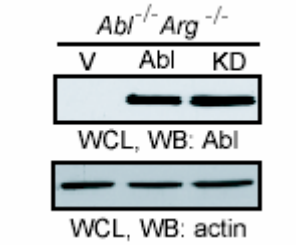


Figure 2-2: continued

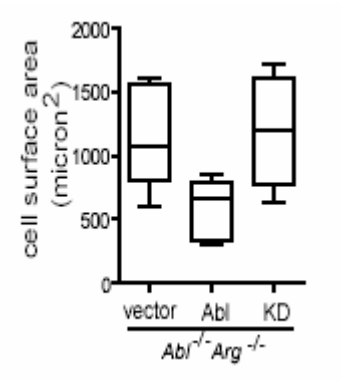
Figure 2-3: Expression of Abl in *Abl/Arg* double knockout fibroblasts inhibits cell spreading

(A) Cells expressing vector (V), wild type Abl (Abl), or kinase defective Abl (KD) were harvested for immunoblotting (WB) with indicated antibody. (B) The indicated cells were detached and held in serum-free DMEM for 45 min. After plating on FN (10μg/ml) coated coverslides for 20 min, cells were fixed and stained with anti-Abl /Alex 568 conjugated secondary antibody (red) and Alex 488-conjugated phalloidin (green). Scale bars equal 25 μm. (C) Quantitation of cell spreading in (B) by measuring cell surface area shown as box plots. Values shown are from at least 100 cells for each sample.

2-3A


Abl-/- Arg -/-
V
Abl
KD
WCL, WB: Abl
WCL, WB: actin

2-3B


cell surface area (micron2)
2000
1500
1000
500
0
vector
Abl
KD
Abl-/- Arg -/-

2-3C

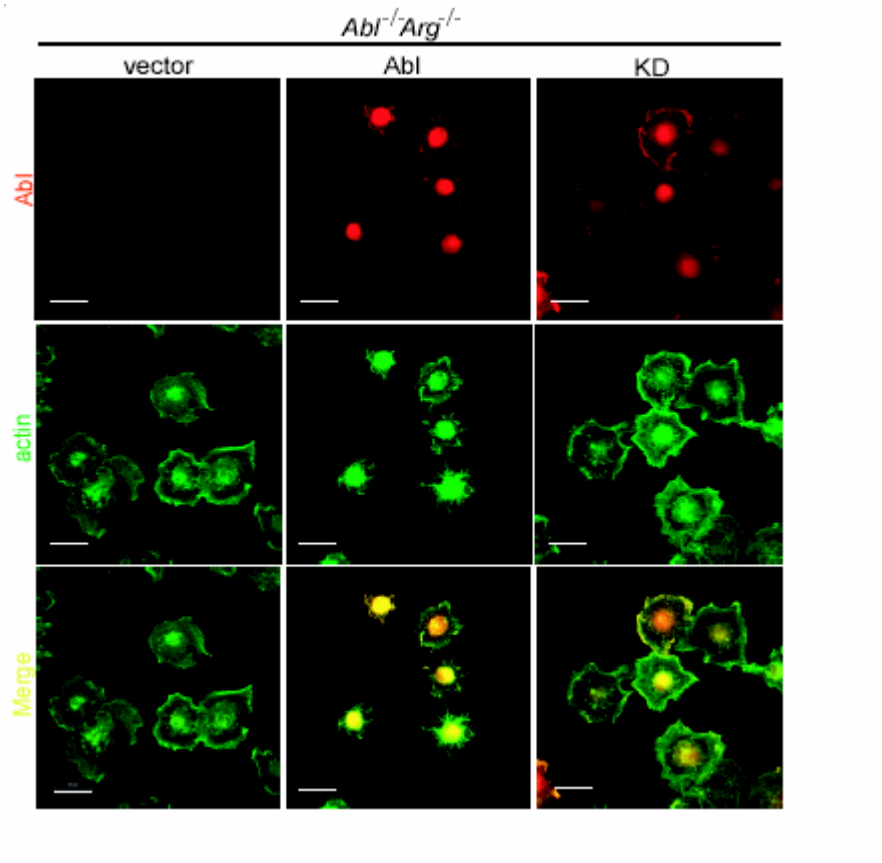
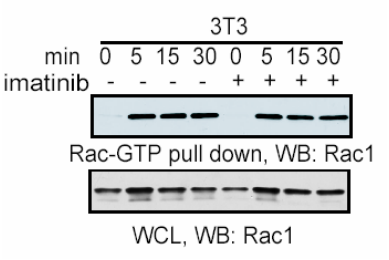


Figure 2-3: continued

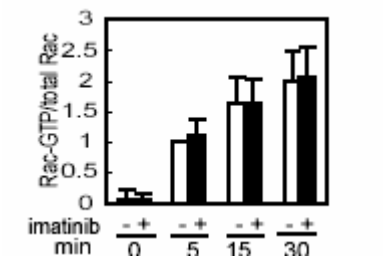
Figure 2-4: Imatinib does not affect GTP-Rac or GTP-Rho levels during cell spreading

(A) 3T3 fibroblasts were serum starved for 18 hr prior to detachment. Cells were held in serum free DMEM for 30 min and then treated with imatinib (5 μM) or vehicle (0.1% DMSO) for 45 min. Cells were plated on FN-coated dishes for the indicated time and harvested for Rac pull-down with GST-PBD as described in Materials and Methods. (B) Quantitation of data in (A) as described in Materials and Methods. The ratio of GST-PBD pull-downed Rac over total Rac of the vehicle-treated 5-min sample was set to 1. Values shown are from three independent experiments. (C) Cells were processed and harvested as in (A) and Rho was pulled down with GST-RBD (Rho-binding domain of Rhotekin) as described in Materials and Methods. (D) Quantitation of GST-RBD pulldown Rho as in (B). Values are from three independent experiments.

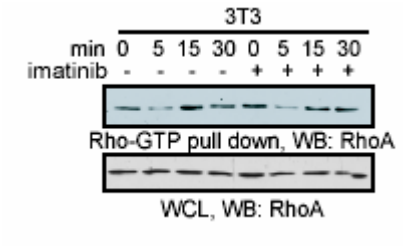
2-4A



2-4B



2-4C



2-4D

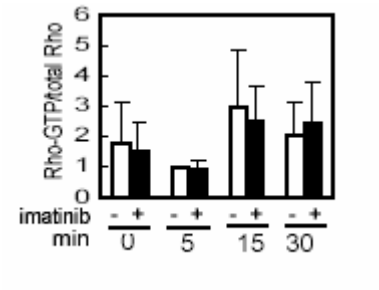
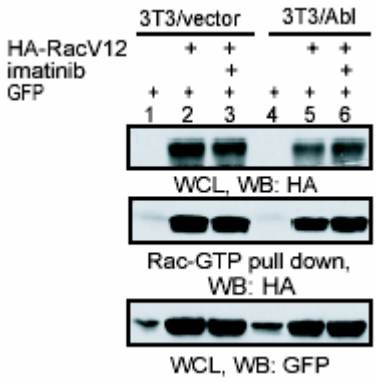
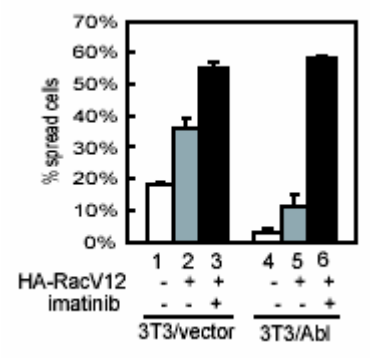


Figure 2-5: Abl inhibits the spreading of cells expressing RacV12
(A) 3T3 cells were infected with retrovirus encoding vector or Abl, and transfected 48 hours later with HA-RacV12 and/or GFP. Transfected cells were detached and held in serum-free DMEM for 30 min, treated with imatinib (5μM) or vehicle for 45 min and plated on FN (10μg/ml)-coated plates, collected at 20 min after plating for immunoblotting (WB) and GST-PBD pull down as described in Materials and Methods. (B) Cells prepared as in (A) were plated on FN (10μg/ml)-coated coverslides for 20 min, fixed and stained with anti-Abl/Alex 568-conjugated secondary antibody. In the 3T3/vector sample, GFP positive cells were evaluated for spreading. In the 3T3/Abl sample, cells that were GFP positive and with higher levels of Abl were scored. At least 100 cells were counted in each sample from three independent experiments. (C) The indicated cells were transfected with GFP-RacV12, treated and cell spreading determined as in (B). At least 100 cells were counted in each sample from three independent experiments.

2-5A


3T3/vector
3T3/Abl
HA-RacV12
imatinib
GFP
1 2 3 4 5 6
WCL, WB: HA
Rac-GTP pull down,
WB: HA
WCL, WB: GFP

2-5B


% spread cells
70%
60%
50%
40%
30%
20%
10%
0%
1 2 3 4 5 6
HA-RacV12
imatinib
3T3/vector
3T3/Abl

2-5C

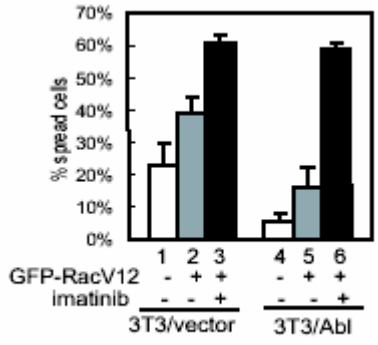
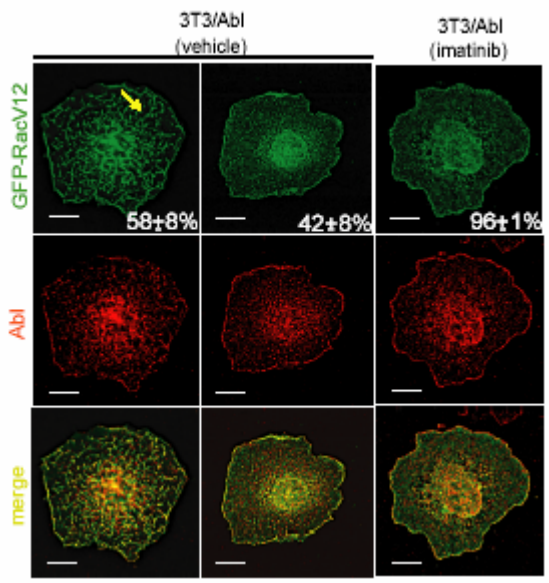

% spread cells
70%
60%
50%
40%
30%
20%
10%
0%
1 2 3 4 5 6
GFP-RacV12
imatinib
3T3/vector
3T3/Abl

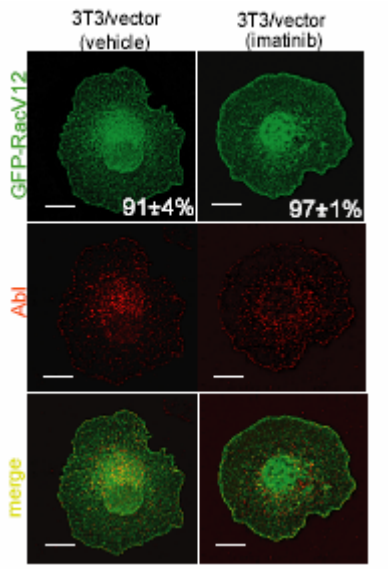
Figure 2-6: Abl Kinase promotes RacV12 localization to dorsal membrane protrusion

The indicated 3T3/vector or 3T3/Abl cells were transfected with GFP-RacV12 (A and B), GFP-Rac (C), or GFP (D), treated with imatinb (5 μM) or vehicle (0.1% DMSO) and then allowed to spread on FN. Cells were fixed between 60 to 90 min after plating, stained with anti-Abl/Alex 568-conjugated secondary antibody (red). Images of fully spread cells were captured by deconvolution microscopy. Representative Z sections from the top of the cells are shown: GFP (green), Abl (red) and merge (yellow). Cells with or without dorsal localization of GFP-RacV12, GFP-Rac, or GFP were determined as describe in Materials and Methods. (A) GFP-RacV12 transfected 3T3/Abl cells. Left panels: a cell with GFP-positive dorsal membrane protrusions (yellow arrow), representing ~58% of spread cells among 50 cells counted. Middle panels: a cell without GFP-positive dorsal membrane protrusions, representing ~42% of spread cells among 50 cells counted. Right panels: an imatinib- treated GFP-RacV12 transfected 3T3/Abl cell. Of 50 cells examined, ~96% showed GFP signals at the peripheral membrane and in the cytosolic space. Scale bars equal 10 μm. (B) GFP-RacV12 transfected 3T3/vector cells. Left panels, a cell with GFP signal at the peripheral membrane and in the cytosolic space, representing 91% of spread cells among 50 cells counted. Right panels, an imatinib treated GFP-RacV12 transfected 3T3/vector cells. Of 50 cells counted, 97% showed GFPsignals at the peripheral membrane and in the cytosolic space. Scale bars equal 10 μm. (C) GFP-Rac transfected 3T3/Abl cells. Left panels, a cell with GFP-positive dorsal membrane protrusions (yellow arrow), representing ~39% of spread cells among 50 cells counted. Middle panels: a cell without GFP-positive dorsal membrane protrusions, representing ~61% of spread cells among 50 cells counted. Right panels, imatinib treated GFP-Rac transfected 3T3/Abl cells. Of 50 cells examined, ~98% showed GFP signals at the peripheral membrane and in the cytosolic space. Scale bars equal 10 μm. (D) GFP transfected 3T3/Abl cells. Left panels: a cell with GFP signal diffusely throughout the cell, representing ~97% of spread cells among 50 counted. Right panels: imatinib treated GFP transfected 3T3/Abl cells. Of 50 cells examined, ~97% showed GFP signals diffusely throughout the cell. Scale bars equal 10 μm.

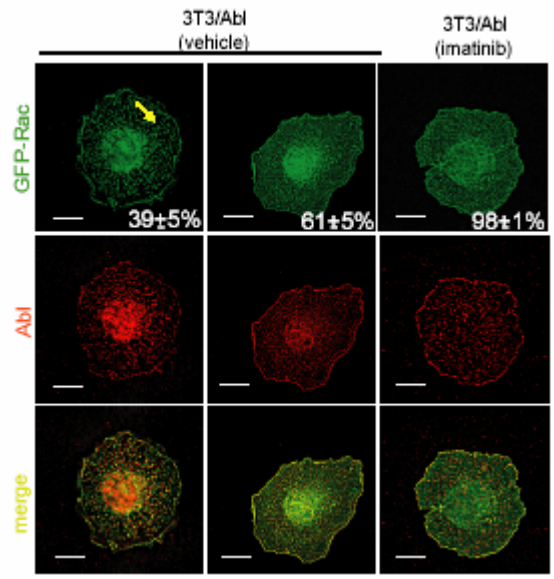
2-6 A


3T3/Abl
(vehicle)
3T3/Abl
(imatinib)
GFP-RacV12
58±8%
42±8%
96±1%
Abl
merge

2-6B


3T3/vector
(vehicle)
3T3/vector
(imatinib)
GFP-RacV12
91±4%
97±1%
Abl
merge

2-6C



2-6D

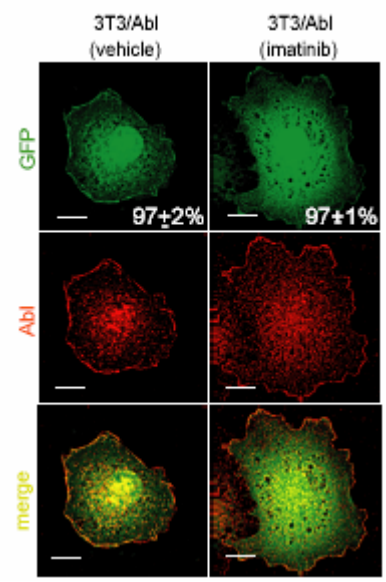
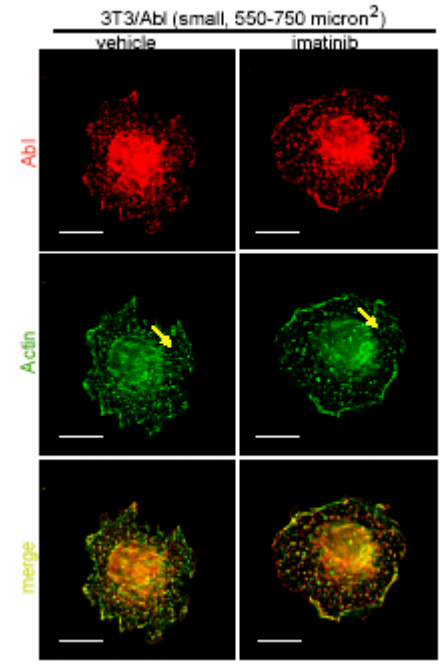


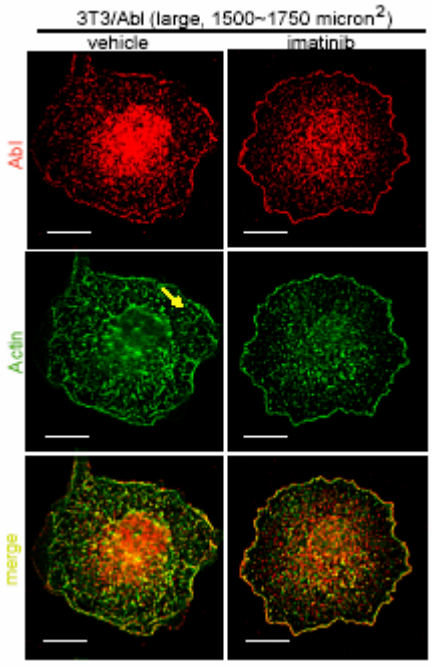
Figure 2-6: continued

Figure 2-7: Abl kinase prolongs dorsal ruffling during cell spreading
(A) and (B), 3T3/Abl cells treated with imatinib (5 μM) or vehicle (0.1% DMSO) were allowed to spread on FN (10μg/ml)-coated coverslides, fixed at 20 or 40 min, stained with anti-Abl/Alex 568-conjugated secondary antibody (red) and Alex 488-conjugated phalloidin (green). Z-section images from the top of cells captured by deconvolution microscopy are shown. Yellow arrow points to F-actin-rich dorsal protrusions. (A) A representative image of a small cell (cell area from 550-750 micron^2). Scale bars equal 10 μm. (B) Representative images of a large cell (cell area from 1500-1750 micron^2). Scale bars equal 10 μm. (C) Quantitation of cells showing dorsal F-actin ruffles as described in Materials and Methods. (D) 3T3 cells treated with imatinib (5 μM) or vehicle (0.1% DMSO) were allowed to spread on FN-coated coverslides and fixed. Images were collected by scanning electronic microscopy (SEM) show dorsal ruffles in vehicle and imatinib treated cells. Scale bars equal 5 μm. (E) 3T3 cells treated with imatinib or vehicle as in (D) were fixed at 20 or 40 min after plated on FN-coated coverslides. Individual cells were analyzed for size and dorsal F-actin protrusions as depicted in (A) and (B). Cells were grouped into 11 categories based on size, and in each category, at least 50 cells were counted to determine the percentage of cells with dorsal F-actin ruffles.

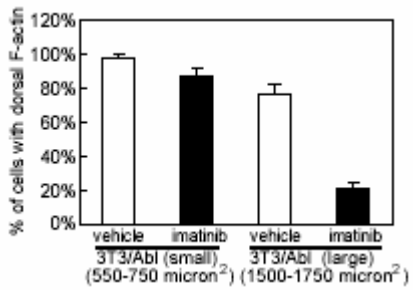
2-7A


3T3/Abl (small, 550-750 micron^2)
vehicle
imatinib
Abl
Actin
merge

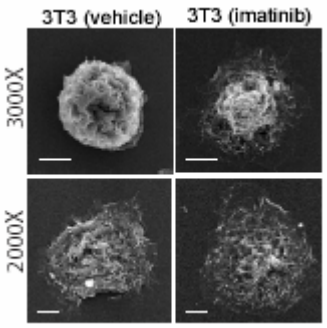
2-7B


3T3/Abl (large, 1500~1750 micron^2)
vehicle
imatinib
Abl
Actin
merge

2-7C



2-7D



2-7E

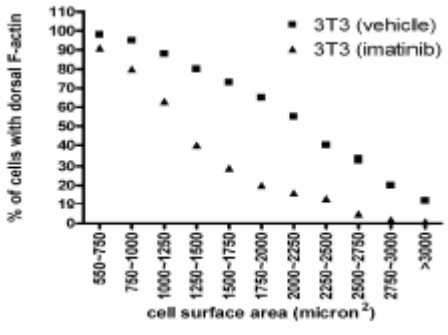
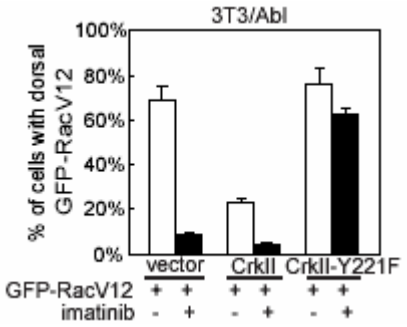


Figure 2-7: continued

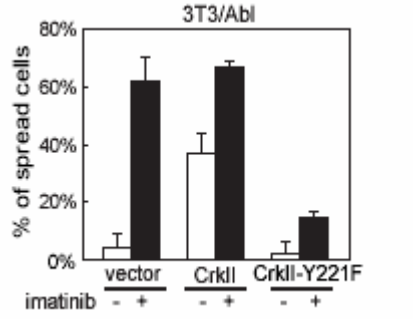
Figure 2-8: Effects of CrkII and dominant negative dynamin-2 on RacV12 dorsal membrane localization

(A) 3T3/Abl cells were cotransfected with GFP-RacV12 and CrkII, or CrkII-Y221F. Transfected cells were treated with imatinib (5uM) or vehicle (0.1% DMSO) and allowed to spread on FN (10μg/ml)-coated coverslides and fixed between 60 to 90 min. Dorsal localization of GFP signals in fully spread cells was scored as in Figure 6. (B) 3T3/Abl cells were cotransfected with GFP and CrkII, or CrkII-Y221F. Transfected cells were treated with imatinib or vehicle and allowed to spread on FN-coated coverslides for 20 min. Percentage of spread cells was determined among at least 100 GFP positive cells. The values and standard deviations were from three independent experiments. (C) 3T3/Abl cells were cotransfected with GFP-RacV12 and dynamin-2, or dynamin-2- K44A. Tansfected cells were treated with imatinib or vehicle and allowed to spread on FN-coated coverslides and fixed between 60-90 min. Localization of GFP signals in fully spread cells was scored as in Figure 6. (D) 3T3/Abl cells were cotransfected with GFP and dynamin-2, or dynamin-2-K44A. Transfected cells were treated with imatinib or vehicle and allowed to spread on FN-coated coverslides for 20 min. Percentage of spreading cells was determined as in (B).

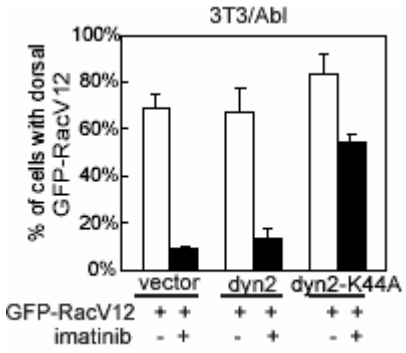
2-8A


3T3/Abl
100%
80%
60%
40%
20%
0%
% of cells with dorsal GFP-RacV12
vector
CrkII
CrkII-Y221F
GFP-RacV12 + + + + + +
imatinib - + - + - +

2-8B


3T3/Abl
80%
60%
40%
20%
0%
% of spread cells
vector
CrkII
CrkII-Y221F
imatinib - + - + - +

2-8C


3T3/Abl
100%
80%
60%
40%
20%
0%
% of cells with dorsal GFP-RacV12
vector
dyn2
dyn2-K44A
GFP-RacV12 + + + + + +
imatinib - + - + - +

2-8D

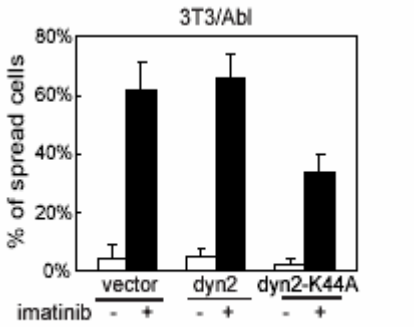


Figure 2-8: continued

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Chapter 3

Phosphoproteomic-based Profiling of Abl Substrates

Abstract

To identify Abl kinase substrates, we generated a 293 cell line that inducibly expresses constitutively active Abl kinase, Abl PP. With this cell line, a mass spectrometry-based method was used to screen for Abl kinase substrates. We followed tyrosine phosphorylation changes of cellular proteins in the 293 cells with Abl PP induction in the presence or absence of the Abl kinase inhibitor, imatinib. From two independent experiments, we found eighteen proteins have increased tyrosine phosphorylation with Abl PP induction in the absence of imatinib. Among the eighteen proteins, eight proteins were identified in both experiments. Six out of the eighteen proteins are established Abl substrates, the remaining twelve are novel Abl substrates. Interestingly, among the eighteen proteins identified, eight of them are known Src substrates.

Introduction

Regulation of the F-actin cytoskeleton by Abl depends on its kinase activity [1], therefore identification of Abl substrate(s) is necessary to elucidate how Abl transduces the extracellular stimuli to regulate the F-actin cytoskeleton. Many Abl substrates have been identified for the regulation of the F-actin cytoskeleton. WAVE2 and Cortactin phosphorylation by Abl play a role in membrane ruffles [2, 3]. Dok-1 phosphorylation by Abl is important in microspike formation [4]. And CrkII phosphorylation by Abl is involved in regulating cell migration [5]. Besides the known Abl substrates, other unknown Abl substrates are likely to be involved in the regulation of cell spreading and other actin-dependent processes.

To identify those substrates, we decided to screen for tyrosine phosphorylated proteins in a 293 cell line in which constitutively active Abl kinase, Abl PP [6], can be induced. We used a previously described mass spectrometry-based approach to identify tyrosine phosphorylated 293 cellular proteins, as well as the phosphorylation sites on these proteins [7]. We followed changes in tyrosine phosphorylation of 293 cellular proteins with Abl PP induction in the presence or absence of Abl kinase inhibitor, imatinib. From two independent experiments, we find that eighteen proteins had increased tyrosine phosphorylation in the presence of Abl PP kinase activity. Among them, eight proteins were identified in both experiments. Six out of the eighteen proteins are known Abl substrates, the remaining twelve are potential Abl substrates that have never been reported. Interestingly, among the eighteen proteins identified, eight of them are known Src substrates.

Material and Methods

Abl PP inducible 293 cell line, cell culture and reagents

Abl mutant, Abl P242E, P249E (Abl PP) [6] inducible 293 cell line was generated with the Flp-In^{TM}T-REX^{TM} system (invitrogen). The AblPP inducible 293 cells were cultured in Dulbecco's modified Eagles medium (DMEM), supplemented with 10% fetal bovine serum (Hyclone), and antibiotics. Doxycycline was used at 2 μg/ml. Imatinib was used at 5 μM.

Immunoblotting and antibodies

Protein samples were fractionated on SDS-PAGE, transferred to nitrocellulose membrane, and blotted with primary antibodies followed by horse peroxidase-conjugated secondary antibodies and visualized by chemiluminescence. Antibodies: anti-Abl, 8E9 (BD Biosciences); anti-phosphotyrosine, 4G10 (Upstate); anti-tubulin (Santa Cruz);

Abl PP induction and immunoprecipitation

Abl PP inducible 293 cells of ~90% confluent were treated with doxycycline, or doxycycline and imatinib at growth conditions for 5 hr. After treatment, $5X10^8$ cells in each sample were collected at 1000 rpm at 4ºC and lysed in 50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, protease inhibitor cocktail (Roche), 1 mM PMSF, 1 mM EDTA, 10 mM sodium vanadate, and 10 mM β-glycerol-phosphate at 4ºC. Anti-phosphotyrosine (PY20) agarose (Sigma) was added to cell lysates at 80 μl of resin per sample and incubated at 4°C for 2 hr. Beads were washed three times with lysis

buffer and three times with 20 mM Tris, pH 7.4, 120 mM NaCl. Proteins were eluted with 8 M Urea and 100 mM NH_4CO_3 for 5 min at 96ºC. Eluate were diluted to 4 M urea and digested with 2.5 µg of trypsin overnight at 37ºC.

Immobilized metal Affinity chromatography (IMAC)

Tryptic peptides were dissolved in 0.1 % acetic acid and subjected to IMAC purification as described [8] using 1 µl of the IMAC resin. Phosphorylated peptides were eluted from the IMAC resin with 10 µl of 100 mM KPO_4, pH 9.5, acidified with acetic acid and then analyzed by µLC-MS/MS.

Mass spectrometry and data analysis

Samples were analyzed by µLC-ES-MS/MS on a Thermo Finnigan LCQ quadruple ion trap mass spectrometer as described [9]. For data analysis, SEQUEST was used for peptide identification.

Results

To perform a proteomic based screening of Abl substrates, we generated a 293 cell line that inducibly expresses constitutively active Abl, Abl P242E, and P249E (Abl PP). After growing in media containing doxycycline for 5 hr, Abl PP inducible cells had robust Abl PP protein expression (Figure 3-1A). Combined treatment of Abl PP inducible cells with doxycycline and the Abl kinase inhibitor, imatinib, did not affect the Abl PP protein induction (Figure 3-1A). As expected, induction of Abl PP protein resulted in tyrosine phosphorylation of many cellular proteins, many of which were inhibited by imatinib (Figure 3-1B). These data indicate that tyrosine phosphorylation of 293 cellular proteins was dependent on Abl kinase activity. To further enrich the tyrosine phosphorylated proteins, we lysed the cells and immunoprecipitated the tyrosine phosphorylated proteins with an anti-phosphotyrosine antibody PY20 (Sigma). Immunoblotting with phosphotyrosine antibody (4G10, Upstate) showed an increase in tyrosine phosphorylation signal after immunoprecipitation compared with that from whole cell lysate (Figure 3-1B). As a control, the tyrosine phosphorylation of immunoprecipitated proteins was not detectable in the presence of imatinib (Figure 3-1B).

After successfully enriching for tyrosine phosphorylated proteins with the anti-phosphotyrosine antibody PY20, we scaled up the experiment to enrich for tyrosine phosphorylated proteins from $5X10^8$ cells after Abl PP induction. The procedure of the large scale experiment is shown in Figure 3-2A. We started with $5X10^8$ cells for each sample and treated cells with doxycycline to induce Abl PP for 5 hr. As a control,

we treated $5X10^8$ cells with a combination of doxycycline and imatinib for 5 hr. Cell lysates were incubated with anti-phosphotyrosine coupled agarose beads and after extensive washing eluted with urea. One tenth of the urea eluates was fractionated on a SDS-PAGE gel to visualize the immunoprecipitated proteins by silver staining (Figure 3-2B). Consistent with the result of anti-phosphotyrosine immunoblot, silver staining of the gel showed numerous proteins enriched by phosphotyrosine antibody coupled beads after Abl PP induction in the absence of imatinib. On the contrary, very few proteins were enriched in the sample after Abl PP induction in the presence of imatinib (Figure 3-2B).

The rest (90%) of the urea eluate from the anti-phosphotyrosine coupled beads underwent trypsin digestion, and the resulting phosphorylated peptides were further enriched with the IMAC column. The eluates from the IMAC column were finally analyzed by MS/MS mass spectrometry. Altogether, we performed the experiment outlined in Figure 3-2A twice. By comparing the results from the samples with and without imatinib treatment, we found twenty-six phosphopeptides were enriched after Abl PP induction in the absence of imatinib in both experiments (Table 3-1). Ten of the twenty-six phosphopeptides were identified in both experiments (Table 3-1, underlined phosphopeptides). The twenty-six tyrosine phosphopeptides corresponded to eighteen proteins. Among them, six were known Abl substrates and twelve were novel substrates not reported previously. Interestingly, among the identified proteins, eight of them were also known Src kinase substrates (Table 3-1).

Discussion

The mass spectrometry-based method we used in this study provides rapid identification of tyrosine phosphorylated proteins and the tyrosine phosphorylation sites of these proteins. From two independent experiments, we have found altogether eighteen proteins to have increased tyrosine phosphorylation induced by constitutively active Abl PP kinase. Eight of the eighteen proteins are identified in both experiments. The other ten proteins were identified only once either in the first or second experiment. The results from the two independent experiments have 50% overlap. Several reasons may explain the 50% overlap between these two experiments: (1) differential digestion of the phosphorylated proteins, (2) differential recovery rate of phosphorylated peptides from the IMAC column, (3) differential preservation of tyrosine phosphorylation during the process of the experiment.

Among the eighteen protein substrates identified, we found twelve of them had not been previously reported. However, we did not identify some of the proteins known to be Abl substrates, such as WAVE2 and CrkII. It is possible that the phosphotyrosine antibody we used (PY20, Sigma) preferentially bound to a subset of tyrosine phosphorylated substrates. Anther possibility is that under the lysis condition we used (1% NP-40), some substrates were not soluble in significant quantities.

Acknowledgements

We thank Xiaodong Huang in Jean Wang's lab at UCSD for generating the Abl PP inducible 293 cell line and Dr. Marcus Smolka from Huilin Zhou's lab at UCSD for IMAC enrichment of phosphorylated proteins and mass spectrometry identification of substrates.

Marcus Smolka is the co-author of Chapter 3. The author of the dissertation is the primary investigator and author of this paper.

Table 3-1: Tyrosine phosphorylated peptides induced by Abl PP kinase

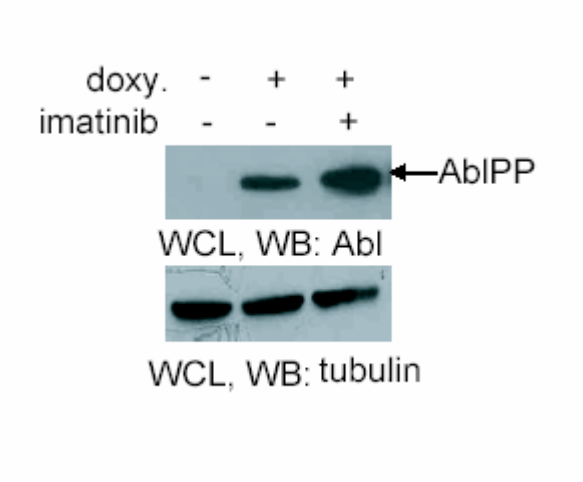
(Result was from two mass spectrometry experiments. Phosphopeptides identified in both mass spectrometry experiments were underlined)

Gene ID	Protein	Phosphopeptides	Abl sustrate	Src substrate
25	Abl	MTGDTpYTAHAGAKF	Yes (ref.[10])	Yes (ref.[11])
9564	p130Cas	RVLPPEVADGGVVDSGVpYAVPPPAERE RHLLAPGPQDIpYDVPPVRG AQQGLpYQVPGPSPQFQSPPAK DVPDGPLLREETpPYDVPPAFAK VQGYVYEAAQPEQDEpYDIPR GLLPSQYGQEVpYDTPPMAVK	Yes (ref.[12])	Yes (ref.[13])
4739	cas-l	KLpYQVPNPQAAPRD		
2017	Cortaction	RLPSSPVpYEDAASFKA	Yes (ref.[14])	Yes (ref.[15])
2549	GAB1	RAPSASVDSSLpYNLPRS		Yes (ref.[16])
3636	SHIP2	KTLSVDpYAPAGPARS		Yes (ref.[17])
6091	robo1	KQEVAPVQpYNICWQNKL THLIQEDILPpYCRPTFPTSNNPR	Yes (ref.[18])	
28988	HIP-55	RFQDVGPQAPVGSVpYQKT		Yes (ref. [19])
93669	LPP-LRFT	RYYEGYpYAAGPGYGGRN NRPPFGQGYTQPGPGpYR		
5903	Ranbp2	RpYIASVQGSTPSPRQ		
8502	plakophilin 4	RQTSNPNGPTPQpYQTTARV		
10152	Abi2	RTLEPVRPPVVPNDpYVPSPTRN	Yes (ref.[20])	
23201	KIAA0280 gene product	RTpYQASSAAFRY		
1796	p62Dok	IAPCPSQDSLpYSDPLDSTSAQAGEGVQR VKEEGpYELPYNPATDDpYAVPPPR	Yes (ref.[4])	Yes (ref.[21])
10006	Abi1	NTPpYKTLEPVKPPTVPNDpYMTSPAR		
266626	CLCP1	ATGNQPPLVGTpYNTLLSR		
28964	Git1	LQPFHSTELEDDAIpYSVHVPAGLYR		Yes (ref.[22])
9898	UBAP2L	FPLDpYYSIPFPTPTTPLTGR		

Figure 3-1: Induction of Abl PP expression and protein tyrosine phosphorylation in the presence or absence of Abl PP

(A) 293 cells grow to ~90% confluence were treated with doxycycline (2μg/ml) or doxycycline and imatinib (5 μg/ml) for 5hr. Whole cell lysates were loaded (50 μg) for immunoblotting with Abl (8E9) and tubulin antibodies. (B) Left, same cell lysates from (A) were loaded for immunoblotting with phosphotyrosine antibody. Right, same cell lysates from (A) were immunoprecipitated with phosphotyrosine antibody (PY20, Sigma), and the bound fractions were loaded for immunoblotting with phosphotyrosine antibody (4G10).

3-1A


doxy. - + +
imatinib - - +
AblPP
WCL, WB: Abl
WCL, WB: tubulin

3-1B

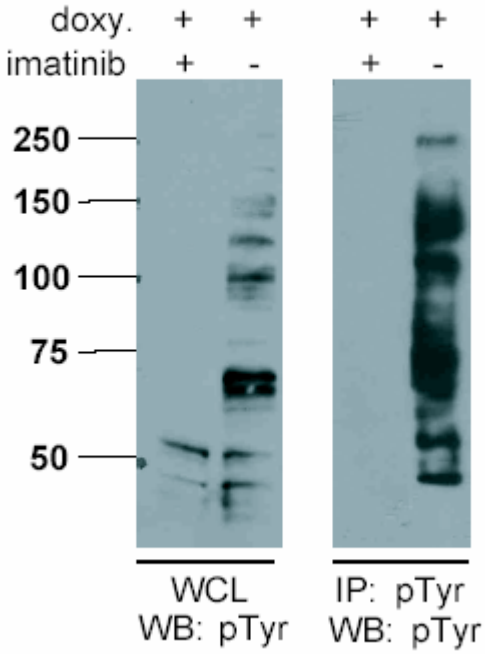
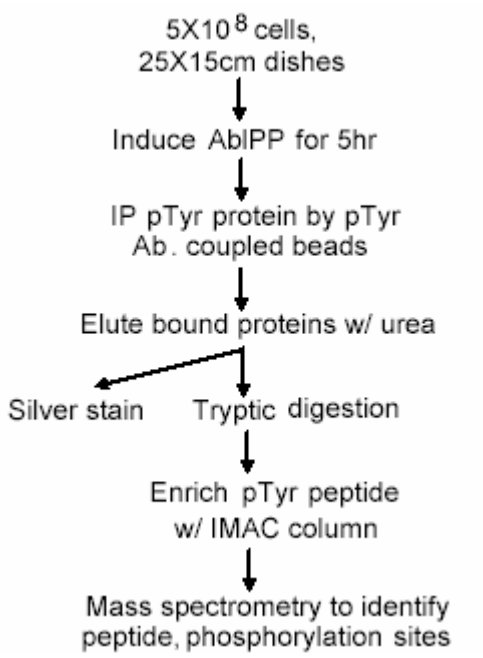

doxy. + + + +
imatinib + - + -
250
150
100
75
50
WCL
WB: pTyr
IP: pTyr
WB: pTyr

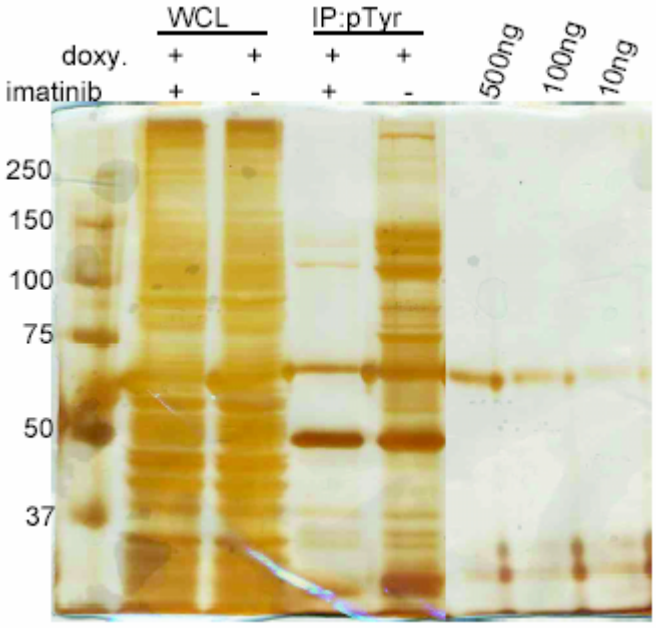
Figure 3-2: Enrichment and silver staining gel of tyrosine phosphorylated proteins

(A) Outline of experiment procedures to enrich tyrosine phosphorylated proteins for mass spectrometry. (B) Silver staining of cell lysates (50 μg/ml) and 1/10 of the urea eluate of phosphotyrosine antibody-coupled beads bound fraction. Samples were from 293 cells treated with doxycycline (2 μg/ml) or doxycycline and imatinib (5 μg/ml) for 5 hr.

3-2A


5X10 8 cells,
25X15cm dishes
Induce AblPP for 5hr
IP pTyr protein by pTyr
Ab. coupled beads
Elute bound proteins w/ urea
Silver stain
Tryptic digestion
Enrich pTyr peptide
w/ IMAC column
Mass spectrometry to identify
peptide, phosphorylation sites

3-2B


WCL
IP:pTyr
doxy. + + + +
imatinib + - + -
500ng
100ng
10ng
250
150
100
75
50
37

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Chapter 4
Discussion

Abl-mediated dorsal ruffling and cell migration

An efficient way for cells to migrate is to form lamellipodia at the leading edge by directional actin polymerization. Therefore, proteins that signal to disrupt directional actin polymerization and lamellipodia formation inhibit spreading. The studies in Chapter 2 analyzed the role of Abl in cell spreading, Abl affects the localization active Rac and thus localization of actin polymerization. This directs actin polymerization from lamellipodia to dorsal ruffles. Given that lamellipodia formation in cell spreading is a similar process as lamellipodia formation in the leading edge of a migratory cell, in cell migration Abl can compromise the actin polymerization at the leading edge needed for lamellipodia formation. This can be another mechanism to explain Abl-mediated inhibition of cell migration. Though the Abl substrate(s) involved in regulating localization of actin polymerization is(are) still under investigation, the downstream effect of the substrate(s) phosphorylation is to regulate localization of active Rac to the dorsal membrane, thus promoting actin polymerization for dorsal ruffling.

Abl-mediated dorsal ruffling and 3-dimensional cell migration

Cultured cell migration is usually two-dimensional (2-D or *in vitro*). *In vivo* cell migration is 3-dimensional (3-D). Contrary to the negative role of dorsal ruffles in 2-D cell migration, dorsal ruffles play an important role in 3-D cell migration [1]. Abrogation of dorsal ruffles by depletion of WAVE1, which is required for dorsal ruffle formation in fibroblasts, greatly inhibited cell migration in 3-D [1]. Therefore, Abl can play a positive role in 3-D or *in vivo* cell migration. Furthermore, 3-D

migration *in vivo*, if not properly regulated, especially in carcinoma cells, can lead to cell invasion. Given that Abl regulates dorsal ruffle formation, it is important to examine the role of Abl in some invasive tumor cell lines. Indeed, in some highly invasive breast cancer cell lines, deregulation of Src-induced Abl activation contributes to the invasive phenotype of these cell lines [2, 3].

Candidate substrates involved in Abl-dependent regulation of F-actin cytoskeleton

In Chapter 3, eighteen proteins were identified to have increased tyrosine phosphorylation after Abl PP induction in 293 cells in the absence of imatinib. Six of these proteins are known Abl substrates (Table 3-1). Among the known substrates, Abl itself is one of them. The phosphorylation site on Abl is Y412, which resides in the Abl kinase activation loop [4]. This is consistent with the finding that Abl undergoes auto-phosphorylation on Y412 upon activation [5]. Robo1 is an Abl substrate established to be involved in repulsive axon guidance [6]. Phosphorylation of Cortactin and Dok-1 by Abl plays a role in dorsal ruffling and microspike formation, respectively [7, 8]. Whether the other two substrates, p130Cas and Abi2 are involved in Abl-mediated regulation of F-actin cytoskeleton needs to be investigated. Give that both p130Cas and Abi2 have connections with Rac GTPase, it is possible that phosphorylation of these two substrates by Abl affects the F-actin cytoskeleton through regulation of Rac activation or localization.

Among the unknown substrates, Gab1 has been shown to play a role in signal transduction downstream of PDGF receptors. PDGF induces rapid Gab1

phosphorylation [9]. Gab1 expression enhances the formation of lamellipodia and cellular protrusions and Gab1-deficient fibroblasts showed a decreased F-actin cytoskeleton reorganization in response to PDGF [9]. Furthermore, the chemotactic response of Gab1-deficient fibroblasts toward gradients of PDGF is compromised as compared to wild-type cells [9]. Given that Abl is activated downstream of PDGF receptors, it is possible that PDGF-induced Gab1 phosphorylation is dependent on Abl, and the phosphorylation of Gab1 can modulate PDGF-induced actin cytoskeleton regulation and chemotaxis. More recently, Gab1 has been shown to be phosphorylated downstream of HGF/c-Met signaling and involved in HGF-mediated tumor cell migration and invasion [10]. Abl plays a role downstream of HGF in HGF-induced chemotaxis [11], and therefore it is possible that Abl phosphorylation of Gab1 plays a role in HGF-mediated tumor cell migration and invasion.

SHIP2 is an inositol phosphatase that plays an important role in regulating cellular levels of lipid secondary messengers. Reattachment of trypsinized cells stimulates tyrosine phosphorylation of SHIP2 and transfection of a catalytic domain deletion mutant of SHIP2 (DeltaRV) inhibits cell spreading [12]. SHIP2 knockdown cells display cell-spreading defects involving a notable absence of focal contact structures and the formation of multiple slender membrane protrusions capped by actin spikes [13]. Moreover, SHIP2 affects endocytosis, illustrated by deregulated EGF receptor internalization induced by SHIP2 knockdown [13]. As a potential Abl substrate, it is possible that Abl phosphorylates SHIP2 to modulate its function in endocytosis of some membrane protein, such as membrane bound Rac-GTP, thus affecting the localization of Rac-GTP and actin polymerization.

HIP-55 (Abp1) has been established to bind to F-actin *in vitro* [14]. Growth factors cause HIP-55 to rapidly accumulate in lamellipodia and in migrating cells, appearing at the actin-rich leading edge of the cells [14]. HIP-55 binds to dynamin, a GTPase that functions in endocytosis *in vitro* and *in vivo.* In neurons, HIP-55 and dynamin have been found to be colocalized at actin-rich sites proximal to the cell body during synaptogenesis. In fibroblasts, both proteins appear at dynamin-rich sites of endocytosis upon growth factor stimulation [15]. The interaction between HIP-55 and dynamin regulates the endocytic signaling pathway [15]. HIP-55 is phosphorylated by Src [14], but whether HIP-55 is phosphorylated by Abl has not been reported. Therefore, it will be interesting to test whether Abl can phosphorylate HIP-55 under physiologically relevant stimulation such as PDGF or adhesion to fibronectin. Given the evidence in Chapter 2 that dynamin is involved in Abl-mediated dorsal localization of active Rac, it is possible that HIP-55 phosphorylation by Abl plays a role in modulating dynamin function, which in turn regulates active Rac endocytosis.

Git1 is a protein that regulates focal adhesion dynamics [16]. It is phosphorylated by Src [17]. Git1 phosphorylation by Abl has not been reported. Abl and Arg knockout cells contain less focal adhesions compared with the Abl kinase reconstituted counterpart during cell spreading (unpublished result). It is possible that Abl-regulated focal adhesion formation is through Git1. Since the dynamics of focal adhesion formation affects the structure and function of the actin cytoskeleton. It is conceivable that focal adhesion dynamics regulated by Git1-Abl interaction can affect F-actin-related cellular processes such as cell spreading and migration. In dendritic spines, GIT1 is a key regulator of spine morphology and synapse formation by

targeting actin regulators and locally modulating Rac activity [18]. In cultured hippocampal neurons, GIT1 is enriched in both pre- and postsynaptic terminals. Mislocalized GIT1 results in numerous dendritic protrusions and a significant decrease in the number of synapses [18]. Given that Abl regulates cell spreading via modulating the function of Rac, it will be interesting to examine whether Abl phosphorylation of Git1 is involved in spine morphology and synapse formation.

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