Cell, Vol. 108, 247–259, January 25, 2002, Copyright ©2002 by Cell Press

Autoinhibition of c-Abl

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Summary

Despite years of investigation, the molecular mechanism responsible for regulation of the c-Abl tyrosine kinase has remained elusive. We now report inhibition of the catalytic activity of purified c-Abl in vitro, demonstrating that regulation is an intrinsic property of the molecule. We show that the interaction of the N-terminal 80 residues with the rest of the protein mediates autoregulation. This N-terminal "cap" is required to achieve and maintain inhibition, and its loss turns c-Abl into an oncogenic protein and contributes to deregulation of BCR-Abl.

Introduction

c-Abl is a nonreceptor tyrosine kinase ubiquitously expressed and highly conserved in metazoan evolution. c-Abl and the product of its paralog gene, Arg (*ABL2*), resemble Src family kinases and consist of a catalytic domain that is preceded by a variable N-terminal region of 60 or 80 residues, an SH3 domain, and an SH2 domain (reviewed in Superti-Furga and Courtneidge, 1995; Van Etten, 1999). In contrast to Src family kinases, c-Abl and Arg lack the short C-terminal regulatory tail and instead have a large C-terminal portion encoded by a single exon and thus called the "last exon region." c-Abl has been implicated in processes of cell differentiation, cell division, cell adhesion, cell death, and stress response (Van Etten, 1999; Wang, 2000). It is found in both the nucleus and cytoplasm of cells and is thought to shuttle between the two subcellular departments, depending on environmental clues (Taagepera et al., 1998). When expressed transiently in tissue culture cells, the ability of c-Abl to become phosphorylated on tyrosine and phosphorylate cellular substrates is considerably weaker than that of the natural oncogenic form BCR-Abl or of c-Abl's viral counterpart, v-Abl.

In humans, chronic myelogenous leukemia (CML) and a subset of acute lymphocytic leukemia (ALL) are causally linked to the presence of the Philadelphia chromosome, which is the result of a translocation between chromosome 22 and chromosome 9. In this translocation, sequences of the first exon of the c-Abl tyrosine kinase gene (*ABL1*) are replaced by sequences from the *BCR* gene. Depending on the breakpoint in the *BCR* gene, the resulting fusion protein, BCR-Abl, can have molecular masses of 210, 190 (the two major forms), or 230 kDa. The consequences of BCR-Abl on signal transduction pathways and the cellular effects have been studied extensively (Raitano et al., 1997). Dependent on the cell type, BCR-Abl expression results in enhanced proliferation, morphological transformation, or abrogation of growth factor or adhesion dependence. In general, the effects are growth stimulatory and antiapoptotic. When forced into the nuclei of cells, however, BCR-Abl induces apoptosis (Vigneri and Wang, 2001).

The common feature critical for all the biological effects of BCR-Abl is its constitutively high level of tyrosine kinase activity derived from the catalytic domain in its *ABL1* moiety. The small molecular inhibitor of BCR-Abl catalytic activity, STI571, binds to the ATP binding pocket of the catalytic domain, but also interacts with less-conserved regulatory structural elements, affecting their function (Schindler et al., 2000). STI571 appears to target an Abl-specific inactive conformation of the catalytic domain, explaining its high selectivity over other tyrosine kinases. STI571 has proved to be a very promising therapeutic agent in clinical trials (Druker et al., 1996; Thiesing et al., 2000). However, point mutations arising in some patients appear able to confer resistance to the drug (Gorre et al., 2001; McCormick 2001). It appears particularly important to understand the mechanism responsible for natural inhibition of the cellular form of the enzyme, of which we remain ignorant.

Mutations have been identified in c-Abl, typically in the SH3 domain, which unleash the catalytic activity and often the oncogenic potential of the Abl protein. It is thought that these "deregulated" forms escape a critical mechanism responsible for tightly regulating the wild-type protein (Pendergast et al., 1991; Mayer and Baltimore, 1994; reviewed in Van Etten, 1999). Several lines of evidence contributed to the hypothesis that a cellular inhibitor is responsible for regulation of c-Abl. First, c-Abl and deregulated forms display equal levels of activity after precipitation or partial purification (Pendergast et al., 1991; Mayer and Baltimore, 1994; Dorey et al., 1999). Moreover, very high levels of expression in cells seem to exhaust regulation, as if through titration of a cellular inhibitor (Pendergast et al., 1991). Expression in a heterologous system, such as the yeast *Schizosaccharomyces pombe*, showed no difference in activity between c-Abl and deregulated forms, suggesting absence of a vertebrate c-Abl inhibitor in fungi (Walkenhorst et al., 1996). Other elements, such as the apparent difference to the regulatory mechanism of Src family kinases, the initially concurrent discovery of the protein-protein interaction potential of the SH3 domain, and the identification of a considerable number of proteins binding to the SH3 domain of Abl, all contributed to the wide acceptance of a cellular inhibitor theory of c-Abl regulation (reviewed in Van Etten, 1999; Brasher and Van Etten, 2000).

The crystal structure of regulated c-Src (reviewed in Sicheri and Kuriyan, 1997) prompted a mutational analysis that supported the possibility of c-Abl also being

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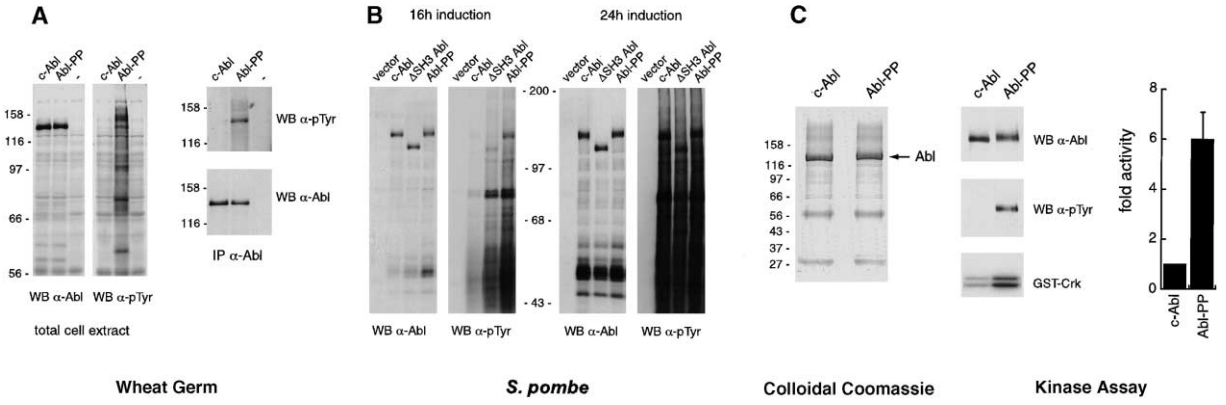


Figure 1. Autoinhibition of c-Abl

(A) c-Abl and Abl-PP were expressed by in vitro translation in wheat germ extract. Total protein extract (left) and anti-Abl immunoprecipitates (right) were separated by SDS-PAGE, blotted to nitrocellulose, and probed with anti-Abl and anti-phosphotyrosine antibodies.

(B) *S. pombe* strain SP200 was transformed with a vector expressing c-Abl, ΔSH3-Abl, or Abl-PP under control of a thiamine repressable promotor. Cells were lysed by boiling in SDS-sample buffer 16 (left) and 24 hr (right) after induction of protein expression by thiamine removal. Lysates were separated by SDS-PAGE, blotted to nitrocellulose, and probed with anti-Abl and anti-phosphotyrosine antibodies.

(C) HEK293 cells were transiently transfected with SV40-driven c-Abl and Abl-PP expression plasmids. After transfection (40 hr), cells were lysed, and Abl protein was immunopurified using covalently coupled anti-Abl antibodies. Abl and copurifying proteins were eluted by pH shock, separated by 4%–15% gradient SDS-PAGE, and visualized by colloidal Coomassie staining (left). The identity of Abl proteins was confirmed by mass spectrometry. Purified c-Abl and Abl-PP proteins were assayed for their catalytic activity by in vitro kinase assay using GST-Crk as exogenous substrate. Bands were excised, and incorporated radioactivity was measured by scintillation counting. The histograph shows the catalytic activity of purified Abl-PP compared to purified c-Abl (mean with SD of two experiments done in triplicate; right). Part of the Abl protein used for the in vitro kinase assay was blotted and probed with anti-Abl and anti-phosphotyrosine antibodies (middle). An autoradiograph showing an example of the incorporation of radioactivity in GST-Crk is presented.

regulated by intramolecular interactions. As in Src family kinases, these would involve binding of the SH3 domain to the linker between the SH2 and catalytic domains, and the catalytic domain itself (Barilá and Superti-Furga, 1998). However, these results did not define the minimal structural requirements for c-Abl regulation, nor did they address the in vitro regulation enigma, so that an exclusion of the existence of a cellular inhibitor responsible for the SH3 domain-dependent regulation was not possible. Moreover, the comparison to the regulation of Src family members lacked explanations for the absence of a regulatory C-terminal tail in c-Abl (Barilá and Superti-Furga, 1998; Brasher and Van Etten, 2000; Van Etten, 1999).

We present here the results of a reinvestigation of the mechanism responsible for c-Abl inhibition. Our data demonstrate that c-Abl is autoinhibited and does not require an SH3 domain-dependent cellular inhibitor. An N-terminal "cap" region binds intramolecularly and is required to achieve and maintain inhibition. The absence of the autoinhibitory "cap" in all BCR-Abl forms and the inhibitory effects upon its reintroduction suggest a new molecular basis for the oncogenic conversion of this long-studied and medically important gene.

Results

Expression of c-Abl in Nonvertebrate Systems

c-Abl and Abl-PP, a deregulated form in which two prolines in the putative intramolecular SH3 binding region connecting the SH2 domain to the catalytic domain are mutated (P242E/P249E), were expressed in wheat germ extract. Analysis of total cellular proteins as well as of immunoprecipitated c-Abl revealed that no tyrosine phosphorylation could be detected when c-Abl is expressed (Figure 1A). However, expression of Abl-PP resulted in the phosphorylation of c-Abl itself and of a number of endogenous proteins, showing that c-Abl is regulated in extracts of plant cells.

We have previously reported that c-Abl and an SH3 domain deletion form of Abl were equally active and toxic in the yeast *S. pombe* (Walkenhorst et al., 1996). We addressed again regulation of c-Abl in *S. pombe*, this time using an inducible promoter of much weaker activity than the one used previously. In this case, SH3 domain-dependent regulation was observed at early induction time points, as judged by a strong difference in tyrosine phosphorylation of cellular proteins between c-Abl- and Abl-PP-containing yeast cells (Figure 1B). After 24 hr, however, when more c-Abl protein had accumulated, this difference was abolished. Thus, c-Abl is regulated in *S. pombe*, but regulation is sensitive to the levels of protein expression.

Regulation of c-Abl In Vitro

We purified c-Abl and Abl-PP from transfected human embryonic kidney (HEK) 293 cells (Figure 1C). After a purification procedure that included a high-salt wash step, the Coomassie blue-stained patterns of c-Abl and Abl-PP proteins were very similar, indicating that any protein(s) that may be responsible for the regulation of c-Abl in vivo does not copurify in detectable amounts or is either too large or too small to be detected. Constitutively active Abl-PP is strongly phosphorylated on tyrosine and serine residues and thus migrates slightly more slowly than does c-Abl (Figure 1C, left; Dorey et al., 1999). To determine the activity of the purified Abl proteins, we tested their in vitro kinase activities using GST-Crk as a substrate. A more than 6-fold difference in activity between c-Abl and Abl-PP was measured

(Figure 1C, right). We also tested a mutant in which the two prolines in the SH2-CD linker are mutated to alanine (Abl-P242A/P249A) to find that it was activated to a similar extent as Abl-P242E/P249E (data not shown). To confirm that the difference in activity involved SH3-domain-dependent regulation, we tested various other mutants, including point mutations within the SH3 domain, v-Abl, and a mutant in which a putative intramolecular salt bridge between the SH3 and catalytic domain is disrupted (Abl K313E; Barilá and Superti-Furga, 1998). All deregulated forms were 7–16 times more active than wild-type c-Abl in in vitro kinase assays (data not shown). Similar results were also obtained with other anti-Abl antibodies and other substrates (data not shown). These results show that other cellular proteins are not necessary to maintain c-Abl in a state of catalytic inhibition.

Although it is unclear why it has been difficult to detect such differences previously (Pendergast et al., 1991; Mayer and Baltimore, 1994; Dorey et al., 1999) and several parameters may exert an influence on c-Abl regulation in vitro, we found that the nature of the divalent salt used in the kinase activity reaction has a profound effect on the catalytic activity. While the use of $MgCl_2$ at 10 mM concentration allows the detection of significant differences in catalytic activity of c-Abl versus Abl-PP, the same samples assayed in the presence of 10 mM $MnCl_2$ resulted in no detectable difference in catalytic activity (data not shown).

Minimal Region Required for c-Abl Regulation

The results on c-Abl regulation in vitro prompted an investigation of the minimal part of the protein required for regulation. The "last exon region" has previously been implicated in c-Abl regulation and represents the binding site for several cellular proteins, including F and G actin (Goga et al., 1993; McWhirter and Wang, 1993; Van Etten et al., 1994; Woodring et al., 2001). We constructed a deletion mutant lacking the last exon region of c-Abl (Abl M1-K531; Figure 2A). We also engineered a mutant lacking, additionally, the N-terminal amino acids preceding the SH3 domain, known to be dispensable for regulation of Src family kinases (Abl P82-K531; Figure 2A).

After transient expression in HEK293 cells, cellular proteins were analyzed for phosphotyrosine content as a measure of in vivo protein activity, and Abl proteins were immunoprecipitated to test for tyrosine phosphorylation as well as catalytic activity in vitro. Deletion of the last exon region (Abl M1-K531) did not lead to activation of Abl (Figures 2B and 2C), but the additional double proline mutation in the SH2 catalytic domain linker (Abl-PP M1-K531) resulted in strong activation. These results suggest that the SH3 domain-dependent regulation is operational within the short form of Abl. Deletion of the last exon region abolished the ability of Abl to phosphorylate GST-Crk but not GST-Jun, probably due to the loss of the Crk binding site (Ren et al., 1994).

Abl P82-K531 showed elevated tyrosine phosphorylation levels in total extracts and was highly active in vitro after immunoprecipitation when compared to its counterpart containing the normal N-terminal sequences (Abl M1-K531; Figure 2). This suggests an involvement of the first 81 residues of the c-Abl protein in regulation, which is in sharp contrast to Src family kinases, where residues N-terminal to the SH3 domain are dispensable for intramolecular regulation (Koegl et al., 1995; Sicheri and Kuriyan, 1997). Taken together, our results demonstrate that the minimal region necessary and sufficient for the regulation of c-Abl comprises the SH3, SH2, catalytic domain as well as the N-terminal residues.

Regulation of c-Abl by Its N Terminus

To obtain functional insight into the role of the N-terminal region in the regulation of full-length c-Abl, we constructed additional mutants and analyzed their catalytic activity in vitro and in vivo (Figure 3). As expected from the results with the short Src-like form (Abl P82-K531), Abl ΔM1-D81 was strongly active also in the presence of the last exon region and led to efficient phosphorylation of cellular proteins, Abl autophosphorylation, and high catalytic activity in an in vitro kinase assay (Figures 3B and 3C). Introduction of the deregulating mutation in the SH2 catalytic domain linker in Abl ΔM1-D81 (Abl-PP ΔM1-D81) had only a small further effect, suggesting that through deletion of the N-terminal 81 residues, Abl becomes fully deregulated. The effect of individually deleting the first 45 residues (exon 1b, Abl ΔM1-H45) or the following 36 residues (Abl ΔE46-D81) was less pronounced (Figures 3B and 3C). Thus, the N-terminal amino acids encoded by the Abl type 1b exon and the first part of exon 2 are both required to keep c-Abl in its regulated state.

We also tested if the absence of this regulatory N-terminal region activated the oncogenic potential of c-Abl. In focus-formation assays using NIH3T3-P cells, Abl ΔE46-D81 was a reliable oncogene, reaching a transformation efficiency comparable to point mutations in the SH3 domain (Table 1; Barilá and Superti-Furga, 1998).

The N-Terminal Region Interacts with c-Abl In *trans*

We tested the ability of the N terminus of Abl to interact with the rest of c-Abl. GST fusion proteins containing the first exon (amino acids 1–45; GST 1b), first and part of the second exon (amino acids 1–80; GST 1b + 2), and only second exon (amino acids 46–80; GST 2) of c-Abl type 1b were prepared and used to pull down transiently expressed c-Abl, Abl ΔM1-D81, and Abl-PP ΔM1-D81 proteins in extracts derived from transfected HEK293 cells (Figure 4A). Since in the *ABL1* gene two alternative first exons are spliced to give rise to c-Abl type 1a and 1b (Shtivelman et al., 1986), we also tested similar constructs of c-Abl type 1a (amino acids 1–26; GST 1a and 1–61; GST 1a + 2). All five Abl GST-fusion proteins were able to pull down Abl ΔM1-D81, suggesting a direct interaction between the Abl N-terminal amino acids and Abl ΔM1-D81 in *trans*. In contrast, c-Abl was not bound by the GST-fusion proteins, suggesting that the binding site in c-Abl is "covered" by the N terminus in *cis*. Abl-PP ΔM1-D81 bound the GST-fusion proteins only weakly. Thus, the N-terminal region appears to bind the rest of Abl in a conformation-dependent manner.

To define the region in c-Abl to which the N-terminal region binds, GST fusion proteins of the first exon type 1a and type 1b and of the second exon were tested for their

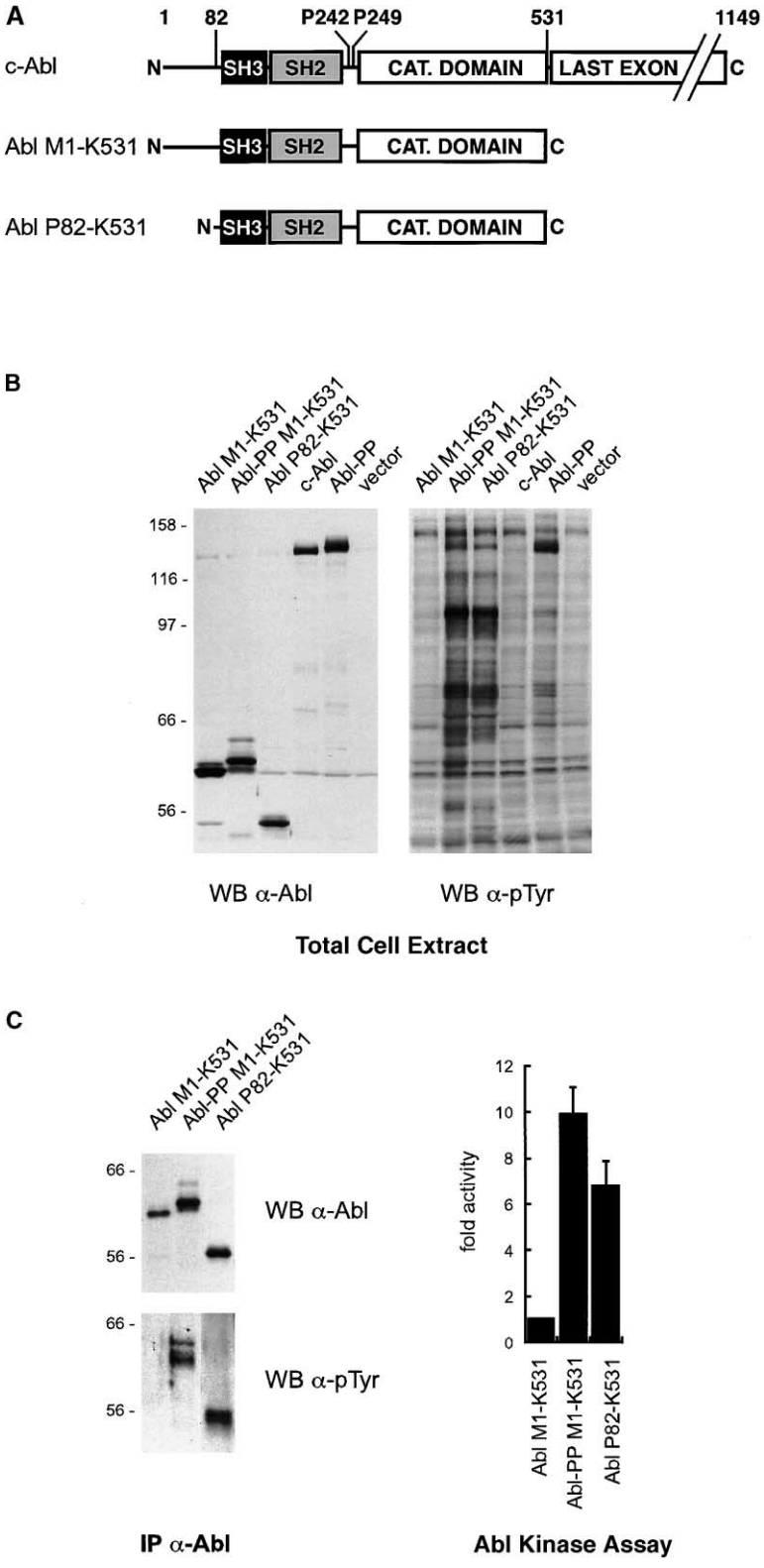


Figure 2. The core Element for c-Abl Regulation

(A) Schematic diagram representing the different generated Abl proteins. The SH3, SH2, and catalytic domains as well as the last exon region are shown as boxes. P242 and P249 indicate the two proline residues in the SH2 catalytic domain linker that are mutated in Abl-PP and derivatives.

(B) HEK293 cells were transiently transfected with the indicated SV40-driven Abl expression constructs. After transfection (40 hr), cells were lysed, and total protein extract was analyzed by anti-Abl and anti-phosphotyrosine immunoblotting.

(C) Abl protein was immunoprecipitated from total cell extract using anti-Abl antibodies, blotted to nitrocellulose, and probed with anti-Abl and anti-phosphotyrosine antibodies (left). Abl immunoprecipitates were assayed for catalytic activity by in vitro kinase assay using GST-Jun as substrate. The histograph shows the catalytic activity (mean with SD of three experiments done in duplicate) of the Abl constructs relative to Abl M1-K531.

ability to pull down transiently expressed c-Abl proteins (Figure 4B), including the SH3-SH2-linker catalytic domain portion (SH3-SH2-CD), the SH3-SH2 domains, or the catalytic domain only (CD). The SH3-SH2-CD protein was bound by all three GST fusion proteins. The catalytic domain was bound by first exons 1a and 1b only, while the second exon bound preferentially to the portion of Abl containing the SH3 and SH2 domains. Thus, different parts of the N-terminal region appear to bind different parts of c-Abl, as if clamping the protein together. Because of its position at the N terminus of c-Abl, we refer to this region as the "cap."

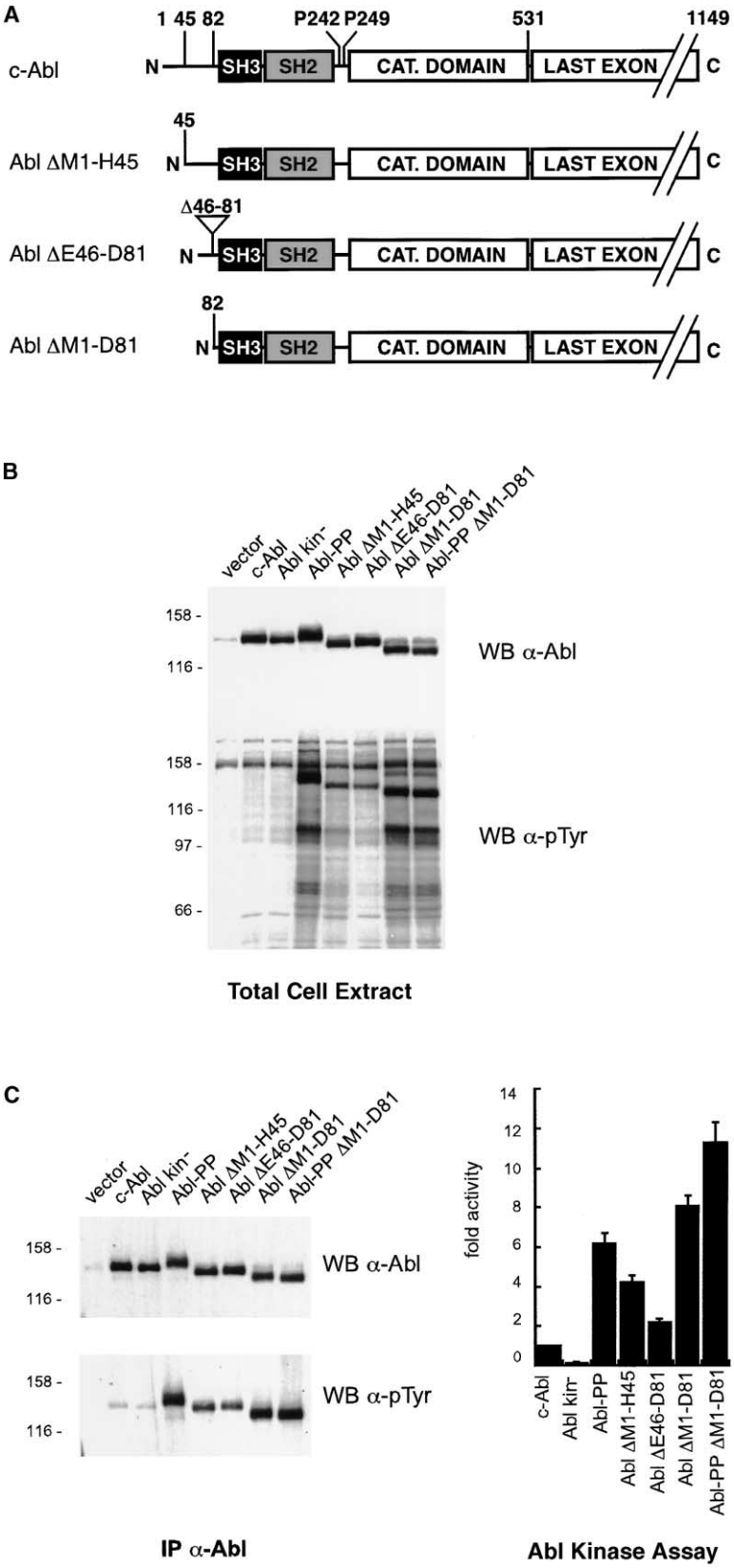


Figure 3. Autoinhibition of c-Abl by Its N Terminus

(A) Schematic diagram representing the different generated Abl proteins. The SH3, SH2, and catalytic domains as well as the last exon region are shown as boxes. P242 and P249 indicate the two proline residues in the SH2 catalytic domain linker that are mutated in Abl-PP and derivatives.

(B) HEK293 cells were transiently transfected with c-Abl or the indicated Abl expression constructs. After transfection (40 hr), cells were lysed, and the resulting total protein extract was analyzed by anti-Abl and anti-phosphotyrosine immunoblotting.

(C) Abl protein was immunoprecipitated from total cell extract using anti-Abl antibodies, blotted to nitrocellulose, and probed with anti-Abl and anti-phosphotyrosine antibodies (left). Abl immunoprecipitates were assayed for catalytic activity by in vitro kinase assay using GST-Crk as substrate. The histograph shows the catalytic activity (mean with SD of three experiments done in duplicate) of the indicated Abl constructs relative to c-Abl.

Mutational Analysis of the N-Terminal Region

To understand better the nature of the interaction between the cap and the rest of Abl, we performed alanine-scanning mutagenesis of groups of four, mostly polar, residues, in the common second exon region. We also mutated residues that appeared conserved between the 1a and 1b alternative exons (Figure 5A, cap 1–6). After transient expression in HEK293 cells, Abl proteins were

Table 1. Transformation Potential of Abl ΔE46-D81

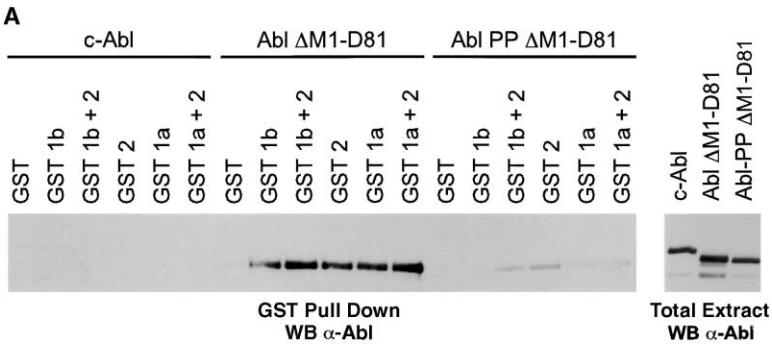
	Neo Colonies (Mean + SD)	Foci in 5% CS (Mean + SD)	Transformation Efficiency (%)
pSLX c-Abl	5595 ± 505	13 ± 3	0.2
pSLX Abl ΔE46-D81	2170 ± 170	215 ± 35	9.9
pSLX Abl-PP	2135 ± 185	615 ± 125	28.8

NIH3T3-P cells transfected with the indicated Abl constructs in vector pSLX were grown for 16 days in DMEM containing 5% calf serum to allow the formation of foci. Duplicate dishes were grown under neomycin selection to determine the transfection efficiency. The transformation efficiency was calculated by dividing the number of foci by the number of neomycin resistant colonies of each construct. Results shown are the averages of two experiments done in duplicate.

immunoprecipitated to test for tyrosine phosphorylation content as well as for catalytic activity in vitro. The cap 1 and cap 6 mutants were phosphorylated and exhibited an increased catalytic activity (Figure 5B). To test the ability of the cap 1 and cap 6 mutants to stimulate cellular signal transduction and elicit transcriptional responses in the nucleus, we cotransfected various Abl-expressing plasmids with different growth-sensitive promoters driving the luciferase gene (Alexandropoulos and Baltimore, 1996; Raitano et al., 1995; Renshaw et al., 1996; Barilá and Superti-Furga, 1998). To standardize the experiment for transfectional efficiency and extract preparation, we used the ubiquitin promoter driving the *Renilla* luciferase. Expression of c-Abl missing the cap region led to a significant stimulation of both a serum-responsive elements (SREs)-containing promoter and a TPA-responsive elements (TREs)-containing promoter, although to an extent that was slightly lower than Abl-PP (Figure 5C). While the cap 1 and the cap 6 mutants seemed to stimulate the two promoters with different efficiencies, they were both clearly more active than the regulated wild-type c-Abl control. Mutation of the cap thus elicits the ability of Abl to stimulate signal transduction pathways, consistent with the ability to transform fibroblasts of the deletion mutant (Table 1). To establish a possible correlation between the role of cap 1 and cap 6 residues in regulation and their binding properties, GST fusion proteins were prepared of Abl exon 1b with the cap 1 mutation (GST 1b cap 1) and exon 2 with the cap 6 mutation (GST 2 cap 6) and used to pull down transiently expressed SH3-SH2-CD and CD Abl proteins from HEK293 cell extracts (Figure 5D). Mutation of both cap 1 and cap 6 residues prevented binding to the SH3-SH2-CD protein, and the relatively strong binding of exon 1b to the catalytic domain was strongly reduced by mutation of cap 1 residues. These results show that both conserved residues of exon 1 and residues of exon 2 are required for the binding to Abl and its regulation.

The N-Terminal Region Is Required to Maintain the Regulated State

The N-terminal region may only be required to assemble the regulatory apparatus, possibly during folding, and then becomes dispensable. Alternatively, the N-terminal region may target c-Abl to particular subcellular sites and only indirectly affect c-Abl regulation. To rule



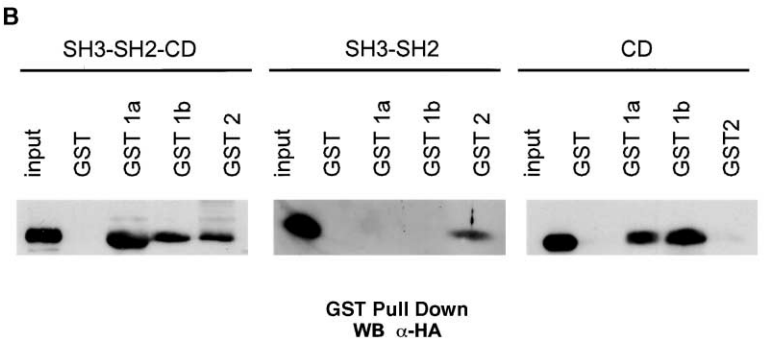


Figure 4. In Vitro Binding of the N-Terminal Region to Abl Protein

(A) c-Abl, Abl ΔM1-D81, or Abl-PP ΔM1-D81 were expressed in HEK293 cells. Cell lysates were incubated with the indicated GST fusion proteins bound to glutathion-Sepharose beads. Adsorbates were analyzed by SDS-PAGE followed by anti-Abl immunoblotting (left). Inputs are shown for Abl (right); identical amounts of GST fusion proteins were used for each pull-down as quantified by Coomassie blue staining (data not shown).

(B) HA-tagged pieces of Abl were expressed in HEK293 cells. Cell lysates were incubated with the indicated GST fusion proteins, as described in (A). Adsorbates were analyzed by SDS-PAGE, followed by anti-HA immunoblotting.

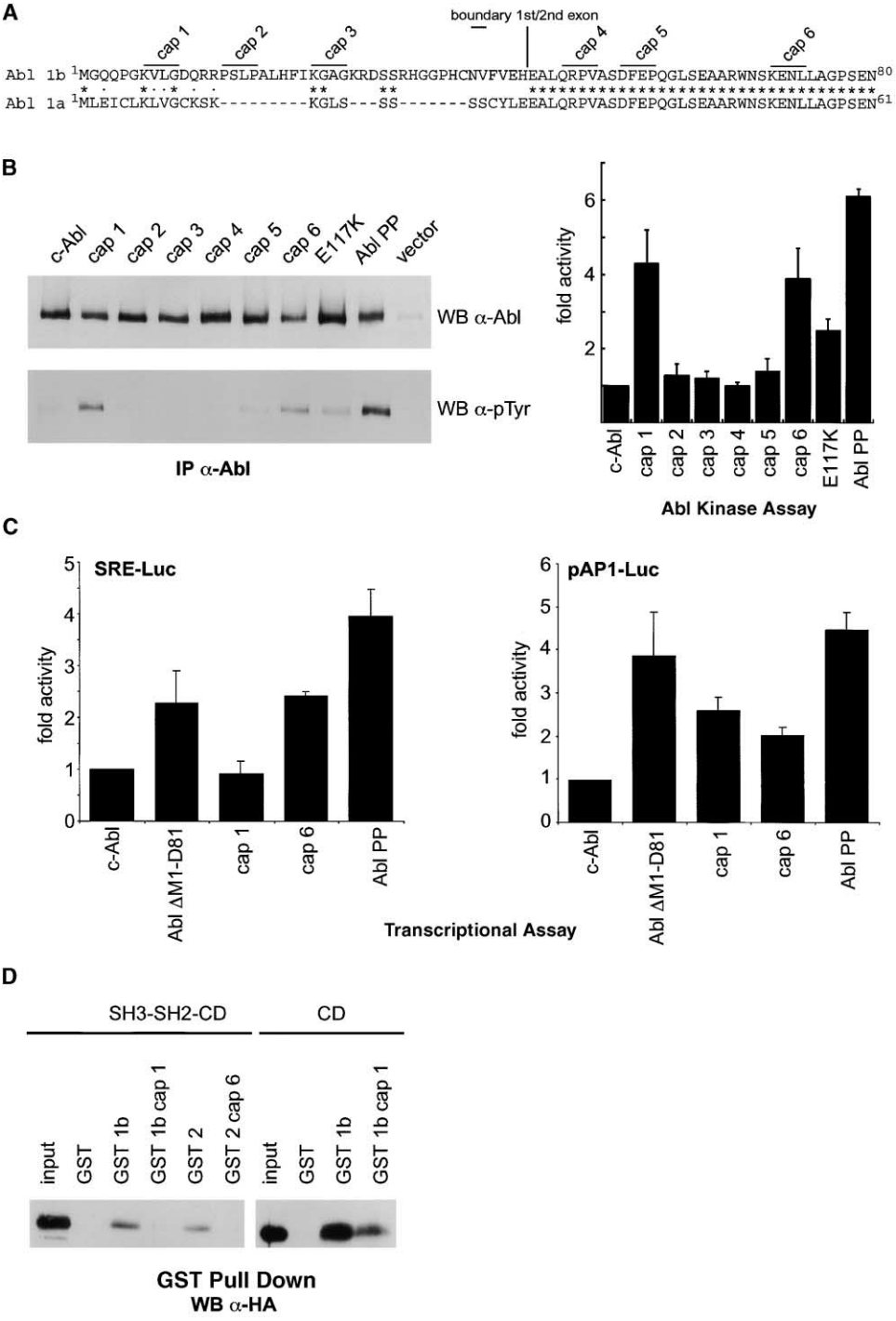


Figure 5. Mutagenesis Analysis of the N-Terminal Cap

(A) Alignment of the c-Abl type 1a and 1b N-terminal regions. Indicated are the residues (groups of four residues in a row) that are mutated to alanine for the different cap mutants (cap 1–6).

(B) Abl cap mutant proteins and the indicated Abl constructs were transiently expressed in HEK293 cells. Anti-Abl immunoprecipitates were analyzed by anti-Abl and anti-phosphotyrosine immunoblotting (left) and assayed for catalytic activity by in vitro kinase assay using GST-Crk as substrate (right). The histograph shows the fold of activation of the different constructs compared to c-Abl (mean with SD of two experiments done in duplicate).

(C) Transcriptional assay: HEK293 cells were transfected as indicated together with a luciferase reporter plasmid under the control of a promoter containing serum-responsive elements (Alexandropoulos and Baltimore, 1996) or TPA-responsive elements pAP1 (Promega). After transfection (24 hr), extracts were assayed for luciferase activity after standardization. The histogram shows the mean of two independent experiments done in duplicate, with standard deviations.

(D) HA-tagged Abl protein pieces (SH3-SH2-CD and CD) were expressed in HEK293 cells. Cell lysates were incubated with the indicated GST fusion proteins bound to glutathion-Sepharose beads. Adsorbates were analyzed by SDS-PAGE, followed by anti-HA immunoblotting.

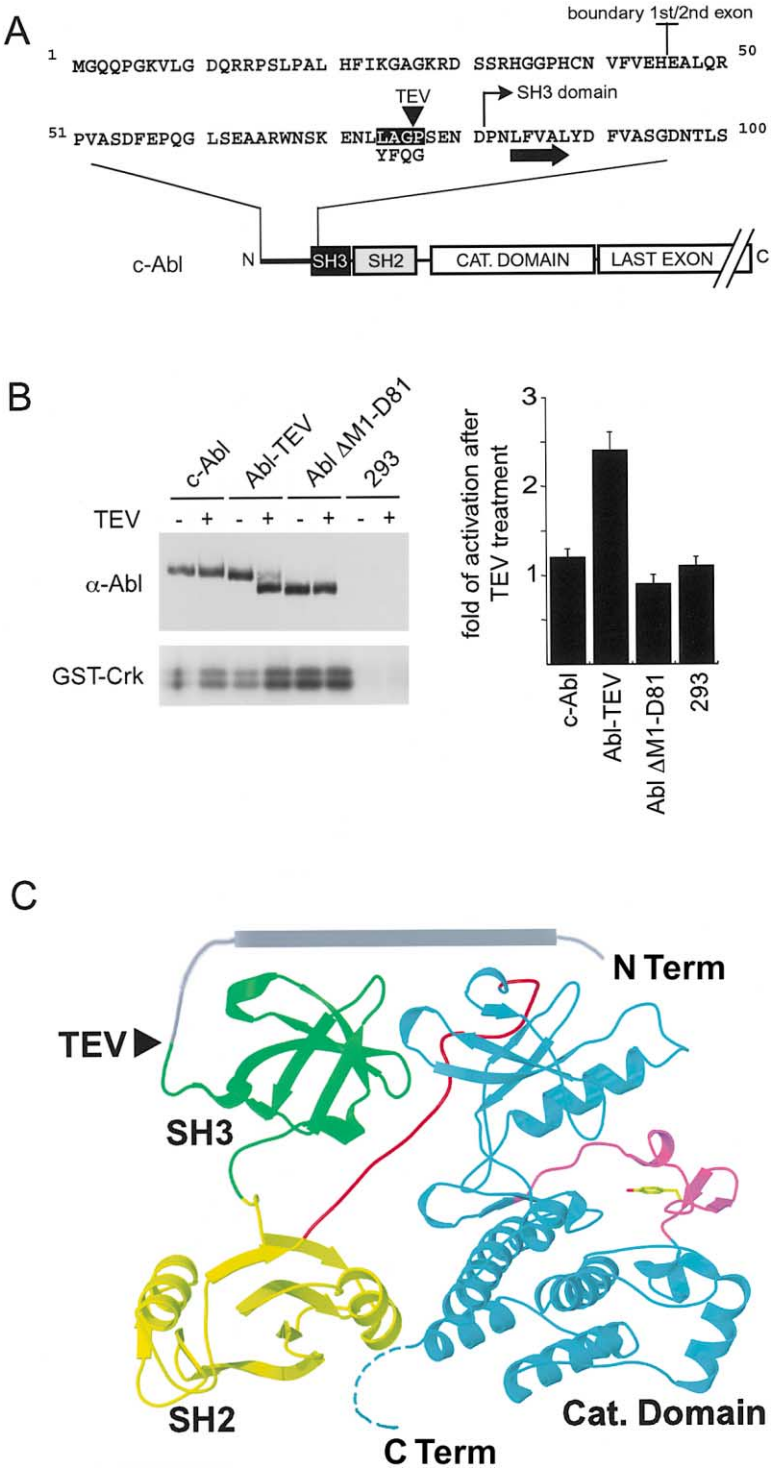


Figure 6. Role of the N-Terminal Cap of c-Abl

(A) Schematic diagram showing the sequence of the N terminus of c-Abl. The SH3, SH2, and catalytic domains as well as the last exon region are represented by boxes. The amino acid sequence of the N terminus of the c-Abl type 1b protein is shown. The bold arrow indicates the beginning of the βa strand of the SH3 domain, the engineered TEV cleavage site is marked in black, and the arrowhead indicates the exact position of cleavage by TEV protease.

(B) Abl-TEV and indicated constructs were expressed in HEK293 cells and immunoprecipitated with anti-Abl antibodies. Immunecomplexes were incubated without (−) or with (+) TEV protease, separated by SDS-PAGE, blotted to nitrocellulose, and probed with anti-Abl antibodies (top). Immunecomplexes were assayed for catalytic activity by in vitro kinase assay using GST-Crk as substrate (bottom). The histograph shows the fold of activation induced by TEV protease (mean with SD of three independent experiments). This was calculated by dividing the catalytic activity of Abl protein after TEV treatment by the catalytic activity without TEV incubation.

(C) Model of the three-dimensional arrangement of c-Abl including the N-terminal regulatory cap based on known structures of the SH3 domain, the SH2 domain, and the catalytic domain of Abl and on regulated Src. The N-terminal cap is represented as a rod structure that may bind to the north face of Abl, thus stabilizing the regulated structure in which the SH3 domain is bound via the SH2 catalytic domain linker to the catalytic domain. The arrowhead indicates the position of the engineered TEV cleavage site.

out these possibilities, we took advantage of the presence of residues resembling the cleavage site for the highly specific tobacco etch virus (TEV) protease roughly at the boundary between the N terminus and the SH3 domain. We engineered a perfect TEV site by mutating four residues to obtain Abl-TEV. The TEV cleavage would occur 7 residues upstream of Phe85, representing the beginning of the βA strand of the SH3 domain (Figure 6A; Musacchio et al., 1994). Mutation of the four residues required to engineer the TEV site did not affect c-Abl regulation (Figure 6B). We transfected HEK293 cells with Abl-TEV as well as with control Abl constructs. Abl proteins were immunoprecipitated and treated or not with TEV protease. TEV treatment caused the appearance of a faster-migrating form of Abl-TEV but not of c-Abl (Figure 6B). Kinase assays performed in parallel revealed a TEV-dependent increase in catalytic activity only in Abl-TEV and not in c-Abl or Abl ΔM1-D81 (Figure

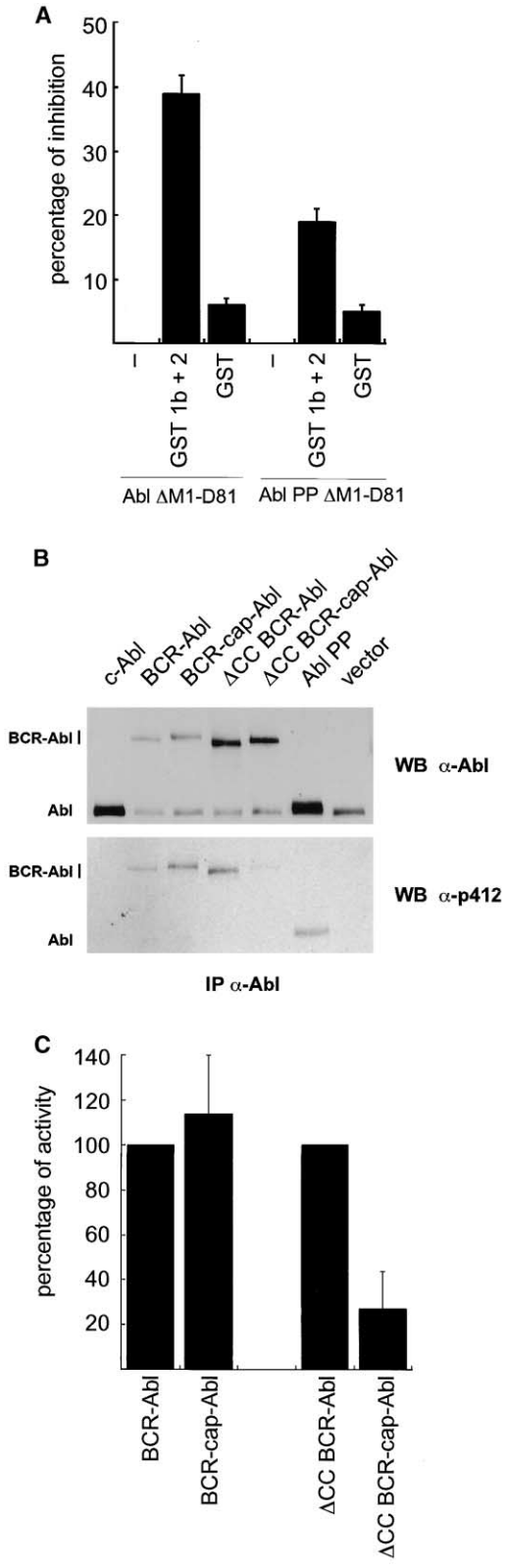


Figure 7. Inhibition of Catalytic Activity by the N-Terminal Cap

(A) Abl ΔM1-D81 and Abl-PP ΔM1-D81 were transiently expressed in HEK293 cells and immunoprecipitated using anti-Abl antibodies. Immunecomplexes were incubated for 2 hr with kinase assay buffer (–) or with equal amounts of GST fusion protein (GST or GST Abl 1b + 2), dialyzed against kinase assay buffer, and subsequently assayed for catalytic activity by in vitro kinase assay using GST-Crk as substrate. The histograph shows the percentage of inhibition (mean with SD of three experiments done in duplicate).

(B) HEK293 cells were transfected with the indicated constructs. After an immunoprecipitation using anti-Abl antibodies, the immune-complexes were analyzed by Western blot anti-Abl (top) or anti-p412 (bottom).

(C) Effect of the cap on the catalytic activity of BCR-Abl or ΔCC BCR-Abl tested by in vitro kinase assay using GST-Crk as a substrate. The activity of BCR-Abl and ΔCC BCR-Abl is set to 100%; the histograph shows the variation of catalytic activity when the cap is restored in BCR-Abl or in ΔCC BCR-Abl (mean of two independent experiments done in duplicate with SD).

6B). The extensive washes of the immunoprecipitated protein likely removed the cleaved N-terminal portion. Thus, cleaving the N-terminal 77 residues of c-Abl in vitro leads to its activation. We conclude that the N-terminal residues are necessary to maintain the inhibited state of c-Abl and that they have a critical role in its regulation.

The N-Terminal Region Inhibits Abl In Vitro and Restores Regulation of BCR-Abl

To test whether the interactions of the cap are sufficient to restore Abl regulation in vitro, we purified a GST fusion protein of the N-terminal region of type 1b Abl (GST 1b + 2) and incubated it with immunoprecipitated Abl ΔM1-D81 and with its counterpart bearing the additional PP mutation in the linker (Figure 7A). While the GST control protein had no effect on catalytic activity, GST 1b + 2 inhibited Abl ΔM1-D81 activity roughly 40%. A significant but reduced level of inhibition was achieved also with Abl-PP ΔM1-D81. This result could indicate a potential direct effect on Abl's catalytic activity, rather than on mere "regulation," and could also reflect the residual binding of GST 1b + 2 to Abl-PP ΔM1-D81 observed previously (Figure 4).

BCR-Abl fusion proteins invariably miss the first exon sequences. If Abl's cap region is so powerful that it can reregulate even active forms of Abl (such as Abl ΔM1-D81), the missing first exon may restore regulation of BCR-Abl if reintroduced in the molecule between the BCR sequences and the beginning of the second exon (BCR-cap-Abl; Figure 7B). In parallel to such constructs, we also tested versions in which the coiled-coil region of BCR-Abl, critically involved in dimerization, is missing (ΔCC BCR-cap-Abl; McWhirter et al., 1993). Expression in HEK293 cells was equally efficient for all BCR-Abl forms (data not shown). Immunoprecipitation was less efficient for BCR-Abl proteins than for the forms bearing the deletion of the coiled-coil region (Figure 7B, top). To monitor BCR-Abl's activity in the cell, we chose to use antibodies specific for tyrosine 412 in the activation loop, likely to reflect the state of activity better than anti-phosphotyrosine, since BCR-Abl is tyrosine phosphorylated also at other sites that may not be directly dependent on catalytic activity (Pendergast et al., 1993a, 1993b). As expected, if compared to the levels of immunoprecipitated protein, the forms bearing the coiled-coil deletion were less phosphorylated at Tyr412, reflecting the importance of dimerization for BCR-Abl activity (Fig-

ure 7B; McWhirter et al., 1993; Smith and Van Etten, 2001). Introduction of the first exon sequences did not have a measurable effect on Tyr412 phosphorylation of BCR-Abl but caused a dramatic reduction in the activity of BCR-Abl bearing the coiled-coil deletion. To test whether this decrease in activation loop phosphorylation also reflected a reduced catalytic activity in vitro, we performed kinase assays with the different immunoprecipitated BCR-Abl proteins. The cap-bearing version of ΔCC BCR-Abl showed about 25% of the activity of its normal counterpart (Figure 7C). Together, these data show that the first exon region is capable of restoring regulation to a dimerization-deficient BCR-Abl, confirm the dominant features of dimerization in overriding the natural regulation of Abl, and suggest that loss of the first exon may contribute to deregulation of BCR-Abl.

Discussion

The data presented here demonstrate regulation of purified c-Abl in vitro as well as regulation in nonanimal expression systems. Regulation of c-Abl activity is thus an intrinsic property and does not require a particular cellular inhibitor. An intramolecular sandwich involving the SH3 domain, the linker between the SH2 and catalytic domain, and the catalytic domain itself has been proposed to regulate the activity of c-Abl as it does in Src family kinases (Barilá and Superti-Furga, 1998). In Src family kinases, however, the assistance of the C-terminal tail region is essential. What structure in c-Abl substitutes for the C-terminal tail of Src? The last exon region of c-Abl bears nuclear import and nuclear export signals on top of binding sites for a variety of cellular proteins including actin, Crk, Nck, and p53. There is genetic evidence that it plays a role in the regulation of c-Abl in cells (Goga et al., 1993; Woodring et al., 2001). We show, however, that the last exon region of c-Abl is totally dispensable for the SH3 domain-dependent regulation of catalytic activity as defined here and measured in vitro.

If the last exon region is dispensable, what is minimally required? In addition to the SH3, SH2, and catalytic domains, the Src-like "core," we found an unexpected critical role for the first 81 residues of the c-Abl protein. In this respect, c-Abl differs to Src family kinases, where residues N-terminal to the SH3 domain are dispensable for intramolecular regulation (Koegl et al., 1995; Sicheri and Kuriyan, 1997). An immediate suggestion concerning a regulatory role of the N terminus comes from the fact that in the *ABL1* gene, two alternative first exons are spliced to give rise to c-Abl type 1a or 1b (Shtivelman et al., 1986). c-Abl 1a is 19 amino acids shorter, is spliced much less frequently and does not include a myristoylation signal. There is no evidence in the literature that would indicate different levels of activity between the two forms. Transgenes encoding both type I and type IV c-Abl proteins rescue the lethality of *c-abl* mutant mice (Hardin et al., 1996). Our attempts to express Abl bearing the type 1a exon in cells failed, consistent with the report of others (Van Etten, 1999), but when translated in reticulocyte lysates, type 1a Abl is regulated as well as type 1b (K.D., unpublished data). Early reports had addressed the role of the N terminus in the regulation of c-Abl (Franz et al., 1989; Jackson and Baltimore, 1989; Wang, 1988), but the results were not sufficiently conclusive, and attention focused on the discovery and function of the adjacent SH3 domain, obscuring the role of the extreme N terminus of c-Abl.

We suggest a novel model according to which c-Abl is regulated by a set of intramolecular interactions (Figure 6C). While Src family kinases have an interaction of their tail with their own SH2 domain that contributes critically to maintenance of the SH3 domain-dependent regulation, c-Abl has an N-terminal cap that serves an analogous function. This cap appears to bind at several regions "across" the molecule and stabilize the regulated, inhibited conformation. According to our data, the "KV/LV/LG" motif (see cap 1 region in Figure 5A), which is common to both type 1a and 1b exons and required for binding, must undergo relatively strong interactions with the catalytic domain. The first part of the common second exon does not interact with the catalytic domain but interacts with the SH3 and/or SH2 domains. Figure 6C shows the cap as if binding to the "north face" merely for graphic convenience. In fact, because the two regions known to be critical for binding (the cap 1 and cap 6 residues) are spaced differently in the 1a and 1b forms of Abl, some of the 19 additional residues of the type 1b cap may "loop out" from whatever is the minimal "bridge" from the SH3 to the catalytic domain.

The experiment with the engineered form of c-Abl in which the presence of the N-terminal region is removed in vitro, has shown that the N terminus is required to maintain and not merely to assemble the regulated conformation. This is also confirmed by the ability of cap sequences to inhibit cap-less Abl in vitro. This mechanism may be exploited by cellular proteins that inhibit c-Abl in *trans*.

In general, cellular proteins may either inhibit or activate c-Abl by favoring or displacing any of the several critical intramolecular interactions. For example, numerous proteins bind the Abl SH3 domain (reviewed in Van Etten, 1999). Moreover, an interdependence of the intramolecular interactions and catalytic activity, as in Src family kinases, seems highly probable (Gonfloni et al., 2000). In this view, phosphorylation of the activation loop with its conformational effects on the catalytic domain and the SH3 and cap-mediated inhibitory intramolecular interactions antagonize each other. The degree of catalytic activity and the degree of SH3 domain availability are the net result of these opposing forces. In fact, the PTP-PEST tyrosine phosphatase, dephosphorylating the activation loop, acts as an inhibitor of c-Abl (Cong et al., 2000), while Src family kinases and Abl itself act as activators by causing phosphorylation (Plattner et al., 1999; Brasher and Van Etten, 2000; Dorey et al., 2001). We have recently found that c-Abl is phosphorylated at Ser94 in the RT loop of the SH3 domain when cotransfected with an active form of Src (K.D., unpublished data). While the effects of this phosphorylation event and the kinase responsible are not yet known, it is possible that this or other modifications can modulate binding of the cap region.

We believe that the cap represents the missing link in c-Abl's intramolecular regulation. The first exon region is lacking in all of the different fusion proteins formed with BCR or TEL resulting from chromosomal transloca-

tions and also in v-Abl. The dimerization properties of BCR and TEL are thought to induce crossphosphorylation and activation of the catalytic domains by induced proximity and thus represent critical "gain-of-function" alterations of c-Abl (Golub et al., 1996; McWhirter et al., 1993; Smith and Van Etten, 2001). Moreover, signaling properties in the BCR portion of BCR-Abl are known to be critical for transformation (reviewed in Sawyers, 1992). Our data suggest that the absence of the N-terminal cap in BCR-Abl (and in TEL-Abl and v-Abl) represents a "loss-of-function" alteration that contributes to the acquisition of constitutive tyrosine kinase activity in these oncogenic forms. Thus, the cap of c-Abl may represent what the C-terminal tail represents for c-Src. A crystal structure of c-Abl including the cap will be essential to elucidate the precise molecular mechanism of regulation, and future work will address how the cap may modulate c-Abl differentially in the different splice variants and in the context of cellular signaling networks.

Experimental Procedures

DNA Constructs

pSGT vector and pSGT-Abl constructs were previously described (Barilá and Superti-Furga, 1998). Abl M1-K531, Abl-PP M1-K531, and Abl P82-K531 were obtained by PCR with c-Abl type 1b as template and subcloned in pSGT vector. For preparation of Abl ΔM1-H45, Abl ΔE46-D81, Abl ΔM1-D81, and Abl-PP ΔM1-D81, pSGT-c-Abl or pSGT-Abl-PP were digested with EcoRI and KpnI, and the released N-terminal fragment was replaced by a PCR product containing the desired deletion. Point mutations and cap mutants (cap 1–6) were obtained using the quick-change site-directed mutagenesis kit (Stratagene) and pSGT-c-Abl type 1b DNA as template. All mutagenesis constructs were confirmed by sequencing. The SH3-SH2-CD (N80-K531), SH3-SH2 (N80-P235), or CD (D252-K531) Abl protein parts were amplified using hAbl as a template and subcloned into a CMV driven vector containing a HA tag (described in Barilá et al., 2000).

The p210 BCR-Abl is a kind gift from Owen Witte. pSGT BCR-Abl and pSGT ΔCC BCR-Abl have been reconstituted by amplifying, respectively, M1-F1059 and L61-F1059. The resulting BCR-Abl fragments were digested EcoRI/Kpn I (a unique internal site in Abl) and subcloned into pSGT hAbl backbone. To generate pSGT BCR-cap-Abl and pSGT ΔCC BCR-cap-Abl, BCR (M1-S927) or ΔCC BCR (L61-S927) have been amplified using p210 as a template. The PCR products were digested EcoRI/NotI and subcloned into pSGT hAbl. All PCR products were sequenced.

Expression of Abl in Wheat Germ Extract and *S. pombe*

c-Abl and Abl-PP RNA were prepared as described (Dorey et al., 1999) and used for expression of protein in wheat germ extract using a commercial translation system (Promega). pSGT-c-Abl, ΔSH3-Abl, and Abl-PP (Barilá and Superti-Furga, 1998) were subcloned to the yeast pRWP vector, a derivative of pRSP (Superti-Furga et al., 1993) containing a mutation of the nmt1 promoter, making it approximately ten times weaker (Basi et al., 1993), and transformed to *S. pombe* strain SP200 (Superti-Furga et al., 1993). Expression of Abl protein was induced by removal of thiamine as described (Walkenhorst et al., 1996), and yeast cellular protein extracts were made by boiling pelleted yeast cells in SDS sample buffer 16 and 24 hr after induction.

Transfection and Immunoprecipitation

HEK293 cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum. Cells were transfected with pSGT-Abl DNAs using the calcium phosphate method. After transfection (40 hr), cells were lysed in IP buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 5 mM EDTA, 5 mM EGTA, 25 mM NaF, 1 mM orthovanadate, 1 mM PMSF, 10 μg/ml TPCK, 5 μg/ml TLCK, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor), and insoluble material was removed by centrifugation (10 min at 13,000 rpm). Abl protein was immunoprecipitated from total cell lysates in IP buffer using anti-Abl antibody (Ab-3, Oncogene Science or K12, Santa Cruz). Immune complexes were recovered using protein G-Sepharose beads, assayed for Abl catalytic activity, and analyzed by SDS-PAGE followed by anti-Abl (Ab-3 or K12) and anti-phosphotyrosine (4G10, Upstate Biotechnology) immunoblotting. Anti-p412 antibodies that specifically recognize Abl when phosphorylated in the activation loop were a kind gift from Dr J. Wu (Cell Signaling Technology [CST], Beverly, MA).

Large-Scale Purification of Abl Protein

c-Abl and Abl-PP protein were expressed by transient transfection in HEK293 cells. After transfection (40 hr), cells were lysed in IP buffer and insoluble material was removed by centrifugation at 100,000 × g for 1 hr. Cell lysates were incubated with anti-Abl antibodies (Ab-3, Oncogene Science) covalently coupled to protein G-Sepharose beads. Beads were washed with IP buffer followed by phosphate buffer (50 mM NaPhosphate [pH 6.3], 0.1% Triton-X100, 500 mM NaCl). Bound proteins were used for Abl kinase assay directly or eluted by pH shock (50 mM glycine [pH 2.5], 0.1% Triton-X100, 150 mM NaCl) and run on a 4%–15% gradient SDS-PAGE gel (Biorad). Abl and copurifying proteins were detected by brilliant blue colloidal Coomassie staining (Sigma).

Abl Kinase Assay

The catalytic activity of purified Abl protein bound to protein G-Sepharose beads was determined as follows. Beads were washed three times with IP buffer, two times with IP buffer without NaCl, and two times with kinase assay buffer (20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 1 mM DTT). Twenty microliters kinase assay mix (1 μg GST-Crk121-226 [Dorey et al., 1999], 0.5 μl [γ-^{32}P]ATP [Amersham], 0.1 mM ATP in kinase assay buffer) was added, and the mixture was incubated at room temperature for 10 and 20 min. The kinase reaction was stopped by adding SDS sample buffer and was analyzed by SDS-PAGE. Quantification of the reaction was done by cutting the desired bands from gel followed by liquid scintillation counting and/or phosphoimager analysis. For the in vitro inhibition of Abl, GST or GST 1b + 2 protein were bacterially expressed, purified, and dialysed against kinase assay buffer. Equal quantities of purified proteins were added directly to the kinase assay mixture, samples were incubated for 2 hr at 4°C, and then assayed as above.

Focus Formation Assay

Abl DNAs were subcloned from pSGT to pSLX vector (Renshaw et al., 1995) by BamHI digestion. Focus formation assay was performed in NIH3T3-P cells (Renshaw et al., 1992) using 0.5 μg of pSLX-Abl DNA essentially as described (Barilá and Superti-Furga, 1998; Renshaw et al., 1995). Percentage of foci was calculated by dividing the number of foci by the number of neomycin-resistant colonies of each construct.

Transcription Assay

HEK293 cells 70% confluent in 6 cm dishes were transfected with 0.01 μg of Ubi-Renilla vector for normalization of the luciferase assay (a kind gift of Carsten Weiss), 2 μg of SRE-luciferase plasmid (Alexandropoulos and Baltimore, 1996) or pAP1-luciferase (Promega), and the different constructs as indicated. After 24 hr, cells were lysed in 300 μl of passive lysis buffer (Promega). Five microliters of extract was used for the luciferase assay, performed under standard conditions.

In Vitro Binding Assay

DNA fragments corresponding to c-Abl amino acids, M1-H45 (1st exon type 1b), E46-N80 (beginning of the 2nd exon until the start of the SH3 domain), and M1-N80 (1st exon type 1b + 2nd exon) were amplified by PCR using c-Abl type 1b as template. M1-E26 (1st exon type 1a) and M1-N61 (1st exon type 1a + 2nd exon) were generated using c-Abl type 1a as template. All PCR products were cloned in pGEX-2T vector. Bacterially produced Abl GST fusion proteins were prebound to glutathion-Sepharose beads and incubated with 2 mg of HEK293 cell lysate containing c-Abl, Abl ΔM1-D81, Abl-PP ΔM1-D81, or HA-tagged Abl protein parts (SH3-SH2-CD, SH3-SH2, or CD) for 3 hr at 4°C. Bound proteins were analyzed

by SDS-PAGE, followed by anti-Abl or anti-HA (12CA5, Boehringer Mannheim) immunoblotting.

TEV Cleavage of Abl Protein

An artificial TEV cleavage site was engineered in c-Abl just before the SH3 domain using the quick-change site-directed mutagenesis kit (Stratagene). c-Abl amino acids 74–77 (LAGP) were replaced by YFQG, introducing a perfect TEV consensus site, giving rise to Abl-TEV. After expression of Abl-TEV in HEK293 cells and immunoprecipitation using anti-Abl antibodies (Ab-3, Oncogene Science), the immune complexes bound to protein G-Sepharose beads were incubated for 2 hr at 16°C in 10 mM Tris (pH 8.0), 100 mM NaCl, 0.1% Igepal, 0.5 mM EDTA with or without 10 units of TEV enzyme (GIBCO). Subsequently, beads were processed as described for Abl kinase assay.

Acknowledgments

We would like to thank J. Kretzschmar for expert technical assistance, J.Y. Wang, K. Smith, R. Van Etten, J. Wu, A. Merino, and O. Witte for various reagents and suggestions, J.R. Engen for preparing the Abl model, S.A. Courtneidge for suggesting the wheat germ expression experiment several years ago, and M. Saraste for urging us to define the minimal requirements for regulation in vitro. We thank R. Mangano and O. Hantschel for help throughout the work. M. Way, M. Saraste, B. Nagar, A. Nebreda, R. Klein, and all members of the laboratory are thanked for critical reading of the manuscript. H.P. is supported by a European Community fellowship. This work is dedicated to the memory of Matti Saraste.

Received May 29, 2001; revised January 9, 2002.

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