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Authors

Brasher, BB
Roumiantsev, S
Van Etten, RA

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Mutational analysis of the regulatory function of the c-Abl Src homology 3 domain

Bradley B Brasher^{1,3}, Sergei Roumiantsev^{2,3} and Richard A Van Etten^{*2}

¹Enanta Pharmaceuticals, 500 Arsenal Street, Watertown, Massachusetts, MA 02472, USA; ²The Center for Blood Research, Department of Genetics, Harvard Medical School, Boston, Massachusetts, MA 02115, USA

The catalytic activity of the c-Abl tyrosine kinase is tightly regulated by its Src homology 3 (SH3) domain through a complex mechanism that may involve intramolecular binding to Pro242 in the linker region between the SH2 and catalytic domains as well as interactions with a trans-inhibitor. We analysed the effect of mutation or replacement of SH3 on c-Abl tyrosine kinase activity and transformation. Random mutagenesis of SH3 identified several novel point mutations that dysregulated c-Abl kinase activity *in vivo*, but the RT loop was insensitive to mutational activation. Activating SH3 mutations abolished binding of proline-rich SH3 ligands *in vitro*, while mutations at Ser140 in the connector between the SH3 and SH2 domains activated Abl kinase activity *in vivo* and *in vitro* but did not impair SH3 ligand-binding. Abl was regulated efficiently when its SH3 domain was replaced with a heterologous SH3 from c-Src that binds a different spectrum of proline-rich ligands, but not by substitution of a modular WW domain with similar ligand-binding specificity. These results suggest that the SH3 domain regulates Abl principally by binding to the atypical intramolecular ligand Pro242 rather than a canonical PxxP ligand. Coordination between the SH3 and SH2 domains mediated by the connector region may be required for regulation of Abl even in the absence of SH2 ligand binding. *Oncogene* (2001) 20, 7744–7752.

Keywords: Abelson; tyrosine kinase; retrovirus; chronic myeloid leukemia

Introduction

c-Abl is a large, non-receptor tyrosine kinase of metazoans whose precise functions are unknown, but roles for c-Abl in growth factor and integrin signaling, cell cycle regulation, neurogenesis, and responses to DNA damage and oxidative stress have

been suggested (Van Etten, 1999). The kinase activity of c-Abl is tightly regulated *in vivo* by an unknown mechanism that is likely to be distinct from that utilized by Src family kinases. c-Src is negatively regulated by tyrosine phosphorylation of a carboxy-terminal tyrosine (Tyr527) that binds the Src SH2 domain in an intramolecular fashion (Liu *et al.*, 1993). In this structure, the Src SH3 domain binds the linker region between SH2 and the catalytic domain (the SH2-CD linker) in an atypical interaction involving a single proline (Pro250) (Xu *et al.*, 1997). Mutation or deletion of Tyr527 (Cartwright *et al.*, 1987; Kmiecik and Shalloway, 1987; Piwinicaworms *et al.*, 1987), or mutation of the SH2 or SH3 domains (Hirai and Varmus, 1990; Seidel-Dugan *et al.*, 1992) dysregulates and increases Src kinase activity both *in vitro* and *in vivo*. In contrast, c-Abl lacks phosphotyrosine in its inactive state, and deletion of the carboxy-terminus or mutation of SH2 does not activate Abl (Jackson and Baltimore, 1989; Mayer *et al.*, 1992). Deletion of the SH3 domain stimulates Abl kinase activity *in vivo* (Franz *et al.*, 1989; Jackson and Baltimore, 1989), as does mutation of a proline residue (Pro242) in the Abl SH2-CD linker region that is homologous to Src Pro250 (Barila and Superti-Furga, 1998). While immunoprecipitated c-Abl and SH3-deleted Abl have similar kinase activity *in vitro* (Franz *et al.*, 1989; Mayer and Baltimore, 1994), we have recently demonstrated that the intrinsic catalytic activity of purified c-Abl is increased about sixfold by SH3 mutation (Brasher and Van Etten, 2000). These observations suggest that the SH3 domain regulates c-Abl in an intramolecular fashion, similar to Src.

However, other evidence suggests that c-Abl kinase activity is also suppressed *in vivo* through binding of a cellular inhibitor (Pendergast *et al.*, 1991). Purified unphosphorylated c-Abl has high basal catalytic activity relative to inactive c-Src (Brasher and Van Etten, 2000). Expression of c-Abl in cells at up to 10-fold over endogenous levels does not result in Abl autophosphorylation (Jackson and Baltimore, 1989; Van Etten *et al.*, 1989), but expression at higher levels (20–50-fold) results in tyrosine phosphorylation of Abl and other cellular proteins (Pendergast *et al.*, 1991; Wen and Van Etten, 1997). Furthermore, c-Abl is constitutively active when expressed in *S. pombe*

*Correspondence: RA Van Etten, The Center for Blood Research, 200 Longwood Avenue, Boston, MA 02115, USA;

E-mail: vanetten@cbr.med.harvard.edu

³The first two authors contributed equally to this work

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(Walkenhorst *et al.*, 1996), but not in *S. cerevisiae* (Brasher and Van Etten, unpublished observations) or *Xenopus* oocytes (Dorey *et al.*, 1999). These observations suggest that c-Abl may require an inhibitor to completely suppress its activity *in vivo*. The putative inhibitor is abundant but can be titrated upon overexpression of Abl, does not efficiently immunoprecipitate with Abl, and is absent in fission yeast. Several Abl-binding proteins have been identified as candidates for such an inhibitor, including Rb-1 (Welch and Wang, 1993), Pag/Msp23 (Wen and Van Etten, 1997), AAP1 (Zhu and Shore, 1996), Abi-1 (Shi *et al.*, 1995), and Abi-2 (Dai and Pendergast, 1995). Of these, Pag/Msp23 has been shown to inhibit c-Abl kinase activity upon co-expression *in vivo* (Wen and Van Etten, 1997).

To better understand the role of the Abl SH3 domain, we previously introduced loss-of-function SH3 mutations from *C. elegans sem5* into c-Abl (Van Etten *et al.*, 1995), and found that both P131L and G128R SH3 point mutations blocked binding of proline-rich (PxxP) ligands by the Abl SH3 domain *in vitro*. P131L potentially activated Abl kinase activity *in vivo*, but G128R did not (Van Etten *et al.*, 1995), suggesting that the role of SH3 in Abl regulation is complex. To investigate further the structural features of SH3 that are important for regulation of Abl catalytic activity, we undertook more extensive mutagenesis of this domain. In particular, we hoped to recover kinase-activating SH3 mutations that did not completely abrogate PxxP ligand binding.

Results

Isolation of activating mutations in the Abl SH3 domain by random mutagenesis

In order to isolate mutations that dysregulate Abl, we utilized the Ba/F3 murine pro-B lymphoid cell line (Palacios and Steinmetz, 1985), which has a strict requirement for interleukin-3 (IL-3) for growth and survival. Expression of dysregulated, constitutively active Abl kinases in Ba/F3 cells alleviates their IL-3 requirement (Daley *et al.*, 1992), providing a convenient forward selection for isolating activated Abl mutants. We mutagenized the Abl SH3 domain using error-prone PCR (Leung *et al.*, 1989) and inserted the products into pMFG-*c-abl*, a retroviral expression plasmid containing the murine type IV *c-abl* cDNA. This library was packaged into replication-defective retroviral stock and used to transduce Ba/F3 cells, which were then selected for their ability to proliferate in the absence of IL-3. Proviral SH3 domains from transformed Ba/F3 clones were directly sequenced, yielding over 60 point mutations in 30 independent clones (Figure 1a,b). When the mutant SH3 domains were subcloned and tested for transformation in a secondary assay, the majority of the mutations failed to confer IL-3-independent growth in Ba/F3 cells (Figure 1b, gray bars), presumably because they were coincident with non-SH3 mutations (induced by the retro-

viral reverse transcriptase) that dysregulated Abl in the primary assay. In spite of this background, we isolated several highly activating SH3 point mutants from the screen, including F91L, W118R, P131S/T, Y134C and S140I/R (Figure 1b, black bars, and Figure 2a).

The first four mutations involve conserved hydrophobic SH3 residues that play a direct role in binding PxxP ligands (Feng *et al.*, 1994), and mutation of Pro131 to Leu had previously been shown to activate Abl transformation (Van Etten *et al.*, 1995). Two different amino acid substitutions at both Pro131 and Ser140 were recovered from the screen, while the same W118R mutation was found (alone or in combination with other mutations) in over 50% of IL-3-independent Ba/F3 clones in screens of independent libraries. This resulted in each case from a TGG to AGG transversion that may reflect a hot-spot for mutation by Taq polymerase, and limited the usefulness of PCR mutagenesis for the discovery of additional SH3 mutants. The W118R and P131L mutant SH3 domains lost the ability to bind to a panel of PxxP-containing SH3 ligands *in vitro* (Figure 2b) demonstrating a strong correlation between loss of the SH3 ligand-binding function and Abl activation. In contrast, the S140I mutation, located just outside the SH3 domain in the connector to SH2, was very highly activating *in vivo* but did not affect PxxP ligand binding by the Abl SH3 domain (Figure 2a,b). To assess the effect of SH3 mutations on PxxP ligand binding by Abl *in vivo*, Abl proteins were co-expressed in 293 cells with a GST-3BP1 fusion protein. Wild-type c-Abl bound to GST-3BP1 *in vivo* but not to GST alone, and this interaction was significantly decreased by the P131L mutation, as expected (Figure 2c). Interestingly, the S140I mutation reproducibly increased the binding of Abl to 3BP1 *in vivo* (Figure 2c).

Mutations at other positions in the Abl SH3 domain were isolated several times from the screen (Figure 1b), including D90G/K, H114D/L/A, and N133/D/K/S, but were non-transforming when retested individually (data not shown). Interestingly, we did not recover mutations of Glu117, which may interact with Lys313 of the catalytic lobe to stabilize the interaction of SH3 with the SH2-CD linker (Barila and Superti-Furga, 1998). This suggests that our screen may not have been saturating despite the high frequency of mutations in the library.

The RT loop of the Abl SH3 domain is insensitive to mutational activation

The RT-loop (Figure 1a) is a highly sequence-divergent region among SH3 domains, and plays a role in ligand binding specificity (Dalgarno *et al.*, 1997; Feng *et al.*, 1995; Weng *et al.*, 1995). Furthermore, some RT-loop mutations in c-Src result in elevated kinase activity without loss of PxxP ligand binding (Erpel *et al.*, 1995). We reasoned that substitutions within the Abl SH3 RT-loop might dysregulate Abl kinase activity, possibly by altering binding of an inhibitor. We generated a retroviral library with random mutations

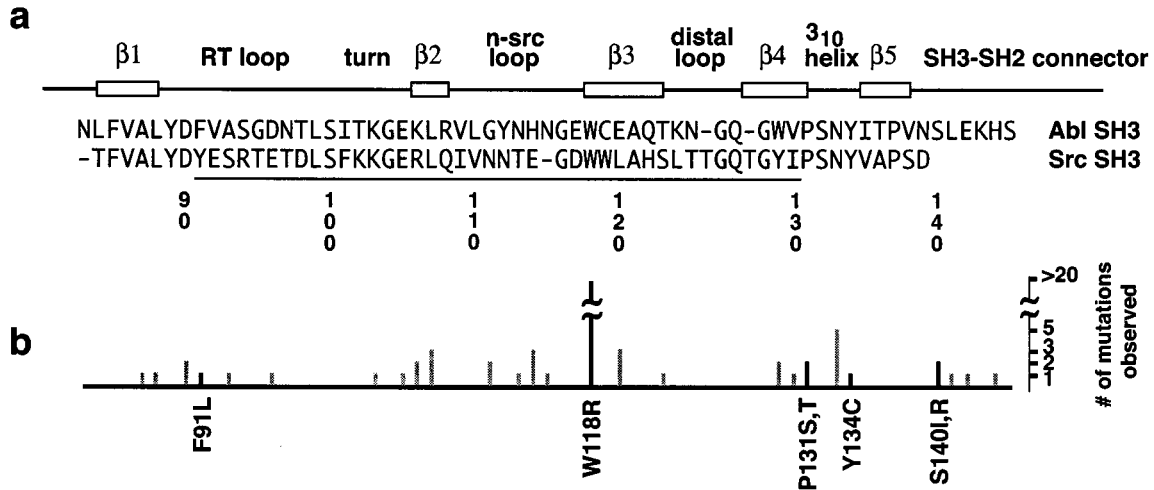


Figure 1 Random mutagenesis of the Abl SH3 domain. (a) Alignment of Abl and Src SH3 domains with structural regions indicated above the sequences, and amino acid position numbers for c-Abl below. (b) Histogram showing the position and relative frequency of mutations recovered in the random mutagenesis procedure, aligned with the amino acid sequence from a. Mutations that were found to activate c-Abl kinase activity and transformation *in vivo* upon rescuing are depicted in black, while non-transforming mutations are in gray

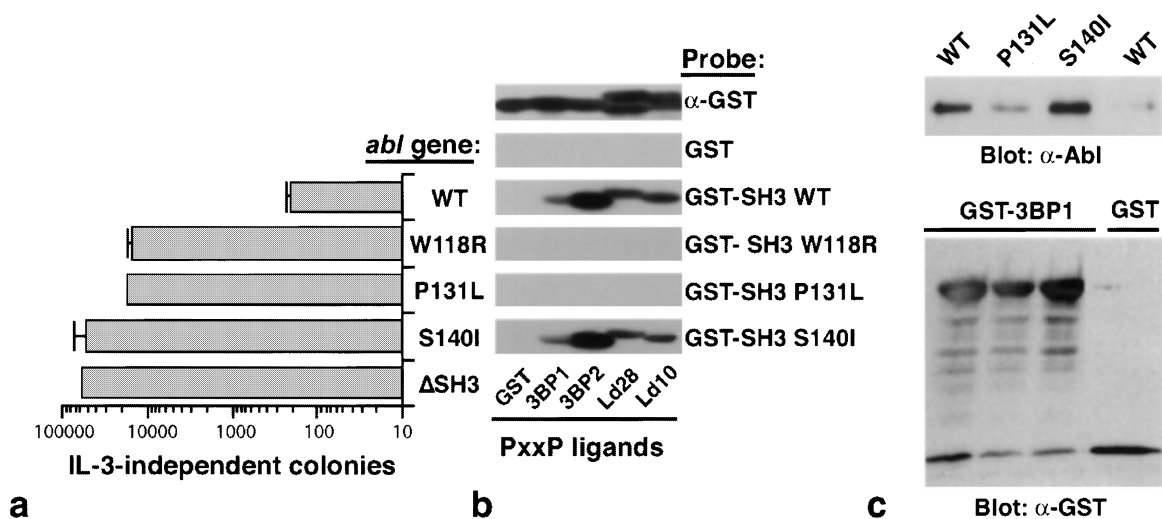


Figure 2 The S140I mutation dysregulates c-Abl without impairing SH3 ligand binding. (a) Transforming activity of Abl SH3 mutants identified in the primary screen was quantitated by the ability to induce IL-3-independent growth of Ba/F3 cells in 0.4% agar medium, as described in Materials and methods. The P131L mutant rather than P131S or T was utilized here, and transformation by the F91L and Y134C mutants were not quantitated. The ΔSH3 mutant contains an in-frame deletion of the SH3 domain. (b) Analysis of mutant Abl SH3 domain ligand binding by Far-Western blotting. Purified GST fusion proteins containing PxxP motifs from the SH3 binding proteins 3BP1 and 3BP2 and the Ld28 and Ld10 ligands from mouse formin were blotted to nitrocellulose filters and probed with purified, biotinylated GST-Abl SH3 domain fusion proteins, followed by detection with peroxidase-conjugated streptavidin and enhanced chemiluminescence. Equivalent amounts of PxxP ligands were present in all lanes as determined by blotting with anti-GST antibodies (top panel). (c) c-Abl and the Abl S140I mutant bind 3BP1 *in vivo*. GST-3BP1 or GST was co-expressed with the indicated Abl proteins in 293 cells, isolated from cell lysates with glutathione agarose beads, and associated proteins analysed by SDS-PAGE and Western blotting with anti-Abl (top panel) and anti-GST (bottom panel) antibodies

in codons corresponding to Abl RT-loop residues 91–99, and transduced Ba/F3 cells as before. Surprisingly, these cells acquired IL-3-independence with very low frequency, similar to cells transduced with retrovirus containing wild-type *c-abl*. We isolated and sequenced SH3 domains from numerous Ba/F3 clones prior to IL-3 deprivation and found that over 50% had one or more RT-loop amino acid substitutions, suggesting the

inefficient Ba/F3 transformation was not the result of a low frequency of mutations in the library. Indeed, we identified mutations at most positions within the RT-loop and confirmed that none activated c-Abl as measured by Ba/F3 IL-3-independent growth (Figure 3a) nor abolished ligand binding as assessed by Far-Western blotting (Figure 3b). Although insensitive to point mutations, the RT-loop is structurally required

for SH3 regulatory function, because a RT-loop deletion mutant (Δ RT) was strongly activating (Figure 3a). Particularly interesting was the lack of activation by the Abl T98D mutation. The majority of known SH3 domains contain aspartate or glutamate at this position, and prefer ligands with an arginine adjacent to the PxxP that interacts with the carboxylate side chain of this residue (Lim and Richards, 1994; Weng *et al.*, 1995). In contrast, Abl SH3 ligands typically have tyrosine or methionine adjacent to the PxxP core as a consequence of the T98 residue (Pisabarro and Serrano, 1996), and the Abl T98D SH3 mutant exhibits altered ligand preference *in vitro* (Weng *et al.*, 1995).

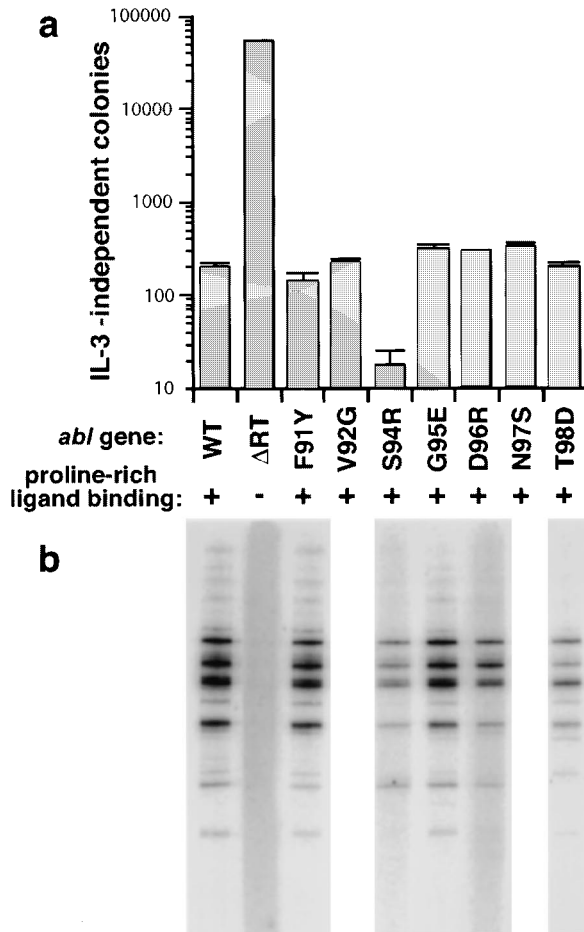


Figure 3 The RT loop of the Abl SH3 domain is insensitive to activating mutations. (a) The Abl SH3 domain RT loop was randomly mutagenized by an oligonucleotide strategy as described in Materials and methods. Depicted are a subset of mutants isolated from Ba/F3 clones prior to IL-3 deprivation and re-tested for quantitative transformation of Ba/F3 cells as described in Figure 2a. Δ RT contains an in-frame deletion of RT-loop residues 93–100. (b) Proline-rich ligand binding is intact for all the RT-loop mutants other than Δ RT. Total protein lysates from NIH3T3 fibroblasts were separated by SDS-PAGE, blotted to nitrocellulose, and probed with purified, 32 P-labeled GST-SH3 fusion proteins as described in Materials and methods. This Far-Western assay demonstrated a modest alteration in the binding profile of T98D SH3, but not as marked as that detected in pull-down assays (Weng *et al.*, 1995). Results for the V92G and N97S mutants were similar to WT but are not shown

The Src SH3 domain can efficiently regulate c-Abl

Because individual RT-loop mutations did not result in significant Abl activation, we tested the effect of replacing the entire SH3 domain with the chicken c-Src SH3. The inhibitory function of Abl SH3 is sensitive to position (Mayer and Baltimore, 1994), so the substitution was made such that the structural relationship of the heterologous SH3 domain to the adjacent Abl sequence was precisely conserved. This substitution increased the length of SH3 by one amino acid and altered a total of 27 residues (Figure 1a, the underlined Src sequence replaced the Abl sequence above it). Surprisingly, expression of this chimeric protein in Ba/F3 cells resulted in IL-3-independent clones at only about three times the frequency of Ba/F3 cells transduced with wild-type *abl* (Figure 4a). By comparison, the Abl SH3-inactivating point mutant P131L (Van Etten *et al.*, 1995) and an inframe SH3 deletion Δ SH3 (also called Δ XB, (Jackson and Baltimore, 1989)) gave up to 100-fold more IL-3-independent clones than wild-type, demonstrating that

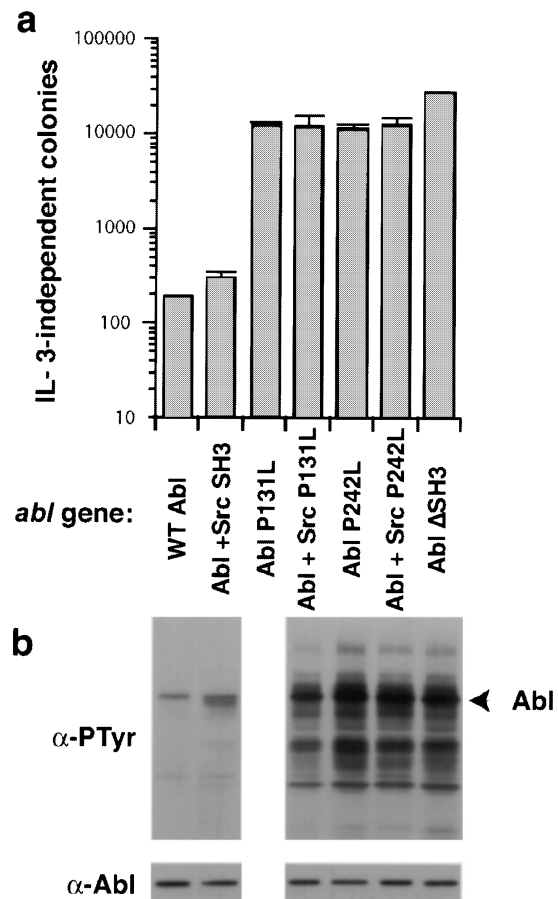


Figure 4 The Src SH3 domain efficiently substitutes for Abl SH3 in regulation of kinase activity. (a) The indicated mutants were tested for the ability to transform Ba/F3 cells as described in Figure 2a. (b) *In vivo* activity of Abl kinases. 293T cells were transfected with indicated pMFG-*abl* plasmids and whole cell extracts analysed by Western blot with anti-phosphotyrosine (top panel) and anti-Abl (bottom panel) monoclonal antibodies. The Abl P131L mutant is not shown, but was indistinguishable from Δ SH3 in phosphotyrosine content

the c-Src SH3 domain functioned relatively well in regulating Abl kinase activity. Lysates from cells expressing wild-type c-Abl or the c-Abl+Src SH3 chimera demonstrated very low *in vivo* tyrosine phosphorylation of Abl and other proteins, while the P131L and Δ SH3-expressing cells had extensive phosphotyrosine (Figure 4b), confirming that the Src SH3 domain efficiently regulates Abl kinase activity *in vivo*.

If Src SH3 suppresses Abl kinase activity by the same mechanism as the Abl SH3, then mutations that deregulate wild-type Abl should also activate the Abl+Src SH3 chimera. To test this, we introduced the P131L mutation into the SH3 domain of the Abl+Src SH3 chimera and transduced Ba/F3 cells as before. The P131L mutation eliminated the ability of Src SH3 to suppress Abl kinase activity and resulted in IL-3-independent Ba/F3 growth (Figure 4a) and increased *in vivo* tyrosine phosphorylation (Figure 4b). Similarly, mutation of Pro242 in the Abl SH2-CD linker to Leu activated both c-Abl and Src SH3-substituted Abl (Figure 4), suggesting that the two SH3 domains regulate Abl kinase activity in a similar manner.

A WW domain with similar ligand-binding specificity cannot substitute for the Abl SH3 domain

WW domains are another modular class of proline-rich ligand-binding domains that are similar to, but distinct from SH3 domains and can be categorized into four classes on the basis of ligand specificity (Kay *et al.*, 2000). WW domains from class I and III can bind similar ligands to the Abl (Bedford *et al.*, 1997) and Src-like SH3 domains, respectively, raising the possibility that WW and SH3 domains compete for the same proline-rich motifs within target proteins. The FBP11 WW domain is a class I domain (Bedford *et al.*, 1997) that binds to ligands with a consensus sequence that often overlaps binding sites for the Abl SH3 domain. We reasoned that if Abl SH3 and FBP11 WW bind similar ligands, then an Abl inhibitor might still associate and inhibit Abl kinase activity if we substituted this WW domain for Abl SH3 in a manner similar to the Src SH3 substitution. Because the orientation of the WW11 ligand-binding surface in the chimeric protein might be important, three different substitutions were made, replacing the SH3 domain and slightly varying lengths of the SH3–SH2 connector region. However, none of the WW domain substitutions regulated Abl kinase activity, as assessed by IL-3-independent growth of Ba/F3 cells (Figure 5a) and *in vivo* tyrosine phosphorylation (Figure 5b). Co-expression of the Abl/WW chimeras with GST-3BP1 demonstrated that the WW11 domain in c-Abl functioned to mediate a significant level of PxxP ligand binding *in vivo* (Figure 5c).

The Abl S140I mutant is resistant to inhibition by Pag in vivo and has increased intrinsic tyrosine kinase activity in vitro

To understand better the mechanism of deregulation of c-Abl by the SH3 mutations, we determined the sensitivity

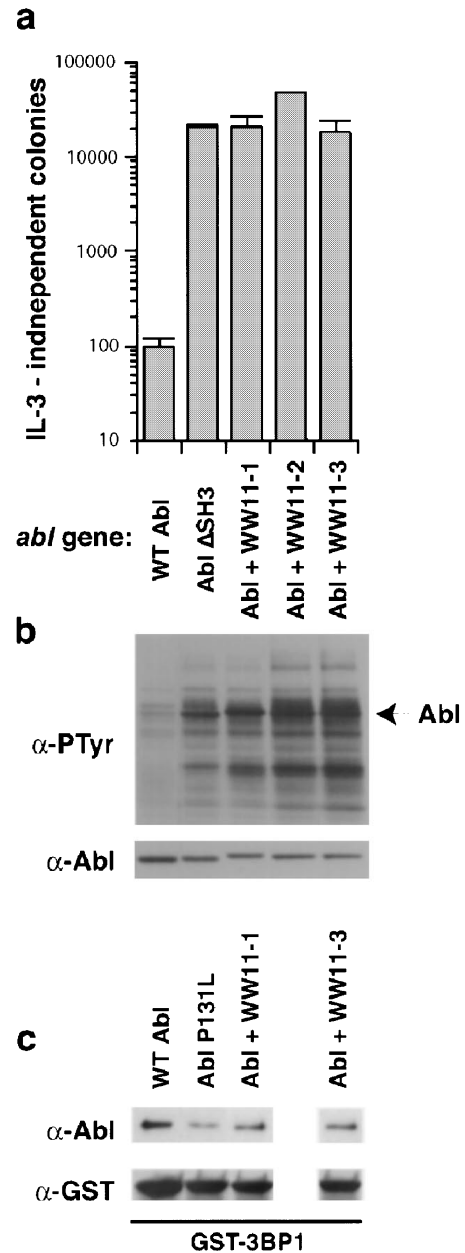


Figure 5 The WW domain of FBP11 cannot substitute for the SH3 domain to regulate Abl kinase activity. (a) Abl+WW11 chimeras were analysed for Ba/F3 transforming activity as described in Figure 2a. (b) Anti-phosphotyrosine Western blot of lysates from 293T cells expressing the Abl+WW11 domain chimeric proteins confirms that these kinases are highly active *in vivo*. Wild-type c-Abl (WT Abl) and Abl Δ SH3 are shown for comparison. (c) The WW11 domain in Abl binds 3BP1 *in vivo*. Abl proteins and GST-3BP1 were co-expressed in 293 cells as in Figure 2c, and complexes detected by isolation on glutathione-agarose beads and Western blotting. Binding of Abl to GST alone was undetectable (data not shown)

of Abl mutants to trans-inhibition by the candidate Abl inhibitor Pag/MSP23 (Wen and Van Etten, 1997). Moderate overexpression of c-Abl in 293 cells dysregulates Abl *in vivo* and induces tyrosine phosphorylation of Abl and other cellular proteins (Pendergast *et al.*, 1991;

Van Etten *et al.*, 1995). Co-expression of Pag in the sense, but not antisense, orientation significantly inhibited Abl-induced tyrosine phosphorylation (Figure 6a), but had no effect on Abl with the P131L SH3 mutation, as previously reported (Wen and Van Etten, 1997). The Abl S140I, W118R, and Src SH3 substitution mutants were all resistant to inhibition by Pag in this co-expression assay (Figure 6a). All the Abl mutants retained the ability to complex with Pag *in vivo* that likely reflects direct binding to the ATP-binding lobe of the Abl kinase domain (Wen and Van Etten, 1997), but the Abl+Src SH3 chimera demonstrated significantly weaker interaction with Pag (Figure 6b).

To assess the effect of the mutations on the intrinsic catalytic activity of Abl, we purified the Abl kinases in

their unphosphorylated state and determined their kinetic parameters in a peptide-based phosphorylation assay (Brasher and Van Etten, 2000). Unphosphorylated c-Abl had significant activity in this assay (K_m 144 μ M, V_{max} 31 pmol/min) that was greatly stimulated by the P131L mutation (Figure 6c), as previously reported (Brasher and Van Etten, 2000). Both the Abl W118R (K_m 43, V_{max} 125) and S140I (K_m 99, V_{max} 123) mutants exhibited high intrinsic catalytic activity, with Abl W118R activity exceeding and S140I activity similar to that of Abl P131L. The Abl+Src SH3 chimera had intermediate intrinsic catalytic activity (K_m 71, V_{max} 62) that was modestly increased over that of c-Abl. These results suggest that the S140I SH3 mutation, although it does not impair PxxP ligand

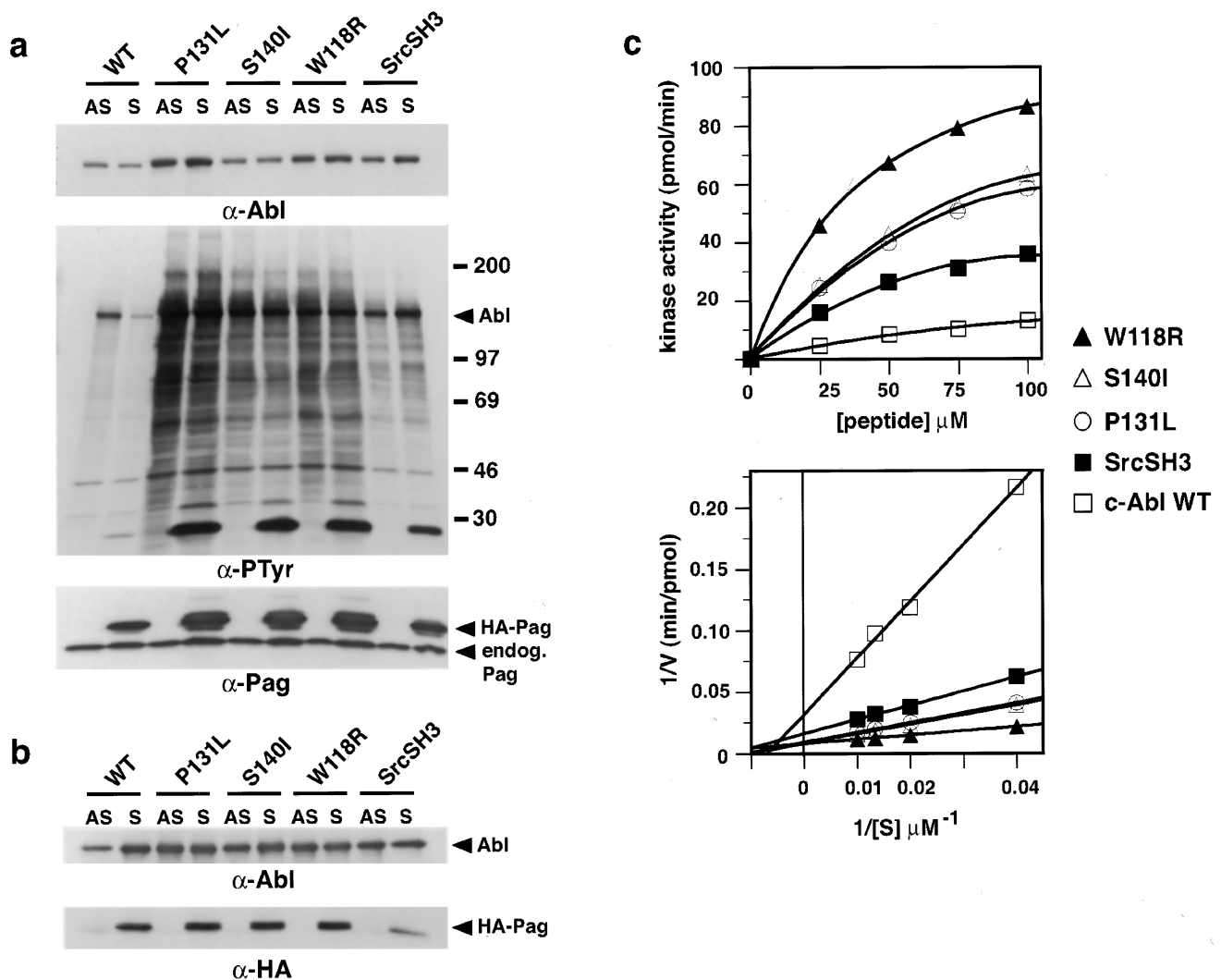


Figure 6 The Abl S140I mutant is resistant to inhibition by Pag/MSP23 and has increased intrinsic catalytic activity. (a) Pag inhibition assay. The indicated Abl proteins were co-expressed with Pag in the sense (S) or antisense (AS) orientation in 293 cells, and levels of cellular phosphotyrosine (middle panel) assessed by Western blotting. Abl protein levels (top panel) were unaffected by co-expression of Pag (bottom panel). (b) Pag co-precipitation assay. Lysates from the experiment in (a) were immunoprecipitated with polyclonal anti-Abl antibodies and examined by Western blotting with anti-Abl (top panel) and anti-hemagglutinin (HA) antibodies (bottom panel). (c) The Abl W118R and S140I mutants have high intrinsic tyrosine kinase activity. Michaelis–Menten (top panel) and Lineweaver–Burke (bottom panel) graphs of the catalytic activity of purified, unphosphorylated Abl proteins in a peptide-based assay. Data shown are representative of three independent protein preparations that gave similar results

binding, interferes with the engagement of the SH2–CD linker by the Abl SH3 domain.

Discussion

We demonstrate here that the SH3 domain in c-Abl maintains a majority of its negative regulatory function in the presence of mutations that significantly modify its PxxP ligand specificity. c-Abl kinase activity is dysregulated when its SH3 is replaced by a modular WW domain that binds a similar spectrum of proteins, yet is fairly efficiently regulated upon substitution with the Src SH3 domain, which has quite different PxxP ligand-binding specificity. Overall, these results support a primary intramolecular regulatory function for the Abl SH3 domain (Barila and Superti-Furga, 1998). Because the sequence of the Abl SH2–CD linker is P₂₄₂TIY, the predicted SH3-linker interaction does not involve a PxxP ligand and may not be affected by mutations (such as RT-loop substitutions) that affect binding of such ligands. An intermolecular salt bridge between Glu117 in the Abl SH3 domain and Lys313 in the catalytic domain that has also been implicated in suppression of c-Abl kinase activity (Barila and Superti-Furga, 1998) is preserved in the Abl + Src SH3 chimera, because the Src SH3 contains an aspartate at position 117 (Figure 1a). It may also be relevant that the Abl RT-loop mutation S94R suppresses Ba/F3 transformation by c-Abl nearly 10-fold relative to wild-type (Figure 3a), as the homologous Arg95 residue in Src contacts and stabilizes the SH2–CD linker, and mutation of this residue can activate c-Src (Potts *et al.*, 1988).

We identified several novel activating SH3 point mutations through a random mutagenesis strategy. The majority of these mutations affected conserved residues implicated in direct contact with proline-rich ligands and abolished ligand-binding by the isolated SH3 domains *in vitro*. These mutants confirm our previous hypothesis that ligand-binding is critical for the regulatory function of the Abl SH3 domain (Van Etten *et al.*, 1995) but do not discriminate between inter- and intramolecular ligands. We also recovered mutations in Ser140 in the SH3–SH2 connector region that strongly dysregulated Abl *in vivo*. Although the S140I mutation does not impair PxxP ligand binding, the Abl S140I kinase demonstrated very high intrinsic catalytic activity as a purified protein, suggesting that the primary effect of this mutation is to interfere with engagement of Pro242 in the SH2–CD linker. In support of this, the S140I SH3 domain displayed increased binding to 3BP1 in the context of the full-length Abl protein *in vivo* (Figure 2c). Recent molecular dynamic modeling of Src kinases demonstrates that the c-Src and Hck SH3–SH2 connector region may act as an ‘inducible snap-lock’, restricting the independent mobility of the SH3 and SH2 domains when the SH2 is bound to phosphorylated Tyr527 in the inactive conformation (Young *et al.*, 2001). Mutation of c-Src Ser142 (homologous to Abl Ser140) to Gly disrupts hydrogen bonding with Glu144 and disorders the $\beta/3_{10}$ helical turn structure of the

connector, uncoupling the SH3 and SH2 domains and rendering c-Src resistant to inhibition by Csk phosphorylation of Tyr527 *in vivo* (Young *et al.*, 2001). Because mutations at the homologous residue are strongly activating in Abl, this suggests that the SH3–SH2 connector may function in a similar manner to constrain the independent orientation of the SH3 and SH2 domains in Abl, which is required for Abl to assume an inactive conformation. This is unexpected because Abl is not tyrosine phosphorylated in the inactive state *in vivo*, and hence the orientation of SH2 is not restricted by binding to a phosphotyrosine ligand. A fixed relationship of SH3 to SH2 might be necessary to orient the Abl SH2–CD linker region into a polyproline-type helix for optimal interaction with the SH3 domain.

Our results do not support a model in which an inhibitor with proline-rich sequences binds to the c-Abl SH3 domain in a canonical fashion. However, the constitutive kinase activity of c-Abl upon purification (Brasher and Van Etten, 2000), expression in fission yeast (Walkenhorst *et al.*, 1996), or overexpression in mammalian cells (Wen and Van Etten, 1997) is difficult to explain if Abl is regulated solely by an intramolecular mechanism. Because the SH2–CD linker in both Abl and Src is a suboptimal SH3 ligand that may not utilize the entire PxxP ligand-binding surface of the SH3 domain, this interaction may require stabilization through the SH2–Y527 interaction in Src, as implied by the significant dysregulation observed with the c-Src Y527F mutant (Kmieciak and Shalloway, 1987; Piwini-ca-worms *et al.*, 1987). An Abl inhibitor might bind outside the peptide-binding surface of the SH3 domain, stabilizing an intramolecular complex between the SH3, SH2–CD linker, and catalytic domains, and compensate for the lack of an SH2-phosphotyrosine interaction. It is interesting that the Abl + Src SH3 chimera is well-regulated *in vitro* but weakly transforming *in vivo*, not unlike the c-Src Y527F mutant, which is 100-fold less efficient than v-Src in transformation of NIH3T3 cells (Hunter, 1987). The Abl + Src SH3 chimera exhibited decreased interaction with and inhibition by Pag/MSP23 *in vivo* (Figure 6a,b), consistent with the lack of interaction of Pag and Src SH3 that was observed in a yeast two-hybrid system (Wen and Van Etten, 1997), and impaired inhibition of this chimeric kinase by Pag *in vivo* could account for the increased transforming activity. However, further enzymological and particularly structural studies will be needed to resolve the long-standing puzzle of the mechanism of c-Abl kinase regulation.

Materials and methods

PCR mutagenesis and library screening

The c-Abl SH3 domain was mutagenized by PCR amplification in the presence of manganese (Leung *et al.*, 1989), PCR products digested at flanking restriction sites, and ligated into the retroviral expression vector pMFG-c-abl. The library was transformed into *E. coli*, plasmid DNA purified from bacteria

and retrovirus stock produced by transfection of 293T cells using the *kat* packaging system (Finer *et al.*, 1994) as described (Li *et al.*, 1999). 10^6 Ba/F3 cells were transduced for 3 h with 1 ml viral supernatant in the presence of IL-3 (5% v/v WEHI-3B conditioned medium (Lee *et al.*, 1982)) and 8 μ g/ml polybrene, then cloned 48 h later by limiting dilution in the absence of IL-3. Genomic DNA from IL-3-independent clones was used in PCR reactions designed to amplify the SH3 sequence from the proviral *abl* cDNA without interference from the endogenous *c-abl* gene. One of the PCR primers contained a 5' biotin tag, allowing immobilization and direct solid-phase sequencing (Dynal Corporation) of products.

For selective mutagenesis of the RT loop of the Abl SH3 domain, oligonucleotides spanning the region including *abl* codons 91 to 98 were synthesized with approximately 5% misincorporation at each nucleotide position (Biopolymer Facility, Genetics Department, Harvard Medical School). Mutation of phenylalanine 91 was limited to tyrosine only, as other substitutions typically cause a loss of PxxP ligand binding (Lim and Richards, 1994). Oligonucleotides were used in non-error-prone PCR reactions to produce SH3 fragments with random RT-loop mutations. The library of mutant SH3 fragments was cloned into pMFG-*c-abl* and used to transduce Ba/F3 cells as described above.

Transformation assay

Mutations identified in the primary library selection were tested in a secondary screen by re-cloning the mutant SH3 domain into pMFG-*c-abl* and repeating the Ba/F3 transduction. Transforming activity of Abl SH3 mutants was quantitated by the ability to induce IL-3-independent growth of Ba/F3 cells in 0.4% agar medium. The number of colonies for each mutant was normalized for retroviral transduction efficiency by determining the proviral copy number by Southern blotting of genomic DNA from the transduced population, with the results expressed as colonies per 10^6 cells transduced at one proviral copy per cell. SH3 mutants that efficiently transformed Ba/F3 cells to IL-3 independence in the secondary screen were characterized further.

Far-Western blotting

The ability of mutant Abl SH3 domains to bind proline-rich ligands was determined by a modified Far-Western technique (Van Etten *et al.*, 1995). Purified GST fusion proteins containing PxxP motifs from 3BP1 and 3BP2 (Ren *et al.*, 1993) and the Id28 and Id10 ligands from mouse formin (Bedford *et al.*, 1997) were blotted to nitrocellulose filters and probed with purified, biotinylated GST-SH3 fusion proteins, followed by detection with peroxidase-conjugated streptavidin (Jackson Immunoresearch) and enhanced chemiluminescence (Amersham). Equivalent amounts of PxxP ligands were present in all lanes as determined by blotting with polyclonal anti-GST antibodies (Figure 2b, top panel (Van Etten *et al.*, 1995)), and all GST and GST-SH3 probes were equally biotinylated as assessed by direct blotting with peroxidase-streptavidin (data not shown).

For the experiment in Figure 3b, GST-Abl SH3 domain fusion proteins were labeled *in vitro* with γ - 32 P-ATP and heart muscle kinase as described (Bedford *et al.*, 1997), and used to probe total protein lysates from NIH3T3 fibroblasts that were fractionated by SDS-PAGE and blotted to nitrocellulose.

GST pull-down assay

293T cells (5×10^6 cells per 6 cm plate) were co-transfected with 10 μ g of Abl expression plasmid (in pcDNA3,

Invitrogen) and 5 μ g of GST-3BP1 fusion or parental GST expression plasmid (in the eukaryotic expression vector pEBG, kind gift of Dr Bruce Mayer, University of Connecticut, USA) using a modified calcium phosphate technique (Mayer and Baltimore, 1994). Forty-eight hours post-transfection, cells were lysed in RIPA buffer, clarified by centrifugation, and incubated with glutathione agarose beads (100 μ l per ml lysate per plate, Molecular Probes) at 4°C for 1 h. Beads were washed three times each with RIPA, high-salt buffer (0.5 M NaCl), and low-salt buffer (20 mM PIPES pH 7.2) before boiling in sample buffer. Associated proteins were fractionated by SDS-PAGE, transferred to nitrocellulose filters, and blotted with anti-Abl monoclonal antibody (3F12, gift of Dr Ravi Salgia, Dana-Farber Cancer Institute, Boston, USA) and polyclonal anti-GST antibodies and enhanced chemiluminescence (Amersham).

In vivo activity of Abl kinases

To assess the *in vivo* activity of Abl mutants and chimeras in Figures 4b and 5b, 293T cells were transfected with indicated pMFG-*abl* plasmids and whole cell extracts analysed by Western blot with anti-Abl (3F12) and anti-phosphotyrosine (4G10, Upstate Biotechnology).

Abl/WW11 chimeras

Constructs WW11-1, WW11-2, and WW11-3 were produced by PCR-generated deletion of murine c-Abl type IV amino acids 83 through 130, 141, or 145, respectively and insertion of a single *Bam*HI site, into which was cloned a 69 amino acid insert (residues 146 through 214 of FBP11 (Chan *et al.*, 1996)) containing two tandem WW11 domains separated by a 15 amino acid spacer.

Pag co-expression assay

Abl mutants (in the vector pcDNA3) and influenza hemagglutinin-tagged Pag/MSP23 (in the vector pCGN, sense and antisense orientations) were co-expressed in 293 cells essentially as described (Wen and Van Etten, 1997), except that 5 μ g Abl plasmid and 7 μ g Pag plasmid were co-transfected per 6 cm plate. Forty-eight hours post-transfection, whole cell lysates were fractionated by SDS-PAGE and analysed by Western blotting with anti-Abl (3F12) and anti-phosphotyrosine (4G10) monoclonal antibodies, and with polyclonal anti-Pag antibodies. For co-precipitation analysis, lysates were precipitated with polyclonal anti-GEX4 Abl antibodies (Van Etten *et al.*, 1995).

Abl purification and in vitro kinase assay

Abl mutants were fused to a hexahistidine tag at the COOH-terminus, expressed by transfection of 293 cells in the presence of the Abl kinase inhibitor STI-571 (50 μ M, Novartis Pharmaceuticals), and purified by single-step affinity chromatography on cobalt-agarose (Talon resin, Clontech) as described (Brasher and Van Etten, 2000). The purified Abl proteins were the predominant species on silver- and Coomassie-stained gels, and lacked detectable phosphotyrosine as assessed by anti-phosphotyrosine Western blotting (data not shown). Kinase assays with the peptide substrate (biotin-GGEAIYAAPFCK) were carried out as described (Brasher and Van Etten, 2000) at 30°C with 50 μ M ATP and 0.01 μ M Abl. Peptide-associated radioactivity was corrected for background and plotted on double reciprocal (1/V vs 1/[S]) graphs to calculate K_m and V_{max} values.

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References

- Barila D and Superti-Furga G. (1998). *Nat. Genet.*, **18**, 280–282.
- Bedford MT, Chan DC and Leder P. (1997). *EMBO J.*, **16**, 2376–2383.
- Brasher BB and Van Etten RA. (2000). *J. Biol. Chem.*, **275**, 35631–35637.
- Cartwright CA, Eckhart W, Simon S and Kaplan PL. (1987). *Cell*, **49**, 83–91.
- Chan DC, Bedford MT and Leder P. (1996). *EMBO J.*, **15**, 1045–1054.
- Dai Z and Pendergast AM. (1995). *Genes Dev.*, **9**, 2569–2582.
- Daley GQ, Van Etten RA, Jackson PK, Bernards A and Baltimore D. (1992). *Mol. Cell. Biol.*, **12**, 1864–1871.
- Dalgarno DC, Botfield MC and Rickles RJ. (1997). *Biopoly.*, **43**, 383–400.
- Dorey K, Barila D, Gavin AC, Nebreda AR and Superti-Furga G. (1999). *Biol. Chem.*, **380**, 223–230.
- Erpel T, Superti-Furga G and Courtneidge SA. (1995). *EMBO J.*, **14**, 963–975.
- Feng S, Chen JK, Yu H, Simon JA and Schreiber SL. (1994). *Science*, **266**, 1241–1247.
- Feng S, Kasahara C, Rickles RJ and Schreiber SL. (1995). *Proc. Natl. Acad. Sci. USA*, **92**, 12408–12415.
- Finer MH, Dull TJ, Qin L, Farson D and Roberts M. (1994). *Blood*, **83**, 43–50.
- Franz WM, Berger P and Wang JYJ. (1989). *EMBO J.*, **8**, 137–147.
- Hirai H and Varmus HE. (1990). *Mol. Cell. Biol.*, **10**, 1307–1318.
- Hunter T. (1987). *Cell*, **49**, 1–4.
- Jackson P and Baltimore D. (1989). *EMBO J.*, **8**, 449–456.
- Kay BK, Williamson MP and Sudol M. (2000). *FASEB J.*, **14**, 231–241.
- Kmieciak TE and Shalloway D. (1987). *Cell*, **49**, 65–73.
- Lee JC, Hapel AJ and Ihle JN. (1982). *J. Immunol.*, **128**, 2393–2398.
- Leung DW, Chen E and Goeddel DV. (1989). *Technique*, **1**, 11–15.
- Li S, Ilaria RL, Million RP, Daley GQ and Van Etten RA. (1999). *J. Exp. Med.*, **189**, 1399–1412.
- Lim WA and Richards FM. (1994). *Nature Struct. Biol.*, **1**, 221–225.
- Liu X, Brodeur SR, Gish G, Songyang Z, Cantley LC, Laudano AP and Pawson T. (1993). *Oncogene*, **8**, 1119–1126.
- Mayer BJ and Baltimore D. (1994). *Mol. Cell. Biol.*, **14**, 2883–2894.
- Mayer BJ, Jackson PK, Van Etten RA and Baltimore D. (1992). *Mol. Cell. Biol.*, **12**, 609–618.
- Palacios R and Steinmetz M. (1985). *Cell*, **41**, 727–734.
- Pendergast AM, Muller AJ, Havlik MH, Clark R, McCormick F and Witte ON. (1991). *Proc. Natl. Acad. Sci. USA*, **88**, 5927–5931.
- Pisabarro MT and Serrano L. (1996). *Biochemistry*, **35**, 10634–10640.
- Piwinica-worms H, Saunders K, Roberts T, Smith A and Cheng S. (1987). *Cell*, **49**, 75–82.
- Potts WM, Reynolds AB, Lansing TJ and Parsons JT. (1988). *Oncogene Res.*, **3**, 343–355.
- Ren R, Mayer BJ, Cicchetti P and Baltimore D. (1993). *Science*, **259**, 1157–1161.
- Seidel-Dugan C, Meyer BE, Thomas SM and Brugge JS. (1992). *Mol. Cell. Biol.*, **12**, 1835–1845.
- Shi Y, Alin K and Goff SP. (1995). *Genes Dev.*, **9**, 2583–2597.
- Van Etten RA. (1999). *Trends Cell Biol.*, **9**, 179–186.
- Van Etten RA, Debnath J, Zhou H and Casasnovas JM. (1995). *Oncogene*, **10**, 1977–1988.
- Van Etten RA, Jackson P and Baltimore D. (1989). *Cell*, **58**, 669–678.
- Walkenhorst J, Goga A, Witte ON and Superti-Furga G. (1996). *Oncogene*, **12**, 1513–1520.
- Welch PJ and Wang JYJ. (1993). *Cell*, **75**, 779–790.
- Wen S-T and Van Etten RA. (1997). *Genes Dev.*, **11**, 2456–2467.
- Weng Z, Rickles RJ, Feng S, Richard S, Shaw AS, Schreiber SL and Brugge JS. (1995). *Mol. Cell. Biol.*, **15**, 5627–5634.
- Xu W, Harrison SC and Eck MJ. (1997). *Nature*, **385**, 595–602.
- Young MA, Gonfloni S, Superti-Furga G, Roux B and Kuriyan J. (2001). *Cell*, **105**, 115–126.
- Zhu J and Shore SK. (1996). *Mol. Cell. Biol.*, **16**, 7054–7062.