Use of a Chemical Genetic Technique To Identify Myosin IIB as a Substrate of the Abl-Related Gene (Arg) Tyrosine Kinase

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Received June 6, 2007; Revised Manuscript Received July 26, 2007

ABSTRACT: Abl family kinases have been implicated in the regulation of cell morphogenesis and migration, but the molecular mechanisms through which they operate are not fully elucidated. We applied the bump−hole technique, pioneered by Shokat and colleagues, to identify direct substrates of Abl and the Abl-related gene (Arg) kinases. This technique required the engineering of Abl/Arg to utilize an unnatural ATP analogue as a phospho-donor. Mutation of T334A and T361A in Abl and Arg, respectively, altered their nucleotide specificity and allowed them to utilize N6-benzyl-ATP as a phospho-donor. These mutations did not affect the catalytic activity or protein substrate specificity of Abl and Arg. An unexpected high level of background labeling necessitated further optimization of this approach. Dialysis, pretreatment with a broad-spectrum Ser/Thr kinase inhibitor, K-252a, and purification of phosphotyrosine-containing proteins allowed for definitive identification of putative substrates. Using mass spectrometry, we identified eight putative substrates. One of these putative substrates, myosin IIB, can be phosphorylated *in vivo* by Arg. Our results indicate that the bump−hole technique can be used to identify Abl family kinase substrates and suggests that myosin IIB may be regulated by tyrosine phosphorylation.

Adhesion and growth factor receptors act through Abl family tyrosine kinases to regulate cell morphogenesis and migration. Genetic studies demonstrate that Abl family kinases are essential for epithelial cell morphogenesis (*1*, *2*), cell migration and chemotaxis (*3*−*5*), F-actin dorsal wave formation (*6*, *7*), axon pathfinding (*8*), and dendrite maintenance (*9*). Abl family kinase signaling pathways also become disrupted in cancer cells (*10*). The Bcr-Abl oncoprotein, resulting from a reciprocal chromosomal translocation involving the *bcr* and *abl* genes, causes chronic myelogenous and acute lymphocytic leukemia in humans (*11*). Cells transformed with Bcr-Abl exhibit altered migratory (*12*) and adhesive behavior (*13*), and reduced responsiveness to chemotactic cues (*14*).

We utilized a chemical genetic technique pioneered by Shokat and colleagues (*15*) to identify putative substrates of Abl/Arg. By examining crystal structures of kinase active sites, Shokat and colleagues identified a bulky amino acid (I338 in v-Src) adjacent to the N6 of adenosine (*15*−*17*), a site that could be easily derivatized. Mutation of I338 to a smaller amino acid (alanine or glycine) allowed the mutant kinase (termed analogue sensitive or *AS*[1]) to use N6-derivatized versions of ATP whereas wild type (WT) kinases could not. Shah et al. used *AS*-Src, N6-benzyl-ATP-γ-^{32}P, and mass spectrometry to identify two novel and three known Src substrates (*18*).

Despite the high degree of conservation between the Src and Abl kinase domains, attempts to generate *AS*-Abl required the construction of a Src-Abl chimera, which utilized N6-benzyl-ATP but maintained Abl's protein specificity (*19*). We hypothesized that mutation of T334 in Abl or T361 in Arg in the context of the full-length protein might generate *AS*-forms of Abl/Arg without requiring a Src-Abl chimera, allowing for labeling and identification of biological substrates of these kinases.

Here we demonstrate that mutation of T334A and T361A alters the nucleotide specificity of Abl and Arg, respectively, show that *AS*-Abl and Arg maintain their protein specificity, illustrate some techniques to optimize the identification of substrates, and show that one of the putative substrates, myosin IIB, is phosphorylated by Arg *in vivo*. Our results demonstrate that this technique can be applied to Abl and Arg and suggest a novel potential mechanism for the regulation of myosin IIB.

EXPERIMENTAL PROCEDURES

Synthesis of N6-Benzyl-adenosine. All syntheses were performed under argon according to Shah et al. (*18*). We

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[1] Abbreviations: Arg, Abl-related gene; ATP, adenosine triphosphate; A*TP, N6-benzyl-ATP; *AS*, analogue sensitive; DMF, dimethyl formamide; TEAB, tetraethyl ammonium bicarbonate; DEAE, diethyl amino ethyl; NMR, nuclear magnetic resonance; NDPK, nucleoside diphosphate kinase; PCR, polymerase chain reaction; GST, glutathione-*S*-transferase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol-bis(beta-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MW, molecular weight; MALDI-TOF, matrix adsorbed laser desorption/ionization time of flight spectrometry; RFP, red fluorescent protein; HSP-70, heat shock protein 70; PI3-kinase, phosphoinositide 3-kinase; GFP, green fluorescent protein; YFP, yellow fluorescent protein; HRP, horseradish peroxidase; PY, phosphotyrosine; WT, wild type; K_m, Michaelis constant; KI, kinase inactive; MIIB, non-muscle myosin heavy chain II isoform B; MIIA, non-muscle myosin heavy chain II isoform A; MIIC, non-muscle myosin heavy chain II isoform C.

10.1021/bi701119s CCC: $37.00
Published on Web 09/25/2007

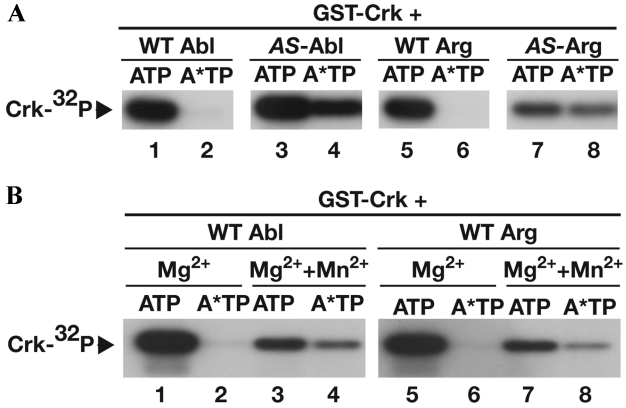


FIGURE 1: Point mutations in Abl and Arg (T334A and T361A) alter nucleotide specificity. (A) *In vitro* kinase assays performed with WT or analogue sensitive (*AS*)-kinases, purified GST-Crk, and either γ-^{32}P-ATP or N6-benzyl-ATP-γ-^{32}P (A*TP). WT kinases cannot utilize A*TP, whereas *AS*-Abl (T334A) and *AS*-Arg (T361A) can utilize both ATP and A*TP. (B) Use of Mn^{2+} in reaction buffer allows WT kinases to utilize A*TP.

dissolved 0.3 g of chloro-riboside in 10 mL of ethanol and then added 900 μL of benzyl amine (5:1 molar ratio of benzyl amine to chloro-riboside) and refluxed overnight. The reaction was monitored by TLC in 7:1:2 butanol:NH_4OH:H_2O. We obtained white crystals from recrystallization in ethanol.

Synthesis of N6-Benzyl-ATP. We dissolved 0.1 g of N6-benzyl-adenosine in 1 mL of trimethyl phosphate. The reaction was cooled on ice, 34 μL of phosphorus oxychloride (1.3 molar excess of N6-benzyl-adenosine) was added, and stirred on ice for 1.5 h. Next we added 1 mL of 500 mM pyrophosphate in DMF and 0.2 mL of tributylamine and stirred vigorously for 5 min. Addition of 5 mL of 1 M triethyl ammonium bicarbonate (TEAB) quenched the reaction, which was stored at -20 °C. We purified N6-benzyl-ATP using a DEAE-Sephadex (Sigma A25120) column with a TEAB gradient from 50 mM to 500 mM. ^{1}H and ^{33}P NMR confirmed proper synthesis. This protocol was adapted as previously described (*18*, *20*, *21*).

Generation of N6-Benzyl-ATP-γ-^{32}P. Nucleoside diphosphate kinase was purified from BL21 cells expressing his-tagged nucleoside-diphosphate kinase (NDPK), a gift from K. Shokat (*22*). NDPK was used to generate radiolabeled A*TP. 100 μL of Ni^{2+}-NTA resin (Qiagen) was poured into a 1 mL column (Pierce) and washed with 2 mL of 150 mM NaCl/100 mM HEPES (HBS). 100 μg of his-tagged NDPK was bound to the Ni-beads and washed with 2 mL of HBS. Then 100 μCi of ATP-γ-^{32}P in 2 mL of PBS was poured onto the column. The column was washed with 2 mL of PBS to remove any unreacted ATP. 0.5 nmol of N6-benzyl-ADP (a gift from K. Shokat) was diluted in 500 μL of PBS + 5 mM $MgCl_2$. 250 μL of N6-benzyl-ADP was added to the column and washed with 750 μL PBS + 5 mM $MgCl_2$. These steps were repeated with the remaining N6-benzyl-ADP. We used scintillation counting to quantitate incorporation of ^{32}P and confirmed that each batch of N6-benzyl-ATP-γ-^{32}P could not be used by WT kinases as in Figure 1A. This entire protocol is slightly modified from Blethrow et al. (*22*). 50% of the ^{32}P phosphate was routinely transferred to form A*TP-γ-^{32}P.

Production of Recombinant Proteins. Wild type and mutant forms of Abl and Arg were constructed from mouse cDNAs using PCR. Point mutations (Abl T334A, T334G, and K290M, and Arg T361A, T361G, and K317M) were generated using site-directed mutagenesis and PCR and confirmed by sequencing. Abl, Arg, and GST-Crk were produced and purified as described previously (*23*). Peptide substrate (AAVIYAAPFAKKK) was synthesized by the Keck facility (Yale), and its sequence was confirmed by mass spectrometry.

Steady-State In Vitro Kinase Assays. Kinase assays contained 25 mM HEPES pH 7.25, 5% glycerol, 100 mM NaCl, 10 mM $MgCl_2$, 2 mM DTT, 1 mM Na_3VO_4, 20 ng/μl BSA, 10 nM kinase, and substrate in concentrations as needed. After 5 min preincubation at 30 °C, the reaction was initiated by adding 50 or 100 μM ATP or A*TP, and 0.75 μCi γ-^{32}P ATP or A*TP. The reaction was quenched by the addition of ice-cold Laemmli SDS buffer after 5 min. The proteins were resolved by SDS PAGE. The gels were dried, exposed for autoradiography, and quantitated using Molecular Dynamics PhosphorImaging System and ImageQuant software (GE Healthcare). The data were fit to the Michaelis−Menten equation using KaleidaGraph (Synergy Software).

Kinase assays to obtain IC_{50} values were performed as above except that increasing concentrations of staurosporine (a gift from D. Austin) or K-252a (a gift from J. Wood) were added prior to addition of ATP.

Preparation of Brain Extracts. Wild type, *abl*−/−, and *arg*−/− mice were sacrificed between postnatal days 28 and 42. The brains were removed and homogenized in homogenization buffer (25 mM HEPES pH 7.25, 10% glycerol, 150 mM NaCl, 5 mM EDTA, and 5 mM EGTA). Phosphatase inhibitors were intentionally excluded to remove endogenous phosphorylation. The homogenate was centrifuged at 20000*g* for 10 min. The supernatant (S1) was collected, and the pellet was resuspended in extraction buffer (homogenization buffer with 2.5% CHAPS). After extracting for an hour at 4 °C, the mixture was centrifuged at 100000*g* for 1 h. The resulting supernatant (S2) was collected. The detergent was removed from the S2 using Bio-Beads SM (Bio-Rad) according to the manufacturer's protocol. Both S1 and S2 were dialyzed against dialysis buffer (25 mM HEPES pH 7.25, 10% glycerol, and 150 mM NaCl) to remove EDTA and EGTA. The MW cutoff of the dialysis tubing was 12 kDa. The concentration of the extracts was obtained using a Bradford assay (Bio-Rad), and the extracts were aliquoted and stored at -80 °C.

Bump−Hole Labeling of Brain Extracts. Brain extracts were used in *in vitro* kinase assays as above, except that 100 μM A*TP was used. 50 μg of brain extracts and 3 μCi A*TP-γ-^{32}P were used for background removal experiments, 500 μg of brain extracts and 3 μCi A*TP-γ-^{32}P were used for proof-of-principle immunoprecipitations, and 10 mg of brain extracts and 10 μCi A*TP-γ-^{32}P were used for mass spectrometry experiments. After 10 min, the reaction was quenched by addition of ice-cold quench buffer (25 mM HEPES pH 7.25, 1% NP-40, 20 mM EDTA, 100 mM NaCl, 0.01% NaN_3, 1 mM Na_3VO_4, and protease inhibitors). An aliquot of the quenched reaction was combined with Laemmli SDS buffer for input. Immunoprecipitation experiments were performed by adding 3 μg of anti-Crk II antibodies (Santa Cruz sc-289) or anti-phosphotyrosine (Upstate 4G10), as indicated, and protein A/G beads (Calbiochem) or using

agarose-coupled-4G10 (Upstate) overnight. The immune complexes were pelleted by centrifugation and washed three times in quench buffer. Finally, Laemmli SDS buffer was added and the samples were boiled and separated by SDS PAGE. Gels were dried and exposed for autoradiography.

Mass Spectrometry. Coomassie stained gels were overlaid on film to identify putative radiolabeled proteins. The bands were excised, and the proteins were trypsinized overnight. The peptides were extracted from the gel and identified by mass spectrometry using MALDI-TOF. The molecular weights obtained from the mass spectrometry were compared to database predictions to identify putative substrates. This was performed by Landon Wilson at the Mass Spectrometry Core Facility at the University of Alabama at Birmingham.

Protein Phosphorylation in Vivo. 293 cells were transfected using Lipofectamine 2000 (Invitrogen) with 4 μg of plasmid containing putative substrate and 2 μg of either WT Abl/Arg or a kinase-inactive point mutant of Abl/Arg. mRFP-fusion proteins were generated by cloning Drebrin 1 BC006714 (Invitrogen), HSP-70 NM005345 (gift from Lynne Regan), ogdh BC013670 (Invitrogen), or syntaxin binding protein 1 BC031728 (Invitrogen) into N1-YFP, where the YFP was replaced by mRFP (*7*). GFP-myosin IIA, GFP-myosin IIB, and myosin IIC-GFP were obtained from R. Adelstein via Addgene (#11347, #10845, #10843) (*24*, *25*). We did not test PI3 kinase (C2 domain-containing gamma polypeptide isoform 2). After 48 h, the cells were lysed in modified RIPA buffer, containing 50 mM Tris pH 7.2, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM Na_3VO_4, 2 mM NaF, 1 mM PMSF, 10 μg/mL pepstatin A, 10 μg/mL chymostatin, 10 μg/mL leupeptin, 1 mM benzamidine, and 50 μg/mL aprotinin. The putative substrates were immunoprecipitated with 3 μg of either anti-RFP (Chemicon) or anti-GFP (Rockland 600_101-215) antibodies and protein A/G beads (Calbiochem). Western blotting was performed using a cocktail of anti-PY antibodies (Upstate 4G10, Santa Cruz PY20, Santa Cruz PY99), GFP (Rockland), or RFP (Chemicon), HRP-conjugated secondary antibodies (Bio-Rad), and chemiluminescence. Blots were quantitated using densitometer and Quantity One software (Bio-Rad). The fold activation was calculated as the ratio of PY to GFP or RFP for WT vs kinase-inactive overexpression.

RESULTS

We successfully extend the bump−hole technique, a chemical genetic technique described previously for Src and other kinases (*15*, *17*, *26*, *27*), to Abl and Arg. For this technique, a hole is engineered in the ATP binding site of the kinase by mutating the residue nearest to N6 of ATP. We identified T334 (Abl) and T361 (Arg) as the residues that correspond to the residue mutated in *AS*-Src (T338 in c-Src or I338 in v-Src) by aligning the kinase domains of Abl, Arg, and Src. We mutated the threonine (334 in Abl, 361 in Arg) to alanine or glycine and purified these kinases from insect cells. Baculovirus-mediated expression in insect cells allows for proper folding and post-translational modifications of these kinases.

Mutation of T334/T361 in Abl/Arg Generates AS-Kinases. We performed *in vitro* kinase assays using GST-Crk, a known Abl substrate (*3*), to determine whether Abl and Arg mutants could utilize N6 benzyl ATP (A*TP). Although WT Abl and Arg could use only ATP, Abl T334A (*AS*-Abl) and Arg T361A (*AS*-Arg) could use either ATP or A*TP (Figure 1A). Abl T334G and Arg T361G are catalytically inactive (data not shown). Surprisingly, WT Abl and Arg can utilize A*TP in assays containing both Mn^{2+} and Mg^{2+} (Figure 1B, lanes 3−4, 7−8). However, inclusion of Mg^{2+} alone prevented use of A*TP by WT kinases. This reduced nucleotide specificity observed in the presence of Mn^{2+} has a precedent in other ATP-binding proteins. For example, Mn^{2+} increases the nucleotide misincorporation rate in DNA polymerases (*28*). This result demonstrates the successful application of the bump−hole technique to Abl family kinases, as *AS*-Abl and *AS*-Arg can utilize A*TP but WT Abl and WT Arg cannot.

Table 1. [a]

substrate	kinase	K_m (μM)	k_{cat}/K_m (min M)$^{-1}$
ATP	WT Abl	18.36	3.03×10^5
	AS-Abl	27.06	1.26×10^5
	WT Arg	43.53	1.32×10^5
	AS-Arg	32.55	8.89×10^4
A*TP	WT Abl	>444	na
	AS-Abl	8.95	3.18×10^5
	WT Arg	>226	na
	AS-Arg	16.53	5.73×10^4
FL Crk	WT Abl	0.50	1.60×10^6
	AS-Abl	0.44	1.91×10^6
	WT Arg	0.50	1.00×10^6
	AS-Arg	0.79	3.74×10^5
Short Crk	WT Abl	2.90	4.48×10^4
	AS-Abl	1.67	8.75×10^5
	WT Arg	6.00	1.67×10^4
	AS-Arg	9.49	4.77×10^4
peptide	WT Abl	36.94	3.35×10^5
	AS-Abl	9.85	2.68×10^5
	WT Arg	122.53	2.86×10^5
	AS-Arg	7.15	2.14×10^5

[a] *AS*-Abl/Arg have similar substrate specificities and catalytic activities to WT Abl/Arg. Steady-state *in vitro* kinase assays with WT or *AS*-Abl/Arg were performed in quadruplicate with various substrates to test if point mutations altered substrate specificity or catalytic activity. The K_m for N6-benzyl-ATP was obtained by increasing the amount of cold A*TP in a series of kinase assays and then fitting the data to the equation for competitive inhibition: $vo = (V_{max}[S])/(K_m(1 + [I]/K_i) + [S])$; the K_i is reported as K_m for A*TP. FL-Crk is glutathione *S*-transferase (GST) tagged to full-length Crk, Short Crk is GST-tagged Crk residues 110−225, and peptide contains Abl's preferred phosphorylation sequence (*32*) (AAVIYAAPFAKKK). The data for WT Abl and Arg phosphorylating Crk and Short Crk were reported previously (*23*).

AS-Abl/Arg Have Normal Catalytic Activities. We performed steady-state kinase assays to compare K_ms and k_{cat}s for WT Abl and Arg with *AS*-Abl and Arg to ensure that the mutations did not adversely affect catalytic activity. *AS*-Abl and *AS*-Arg have a similar K_m for ATP as wild type kinases (Table 1). Interestingly, in the *AS*-kinases, the K_m for A*TP is 2−3-fold lower than for ATP, suggesting a slight preference for A*TP over ATP. Importantly, the catalytic efficiencies (k_{cat}/K_m) for the *AS*-kinases are not impaired by mutation of the threonine. These data suggest that point mutation of threonine to alanine in Abl and Arg alters nucleotide specificity and that these mutants prefer A*TP to ATP, but have no effect on catalytic efficiency.

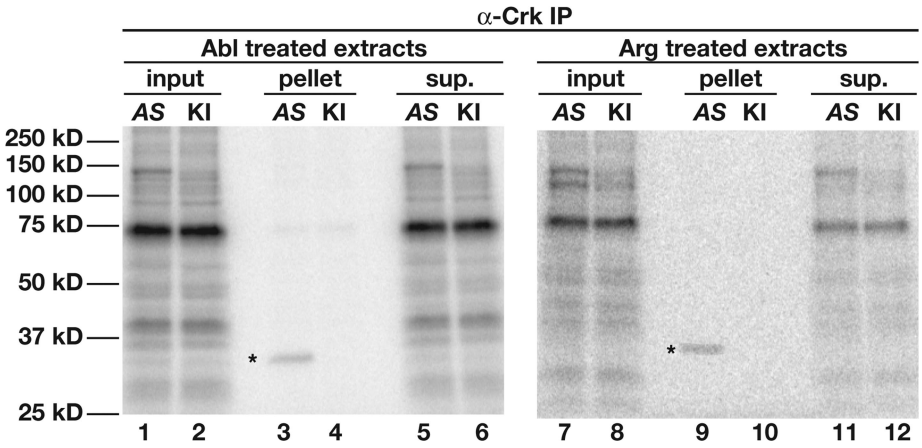


FIGURE 2: *AS*-Abl/Arg phosphorylate Crk, a known substrate, in brain extracts. Brain extracts were treated with A*TP and either *AS*-Abl/Arg (AS) or kinase-inactive (KI) Abl/Arg for 10 min. An aliquot of the reactions was loaded as the preimmunoprecipitation input. The known Abl/Arg substrate, Crk, was immunoprecipitated and is indicated by the asterisk (pellet). An aliquot of the post-immunoprecipitation supernatant was also saved (sup.). Crk was radiolabeled only in *AS*-Abl/Arg not KI-Abl/Arg treated extracts.

Protein Substrate Specificity Is Not Altered. We performed steady-state kinase assays with different substrates of Abl and Arg to confirm that *AS*-Abl and *AS*-Arg maintain protein substrate specificity. *AS*-Abl and *AS*-Arg have K_ms similar to WT kinases for full-length GST-Crk (FL Crk), GST-Crk amino acids 120−225 (Short Crk), and a peptide optimized for Abl family kinases (AAVIYAAPFAKKK) (Table 1) (*23*, *29*−*32*). Surprisingly, the *AS*-kinases have a lower K_m for the peptide than the WT kinases. This may result from slight alterations in the catalytic cleft. Regardless, the K_m and catalytic efficiency of *AS*-Abl and *AS*-Arg are similar to WT Abl and Arg, especially for interacting with full-length proteins (Table 1). These data suggest that the protein specificity of *AS*-Abl and *AS*-Arg are similar to WT Abl and Arg.

Bump−*Hole Labeling of Brain Extracts Yielded Unexpected Background Phosphorylation.* Having confirmed that *AS*-Abl and *AS*-Arg function similarly to WT kinases *in vitro*, we performed a proof-of-principle experiment to test if *AS*-Abl/Arg can phosphorylate a known substrate in brain extracts radiolabeled with exogenously added *AS*-Abl/Arg and A*TP-γ-^{32}P. As a control, we incubated extracts with a kinase inactive point mutant of Abl/Arg (KI) that should not transfer radioactive phosphate to proteins from A*TP-γ-^{32}P. Surprisingly, KI Abl- or KI Arg-treated extracts contained numerous radiolabeled bands (Figure 2, lanes 2 and 8). Regardless, when Crk is immunoprecipitated, it is only phosphorylated in *AS*-Abl/Arg-treated but not control extracts (Figure 2, lanes 3, 4 and 9, 10). This proof-of-principle experiment demonstrates that *AS*-Abl/Arg can phosphorylate a known substrate in a complex protein extract. This finding suggests that the bump−hole technique may identify additional putative Abl/Arg substrates after eliminating the nonspecific background phosphorylation.

Methods for Removing Nonspecific Background Phosphorylation. We hypothesized that the source of the background phosphorylation resulted from reincorporation of the radiolabeled phosphate into endogenous ATP or the use of A*TP by other kinases in the extract. Dialysis of the extract reduced the nonspecific background phosphorylation (Figure 3B, compare lanes 2 and 4 or 8 and 10). This step may remove excess free nucleotides from the extract, eliminating the recipients of any recycled ^{32}P-phosphate. Additionally, since Abl and Arg are tyrosine kinases, we enriched for phosphotyrosine-containing proteins in the substrate reactions by immunoprecipitation with phosphotyrosine specific antibodies (Figure 3C). For example, six discrete radiolabeled proteins were visibly immunoprecipitated in both the *AS*-Abl and *AS*-Arg treated extracts, whereas no radiolabeled proteins were pelleted in the control reactions to which KI-Abl/Arg were added. Surprisingly, the nonspecific, radiolabeled proteins in KI-treated extracts remained in the supernatant (Figure 3C, lanes 4 and 8), suggesting that phosphorylation of these proteins may be on Ser or Thr residues. This data implies that inhibition of Ser/Thr kinases may further reduce nonspecific background.

To test this hypothesis, we sought a general kinase inhibitor that had little effect on Abl and Arg but was potent against Ser/Thr kinases. Staurosporine and K-252a, which inhibit Ser/Thr kinases with an IC_{50} of 10−80 nM, weakly inhibited Abl and Arg *in vitro* (Figure 4). Staurosporine inhibited Abl and Arg *in vitro* with IC_{50} of 4 μM and 13 μM, respectively. K-252a inhibited Abl and Arg with IC_{50} greater than 50 μM. We found that a combination of dialysis and K-252a treatment (10 μM) nearly eliminated the nonspecific background (Figure 3B, lanes 6 and 12).

Identification of Putative Substrates. By combining dialysis, K-252a treatment, and phosphotyrosine immunoprecipitation in our labeling experiments, we eliminated the background and identified the presence of several specific radiolabeled proteins (Figure 5A). These bands were excised and subjected to mass spectrometry. The putative substrates are drebrin 1, myosin IIB, munc18-1, PI-3 kinase (C2 domain-containing gamma polypeptide isoform 2), alpha- and beta-tubulin, HSP-70, and a subunit from alpha ketoglutarate dehydrogenase. Interestingly, six of the eight putative substrates are proteins known to have a role in cytoskeletal reorganization.

Arg Phosphorylates Myosin IIB in Vivo. We have previously shown that coexpression of Abl/Arg with their substrates cortactin or p190RhoGAP leads to increased phosphorylation on the substrate (*7*, *31*). Because Abl and Arg have nearly identical protein substrate specificity *in vitro* (*7*, *23*) we tested whether the putative substrates could be phosphorylated by either kinase in cells. Each putative substrate was expressed with either WT Abl/Arg or KI Abl/

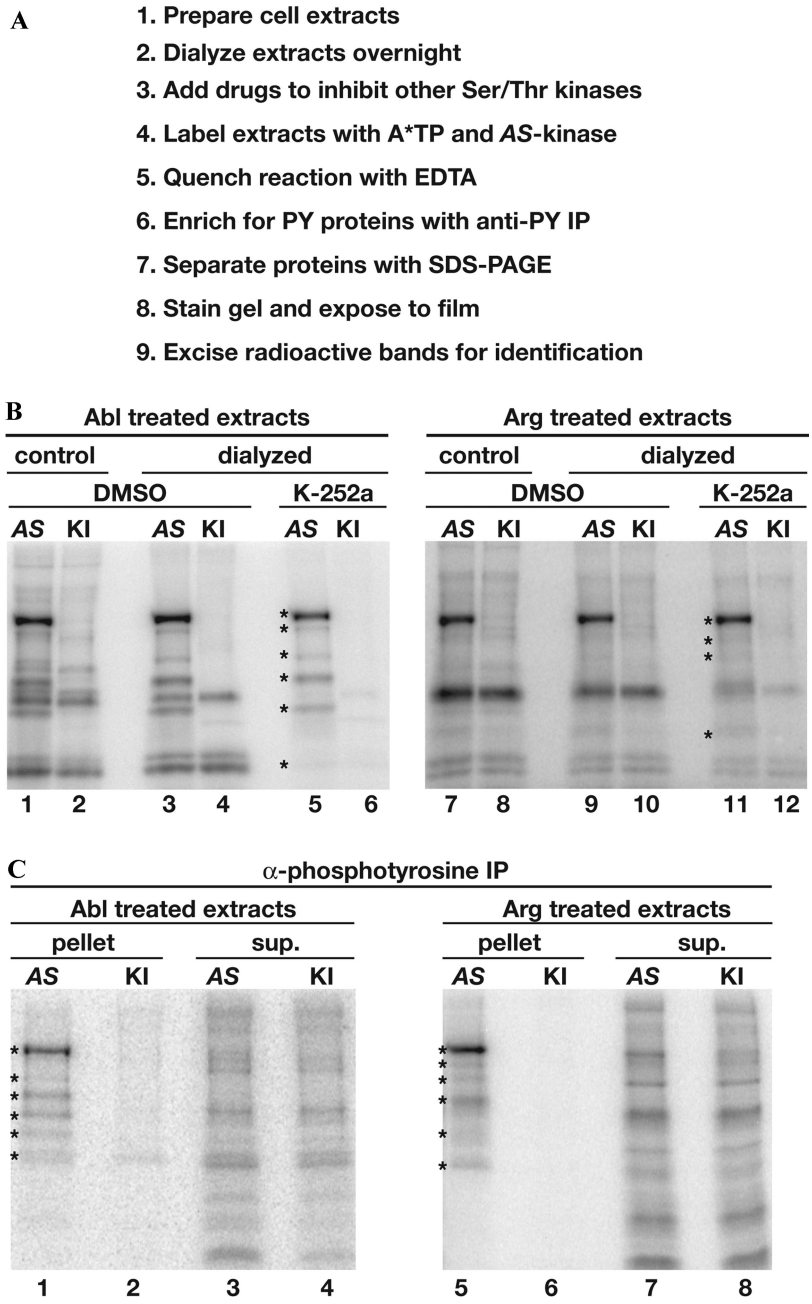


FIGURE 3: Dialysis, drug treatment, and phosphotyrosine enrichment eliminate nonspecific background phosphorylation. (A) Flowchart outlining methods to reduce background phosphorylation. (B) Mouse brain extracts and A*TP were combined with exogenously added *AS*-Abl/Arg (AS) or kinase-inactive (KI) Abl/Arg as a control. Dialysis and pretreatment of the extracts with K-252a (10 μM) eliminated background. Putative substrates are indicated by asterisks. (C) Nonspecific phosphorylation is on serine or threonine residues. Enriching for phosphotyrosine containing proteins with anti-phosphotyrosine immunoprecipitation (pellet) demonstrates that nonspecific phosphorylation remains in the supernatant (sup.).

Arg, and following immunoprecipitation of the putative substrate, its phosphorylation status was assessed by immunoblotting with phosphotyrosine specific antibodies. We found that of the putative substrates tested only myosin IIB was reproducibly tyrosine phosphorylated (6.4-fold over coexpression with KI) when coexpressed with WT Arg (Figure 6A). Myosin IIB was not phosphorylated by Abl in the same assay (data not shown). Because this is the first description of myosin IIB being phosphorylated on tyrosine residues, we also tested whether other isoforms of myosin II were also phosphorylated by Abl or Arg. We found that WT Arg phosphorylates myosin IIA (1.5-fold over coexpression with KI) but not myosin IIC.

DISCUSSION

We have described the optimization of the bump−hole technique to identify putative substrates of Abl family kinases in the mouse brain. We altered the nucleotide specificity by mutating Thr334 in Abl and Thr361 in Arg to alanine. This residue is identical to that mutated by Liu et al. as a probable candidate for altering the nucleotide specificity of Abl (*19*). These single point mutants exhibited similar catalytic activities to WT kinases, but could also utilize an unnatural nucleotide, N6-benzyl-ATP. This result differs from a published report suggesting that a Src-Abl chimera is required to generate an *AS*-Abl (*19*). A major difference between this work and Liu et al. is the purification of full-length kinases

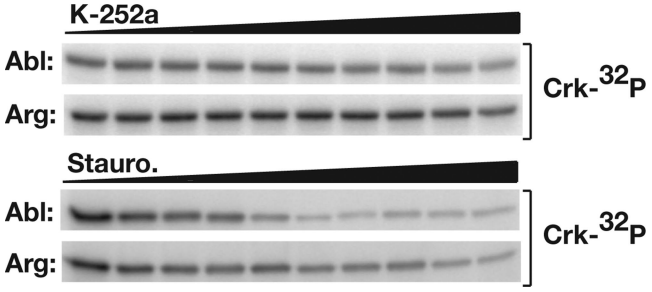
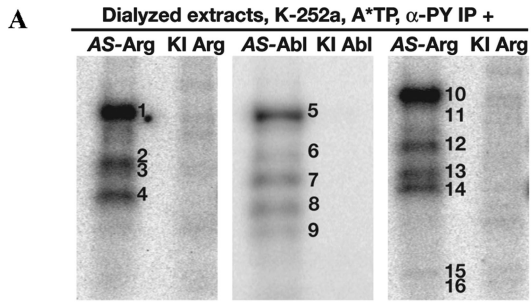


FIGURE 4: K-252a inhibits Abl and Arg with IC_{50} greater than 50 μM. Representative phosphorimage of *in vitro* kinase assays performed with excess GST-Crk and ATP, increasing concentration of K-252a or staurosporine (stauro.). Staurosporine inhibits Abl and Arg with and IC_{50} of 4 and 13 μM, respectively. However, K-252a inhibits Abl and Arg with an $IC_{50} > 50$ μM. Because it potently inhibits many kinases, K-252a is a candidate for eliminating background phosphorylation without affecting Abl and Arg activity.



B **Putative Substrates:**

1. Arg	8. Syntaxin binding protein 1
2. HSP 70	9. No identity
3. PI3K, isoform 2	10. Arg
4. No identity	11 Drebrin 1
5. Myosin IIB (fragment)	12-14. No identity
6. α-ketoglutarate dehydrogenase	15. α-tubulin
7. Myosin IIB (fragment)	16. β-tubulin

FIGURE 5: Identification of novel putative Abl/Arg substrates. (A) Phosphorimage depicts sixteen discrete protein bands labeled by *AS*-Abl/Arg. Bands were excised and identified by mass spectrometry. Substrates 1−4 were labeled in soluble fraction (S1) of brain extracts, whereas substrates 5−16 were labeled in cytoskeletal fraction (S2) CHAPS-extracted brain extracts. (B) Identities of putative substrates. Seventy-five percent of the putative substrates have known roles in cytoskeletal regulation.

from insect cells vs kinase fragments from bacteria. Expression and purification from insect cells may promote folding and post-translational modification required for proper kinase function.

Although WT Abl and Arg could not utilize A*TP *in vitro*, many mouse brain extract proteins were radiolabeled in the presence of A*TP− γ-^{32}P. This suggests that either endogenous kinases or ATPases in the extract can utilize A*TP, and that the radiolabeled phosphate is then reincorporated as ATP-γ-^{32}P. Dialyzing the extracts reduced most of the background, suggesting that removal of the free nucleotides eliminated the opportunity for the ^{32}P-labeled phosphate to be recycled or that the dialysis denatured or eliminated the enzyme responsible for recycling the ^{32}P-labeled phosphate.

The nonspecific background is likely due to Ser/Thr kinases, as enriching for phosphotyrosine-containing proteins by immunoprecipitation eliminates the background in control

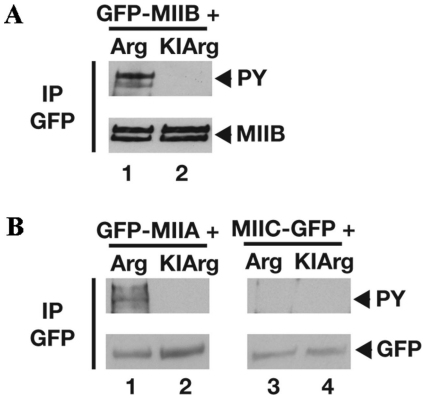


FIGURE 6: Arg phosphorylates myosin IIA and myosin IIB *in vivo*. (A) Myosin IIB and WT Arg or kinase-inactive (KI) Arg were expressed in 293 cells. The phosphotyrosine (PY) content of immunoprecipitated myosin IIB was assessed by immunoblotting. Expression of WT Arg leads to 6.4-fold increase in phosphotyrosine in myosin IIB compared to KI Arg expression, $n = 4$ experiments. (B) Myosin IIA or myosin IIC and WT Arg or KI Arg are expressed as in panel A. Expression of WT Arg leads to a 1.5-fold increase in phosphotyrosine in myosin IIA but not myosin IIC compared with KI Arg expression, $n = 2$ experiments.

treated extracts. The background labeling remained in the supernatant, indicating that the ^{32}P-labeled phosphate was on serine or threonine residues. This led us to identify a broad kinase inhibitor that would eliminate background Ser/Thr kinase activity but not inhibit Abl and Arg. K-252a inhibits Abl and Arg weakly *in vitro* with an IC_{50} greater than 50 μM, whereas it inhibits other kinases between 10 and 80 nM. When we combined dialysis, treatment of extracts with 10 μM K-252a, and phosphotyrosine immunoprecipitation, *AS*-Abl and Arg specifically radiolabeled sixteen bands. By mass spectrometry, we identified eight novel putative substrates of Abl family kinases.

Surprisingly, we did not identify other known substrates (cortactin, Crk, p190RhoGAP, and paxillin) using this technique. These proteins may not be abundant enough in brain extract for us to label, purify, and identify using mass spectrometry. We were able to demonstrate specific phosphorylation of Crk and cortactin (data not shown) after immunoprecipitating these substrates from labeled extracts, suggesting that these and other proteins might be below the threshold for identification by mass spectrometry. This experiment demonstrates an additional advantage of the bump−hole technique: confirming that putative substrates are directly phosphorylated by a particular kinase.

We identified myosin IIB via extract labeling *in vitro* with *AS*-Abl, although only Arg promotes myosin IIB phosphorylation *in vivo*. Myosin IIB is an actin-based motor that forms bipolar filaments, which exert and maintain tension on the cellular actin network. In neurons, myosin IIB is enriched at the leading edge of growth cones (*33*, *34*) where it promotes growth cone motility and neurite outgrowth (*33*, *35*−*38*). It is likely that the *in vivo* localization of myosin IIB favors Arg-mediated phosphorylation, but precludes phosphorylation by Abl. Abl and Arg have nearly identical catalytic activities on substrates *in vitro* (*7*, *23*), so it is not surprising that Abl can phosphorylate myosin IIB *in vitro*. This is the first report demonstrating tyrosine phosphorylation of myosin IIB, though it is known to be phosphorylated on serine and threonine residues. Future studies will explore

whether tyrosine phosphorylation of myosin IIB regulates its motor, actin-binding activity, filament-formation, or interaction with other regulators.

ACKNOWLEDGMENT

We thank Xianyun Ye for technical support, Scott Strobel and Emmanuel Pfund for help in synthesizing N6-benzyl-ATP, Justin Blethrow and Kevan Shokat for N6-benzyl-ADP and nucleoside diphosphate kinase, Lynne Regan for HSP-70 cDNA, Dave Austin for staurosporine, John Wood for K-252a, and Bill Bradley and Chris Mader for critical reading of the manuscript.

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BI701119S