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Phosphorylation and structure-based functional studies reveal a positive and a negative role for the activation loop of the c-Abl tyrosine kinase

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c-Abl is a nuclear and cytoplasmic tyrosine kinase involved in a variety of cellular growth and differentiation processes. In contrast to its oncogenic counterparts, like BCR-Abl, c-Abl is not constitutively tyrosine phosphorylated and its catalytic activity is very low. Here we report tyrosine phosphorylation of endogenous c-Abl and a concomitant increase in catalytic activity. Using Abl −/− cells reconstituted with mutated c-Abl forms, we show that phosphorylation and activity depend on Tyr412 in the activation loop. Tyr412 is also required for stimulation by PDGF or by cotransfection of active Src. Phosphorylation of Tyr412 can occur autocatalytically by a *trans*-mechanism and cause activation of otherwise inactive c-Abl, suggesting a positive feedback loop on c-Abl activity. In the recent structure of the Abl catalytic domain bound to the STI-571 inhibitor, unphosphorylated Tyr412 in the activation loop points inward and appears to interfere with catalysis. We mutated residues involved in stabilizing this inhibited form of the activation loop and in positioning Tyr412. These mutations resulted in tyrosine phosphorylation and activation of c-Abl, as if relieving c-Abl from inhibition. Tyr412 is therefore necessary both for activity and for regulation of c-Abl, by stabilizing the inactive or the active conformation of the enzyme in a phosphorylation-dependent manner. *Oncogene* (2001) **20,** 8075–8084.

Keywords: activation loop; crystal structure; phosphorylation; tyrosine kinase

Introduction

Non-receptor tyrosine kinases rely on a combination of phosphorylation and protein–protein interactions to accomplish a variety of molecular activities (Neet and Hunter, 1996). As in protein kinases with serine/threonine specificity, differential phosphorylation in the so-called activation loop found at the interface between the small N-terminal and larger C-terminal lobes of the catalytic domain of protein tyrosine kinases, is thought to be critical for function and regulation (Hubbard, 1999b; Johnson *et al*., 1996; Smith *et al*., 1999a). The activation loop represents the most flexible part of the catalytic domain and may adopt several conformations depending on its phosphorylation state and in accord with the orientation of the N- and C-lobes to each other. Phosphorylation of the activation loop appears to have several consequences. The major purpose is the stabilization of an 'active' conformation of the loop. Such a conformation may allow access of substrate and ATP to the catalytic center of the kinase domain. Moreover, catalytic activity may be stimulated by allowing positioning of the αC-helix of the N-terminal lobe so that a crucial ion pairing between a glutamic acid in the helix and the lysine in the glycine-rich ATP binding loop can occur. In the insulin receptor, phosphorylation of a tyrosine in the activation loop may additionally defeat inhibition by the hydroxyl group of the tyrosine that otherwise could hydrogen-bond with residues important for catalysis. Importantly, phosphorylation of the activation loop may also affect substrate specificity (Hubbard, 1999a). In several tyrosine kinases, therefore, phosphorylation of one or several tyrosine residues in the activation loop is required to achieve high catalytic activity (Johnson *et al*., 1996).

The cellular form of the Abl tyrosine kinase (c-Abl) is normally in a state of low catalytic activity and has no phosphorylation of tyrosine residues. In contrast, forms rendered constitutively active by leukemiogenic fusion (BCR-Abl, TEL-Abl), deletion of the Src homology 3 domain (SH3), or by several different point mutations, are highly active and tyrosine phosphorylated (Van Etten, 1999). Moreover, overexpression of proteins binding to c-Abl, like Nck, dAbi/Abi-2 and c-Jun, result in tyrosine phosphorylation of c-Abl and concomitant activation of its catalytic potential (Barilá *et al*., 2000; Juang and Hoffmann, 1999; Smith *et al*., 1999b). c-Abl becomes

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active in cells expressing activated forms of Src family kinases (Plattner *et al*., 1999). *In vivo*, Src appears capable of phosphorylating Abl on Tyr412 in the activation loop (Plattner *et al*., 1999). Recent important work by Brasher and Van Etten (2000) unequivocally showed using mutagenesis and *in vivo* kinase assays that Tyr412 and Tyr245 in the SH2-CD linker play a critical role in c-Abl's catalytic activity. However, these sites were not shown to be tyrosine phosphorylation sites *in vivo* nor was their role tested in cellular assays.

The tyrosine phosphorylation sites of two constitutively active fusion forms of the Abl enzyme, BCR-Abl and v-Abl, have been mapped. In BCR-Abl, two major sites are Tyr177 in the BCR moiety, a binding site for the Grb2 adaptor, and the tyrosine in the activation loop corresponding to Tyr412 of c-Abl (Pendergast *et al*., 1993a,b). In v-Abl, there are two major tyrosine phosphorylation sites, Tyr514 (corresponding to Tyr412 or c-Abl) and Tyr385 in the N-terminal lobe of the catalytic domain (Konopka and Witte, 1985). However, there may be other sites as well (Cong *et al*., 1999).

In contrast, despite early reports on tyrosine phosphorylation of the cellular form of Abl, the role of tyrosine phosphorylation of c-Abl has remained elusive (Allen and Wiedemann, 1996; Dikstein *et al*., 1996). We report here that tyrosine phosphorylation of endogenous c-Abl is accompanied by an increase in catalytic activity. Phosphorylation and activity depend on Tyr412 in the activation loop. We performed a study to determine the mechanism of Tyr412 phosphorylation and the functional consequences thereof.

Results

Tyrosine phosphorylation and activation of endogenous c-Abl

To test if endogenous c-Abl could become phosphorylated on tyrosine, we used 3T3 fibroblasts derived from mice deficient for both the *ABL1* and *ABL2* genes (Abl,Arg−/−) cells and their wild-type counterparts (Koleske *et al*., 1998). Cells were treated for 30 min with pervanadate, a potent inhibitor of tyrosine phosphatases. c-Abl was immunoprecipitated and its phosphorylation state analysed using anti-phosphotyrosine antibodies (Figure 1a). A protein band immunoprecipitated by anti-Abl antibodies and running at the appropriate molecular weight became tyrosine phosphorylated after treatment. No such band was visible in the immunoprecipitates from Abl,Arg−/− cells. Pervanadate-induced tyrosine phosphorylation of c-Abl was also detectable using several other human or murine cell lines (data not shown). We tested whether tyrosine phosphorylation correlated with a change in catalytic activity. c-Abl from pervanadate-treated cells showed a 17-fold increase compared to non-treated cells (Figure 1b). Thus c-Abl has the potential to become phosphorylated on tyrosine and this correlates with increased activity.

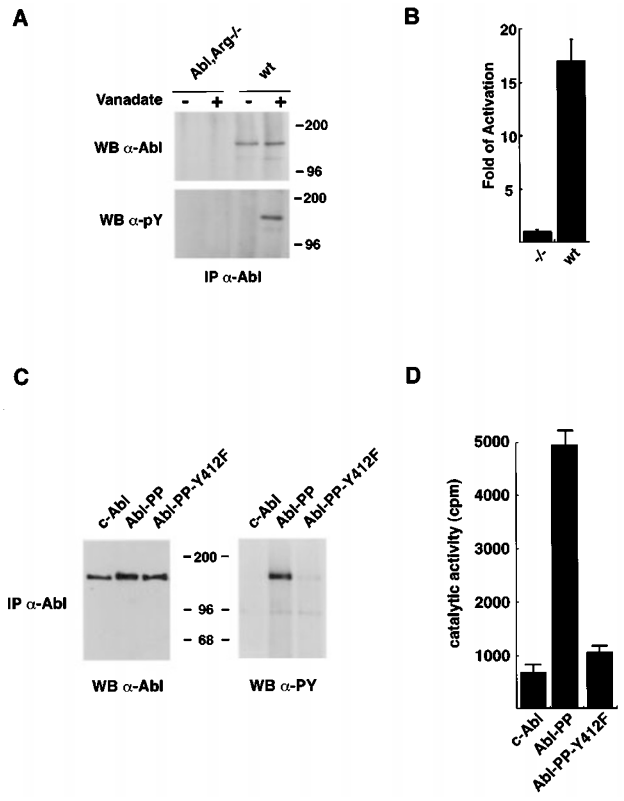

Figure 1 c-Abl is tyrosine phosphorylated when activated. (**a**) Abl,Arg−/− cells or their wt counterpart were treated (+) or not (−) for 30 min with pervanadate. Endogenous c-Abl was immunoprecipitated using anti-Abl antibodies. The immunocomplexes were analysed by immunoblot anti-Abl (upper panel) and anti-phosphotyrosine (lower panel). (**b**) The same immunocomplexes as in (**a**) were used for an *in vivo* kinase assay using GST-Crk as a substrate. The histogram shows the fold of activation of c-Abl activity after pervanadate treatment. The values represent the average of three independent experiments. (**c**) HEK293 cells were transiently transfected as indicated. An aliquot of 400 μg of total lysate was used for immunoprecipitation with anti-Abl antibodies. Immunoprecipitated proteins were analysed by anti-Abl (left panel) or anti-phosphotyrosine immunoblot. (**d**) The same immunocomplexes as in (**c**) were assayed *in vivo* using GST-Crk as a substrate. The values represent the average of three independent experiments

Tyr412 in the activation loop is a major phosphorylation site of Abl and is required for catalytic activity

Attempts to identify the phosphorylation sites on endogenous c-Abl using mass spectrometry failed because of the low abundance of c-Abl protein and of the possible low phosphorylation stoichiometry. We therefore decided to perform a detour using transfected c-Abl and later test possible findings on the protein expressed at more physiological levels. We have identified a double point mutation deregulating c-Abl activity without affecting the integrity of any domain. This regulatory mutation maps to a short stretch of residues that link the SH2 domain to the catalytic domain (P242E, P249E; Abl-PP). In contrast to c-Abl, Abl-PP is constitutively active and is phosphorylated on tyrosine (Barilá and Superti-Furga, 1998). Analysis of the trypsin-digested, Coomassie-stained Abl band by

nanoelectrospray mass spectrometry for the specific detection of phosphopeptides showed unambiguously that Tyr412 in the activation loop was indeed tyrosine-phosphorylated (data not shown). We mutated Tyr412 to phenylalanine in Abl-PP and expressed it in HEK293 cells. Immunoprecipitation of Abl showed that ABL-PP-Y412F protein accumulated well but barely showed any tyrosine phosphorylation (Figure 1c). We assayed the effect of the Y412F mutation on the catalytic activity *in vivo*. Compared to Abl-PP, Abl-PP-Y412F showed a fivefold lower activity in this assay (Figure 1d).

Role of Tyr412 in vivo

To test the role of Tyr412 *in vivo*, Abl,Arg−/− cells were stably transfected with bicistronic vectors expressing either wild-type c-Abl or Abl-Y412F and GFP followed by multiple round of cell sorting by FACS. c-Abl expression levels were equivalent to those observed in wild-type cells (data not shown). The level of Abl-Y412F proteins were always slightly higher than those obtained with wild-type c-Abl (data not shown and Figure 2a) possibly because of a weaker growth-inhibitory effect of this allele (Sawyers *et al*., 1994). c-Abl became tyrosine phosphorylated after pervanadate treatment (Figure 2a). This resulted in an increase in *in vivo* catalytic activity of approximately 17-fold. In contrast, Abl-Y412F was neither tyrosine-phosphorylated nor activated after treatment, demonstrating the importance of Tyr412 and suggesting the requirement of Tyr412 phosphorylation for activity.

c-Abl is activated by PDGF (Cong *et al*., 2000; Plattner *et al*., 1999). To test if Tyr412 is required for c-Abl activation by PDGF, we treated the reconstituted cells with PDGF-BB. Measurement of Abl activity by *in vivo* kinase assay showed a small but reproducible increase of activity, whereas no increase was observed in cells reconstituted with Abl-Y412F (Figure 2c).

Activation of c-Abl by Src requires Tyr412

Members of the Src family can phosphorylate Abl on Tyr412 *in vivo* (Plattner *et al*., 1999). To test whether Src could also elicit c-Abl phosphorylation *in vivo*, HEK293 cells were transiently transfected with Src-Y527F, a constitutively active form of c-Src. Endogenous c-Abl was immunoprecipitated and tyrosine phosphorylation measured using antibodies anti-phosphotyrosine. Expression of Src-Y527F caused tyrosine phosphorylation of cellular c-Abl (Figure 3a). Again, phosphorylation correlated with an increase of Abl's catalytic activity as measured *in vivo* (Figure 3b). Constitutively active c-Abl can phosphorylate the c-Jun transcription factor (Barilá *et al*., 2000). To obtain further evidence for the effect of Src family kinases on the activity of c-Abl, we took advantage of the fact that Src and Abl display distinct abilities to phosphorylate c-Jun. As shown in Figure 3c, when constitutively active Src-Y527F is transfected along with c-Jun in

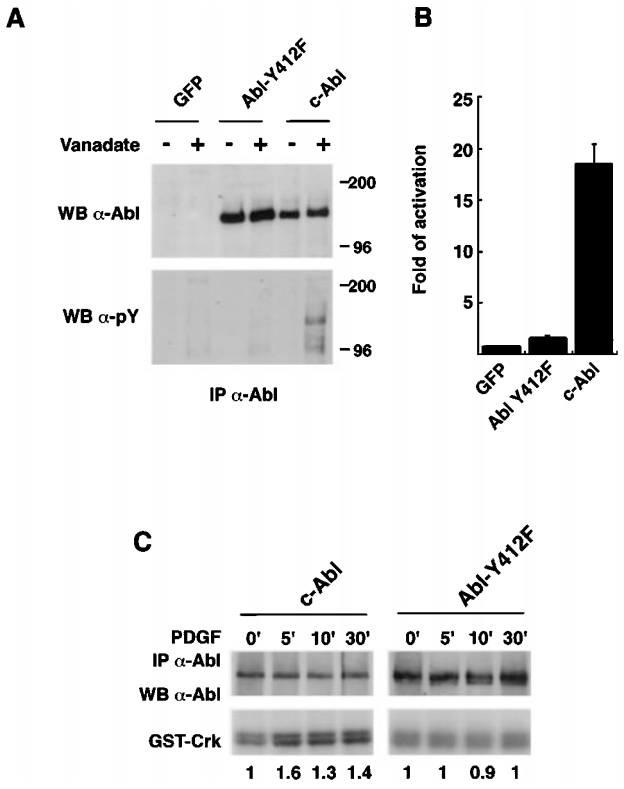

Figure 2 Endogenous Abl is phosphorylated on Tyr412. Abl,Arg−/− cells were reconstituted with GFP, Abl Y412F or c-Abl. (**a**) The cells were treated 30 min with pervanadate and Abl immunoprecipitated with anti-Abl antibodies. The immunocomplexes were analysed by immunoblot anti-Abl (upper panel) and anti-phosphotyrosine (lower panel). (**b**) The catalytic activity was quantified by an *in vivo* kinase assay. (**c**) Cells reconstituted with c-Abl or Abl-Y412F were treated with PDGF-BB, Abl immunoprecipitated and its catalytic activity tested by *in vivo* kinase assay (lower panel). These data represent one of three independent experiments

HEK293 cells, the 39 kDa protein band corresponding to c-Jun does not become tyrosine-phosphorylated (lane 2). In contrast, expression of the constitutively active Abl-PP leads to strong c-Jun phosphorylation (lane 7). Expression of c-Abl, which is regulated, results in only a weak phosphorylation of c-Jun (lane 3). However, transfection c-Abl along with Src-Y527F leads to a significant phosphorylation of c-Jun (lane 4). This is consistent with activation of c-Abl by Src-Y527F and phosphorylation of c-Jun by activated c-Abl. Abl mutated at Tyr412 is not responsive to activation by Src-Y527F, confirming the importance of this residue (lane 6).

Stimulation of cellular signaling pathways by the activity of transfected Src and Abl can be monitored conveniently using a promoter containing serum-responsive elements controlling expression of luciferase (SRE-luciferase, Alexandropoulos and Baltimore, 1996; Barilá and Superti-Furga, 1998; Raitano *et al*., 1997). At low level of expression, Src-Y527F leads to only a modest stimulation of SRE-promoter activity (Figure 3d). A similar level of promoter activity is obtained by expression of more significant amounts of c-Abl, which

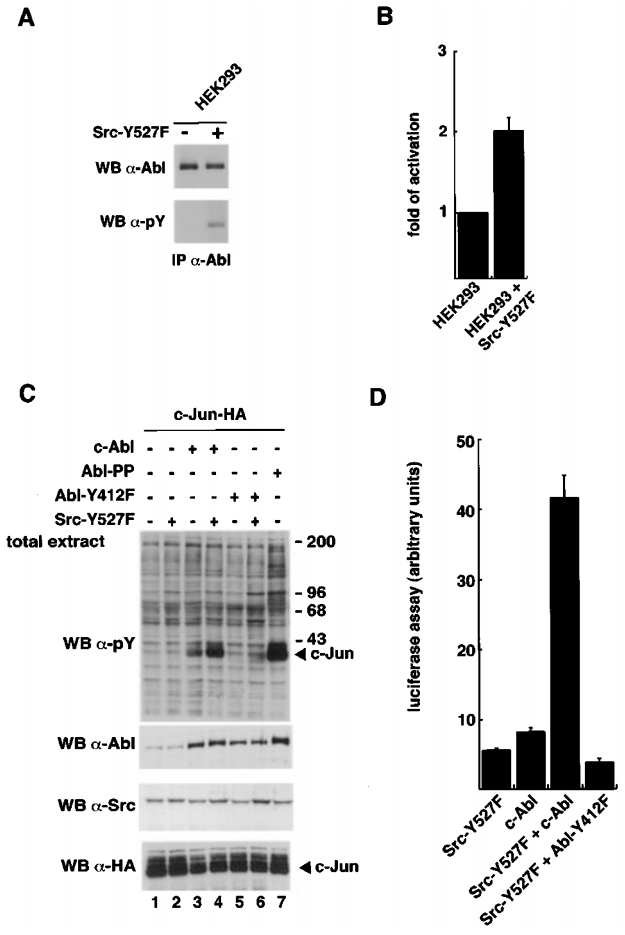

Figure 3 Src activates c-Abl but not Abl-Y412F. (**a**) Endogenous c-Abl was immunoprecipitated from cells non-transfected (−) or transfected (+) with a constitutive active form of Src, Src-Y527F. The phosphorylation state of Abl was treated by immunoblot anti-phosphotyrosine (lower panel). (**b**) The catalytic activity of the immunoprecipitates were quantified by an *in vivo* kinase assay. (**c**) HEK293 cells were transfected with the indicated constructs for a total of 20 μg of plasmids. Protein extracts were separated, blotted and probed with the indicated antibodies. c-Jun is HA-tagged and the band below the 43 kDa marker in anti-phosphotyrosine corresponds to c-Jun phosphorylation. (**d**) Transcriptional assay: HEK293 cells were transfected as indicated together with a luciferase reporter plasmid under the control of a promoter containing serum-responsive element (Alexandropoulos and Baltimore, 1996). Twenty-four hours after transfection, extracts were assayed for luciferase activity. The histogram shows the mean of two independent experiments done in duplicate, with standard deviations

is regulated. In contrast, expression of both proteins results in a more than additive stimulation of promoter activity, suggesting activation of c-Abl by Src-Y527F. c-Abl mutated at Tyr412 is insensitive to the Src-Y527F-induced synergy. Altogether, these data show the importance of Tyr412 for Src-mediated activation of c-Abl.

Autophosphorylation and activation of Abl

The experiments above corroborate the original suggestion of Plattner *et al*. (1999) that Src family kinases may activate c-Abl. Yet, the fact that constitutively active forms of c-Abl are tyrosine phosphorylated, suggests that Abl may also autophosphorylate. To investigate this possibility, we used a combination of catalytically inactive forms and a constitutively active SH3 deletion mutant. Being smaller, Abl-ΔSH3 migrates faster than full-length Abl forms and can be easily distinguished (Figure 4a). After transfection, Abl proteins were immunoprecipitated and tested with anti-phosphotyrosine antibodies. Catalytically inactive Abl was only tyrosine phosphorylated upon coexpression of Abl-ΔSH3, showing that Abl can autophosphorylate by a *trans* mechanism. Abl bearing the Y412F mutation was considerably less tyrosine-phosphorylated than its catalytic competent counterpart, indicating that Tyr412 is indeed the major Abl autophosphorylation site. Because phosphorylation was not abolished, other minor tyrosine phosphorylation sites may exist.

To investigate the consequences of Tyr412 phosphorylation on the catalytic activity of Abl, we expressed various full-length Abl forms in HEK293 cells, along with a short form of Abl-PP that lacks the last-exon region and is constitutively active. When lysates derived from transfected cells are used for immunoprecipitation with anti-Abl antibodies that recognize the C-terminus of the protein, only the full-length forms are precipitated. Co-expression of the short active Abl form resulted in phosphorylation of c-Abl, as expected (data not shown), and an approximately 2.5-fold stimulation of catalytic activity (Figure 4b). In contrast, Abl-Y412F does not become more active in the presence of short active Abl (Figure 4b). This suggests that indeed, *trans*-autophosphorylation activates c-Abl.

Tyr412 is more accessible to autophosphorylation if Abl is in an active conformation

The recent crystal structure of the Abl catalytic domain bound to the small molecular inhibitor STI-571 has shown that Tyr412 in the activation loop faces inward towards the catalytic residues and should therefore not be easily accessible to phosphorylation *in trans* (Schindler *et al*., 2000; Figure 5a). Because no structure is available of the Abl catalytic domain in an active conformation, we have modeled Abl on the structure of the active catalytic domain of Lck (Yamaguchi and Hendrickson, 1996). Studies with Src have shown that the phosphorylation state of the activation loop and its conformation are tightly linked to the assembly of the regulatory apparatus (Gonfloni *et al*., 2000). Because our assay allows monitoring *trans*-phosphorylation of Abl, we set out to test if accessibility of Tyr412 is higher in a form of Abl in which the catalytic domain is in an active conformation. The double proline mutation in the SH2-CD linker that prevents SH3 domain binding, deregulates Abl. We combined this mutation with mutation of Lys290 in the ATP-binding loop. When co-transfected with ΔSH3-Abl, the kinase-

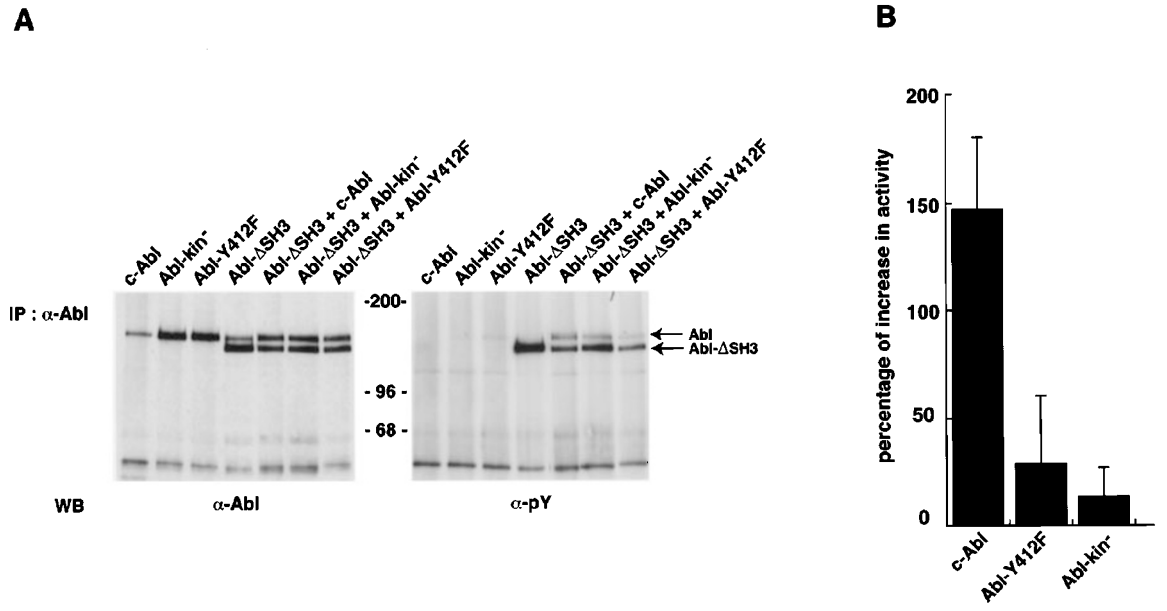

Figure 4 Abl autophosphorylation *in trans* leads to activation. (**a**) HEK293 cells were transfected with 15 μg of DNA (10 μg of full length with 5 μg of vector or Abl-ΔSH3 domain). Four hundred μg of total protein extract was immunoprecipitated using anti-Abl antibodies. Immunoblots were probed with anti-Abl (right panel), stripped and reprobed with anti-phosphotyrosine antibodies (left panel). (**b**) HEK293 cells were transfected with 15 μg of the indicated constructs and either 5 μg of pSGT-Abl-PP-32KIN or empty vector. After immunoprecipitation with anti-Abl antibodies, the samples were tested in kinase assay using GST-Crk. The histogram shows the percentage of activation comparing the catalytic activity with or without pSGT-Abl-PP-32KIN. The data represent the mean of four independent experiments

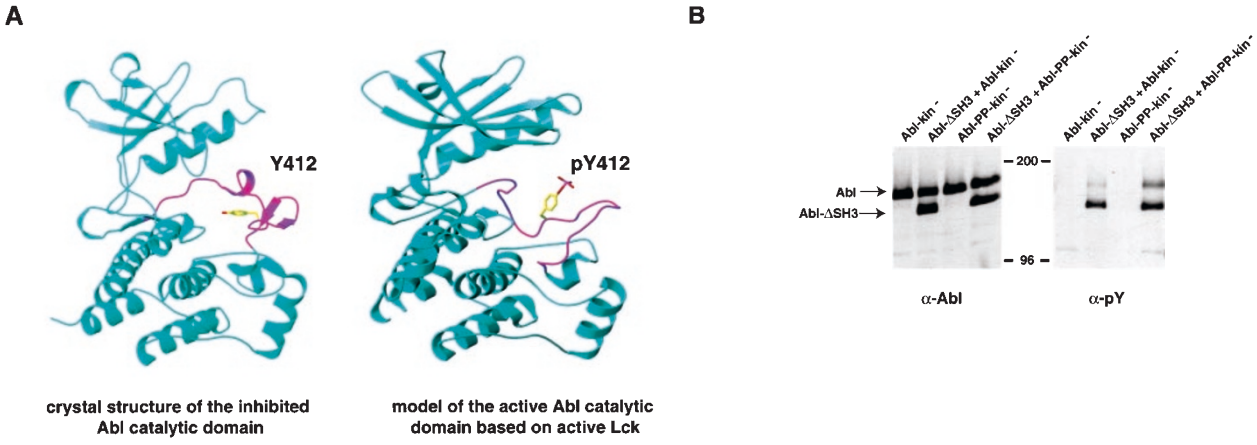

Figure 5 Tyrosine 412 is more accessible to autophosphorylation when the regulatory apparatus is disassembled. (**a**) Comparison of the crystal structure of the inhibited catalytic domain of c-Abl with a model of c-Abl on the active conformation of the Lck catalytic domain (Yamaguchi and Hendrickson, 1996). The model of active c-Abl was prepared by aligning the sequences of the catalytic domains of c-Abl and Lck (48% identity) and preparing a homology model with WHAT_IF (Vriend, 1990). The validity of the model was checked with WHAT_IF and side-chain clashes removed. The figure was prepared using MOLSCRIPT (Kraulis, 1991). (**b**) HEK293 cells were transfected as indicated. Abl was immunoprecipitated from 400 μg of total extract and the immunoprecipitates analysed by anti-Abl (left panel) or anti-phosphotyrosine (right panel) immunoblot

dead Abl-PP was consistently better phosphorylated than kinase-dead c-Abl (Figure 5b).

Activation of Abl by destabilization of the inhibited conformation

In the crystal structure of the Abl catalytic domain bound to STI-571, the activation loop adopts a path that is very similar to the one observed in the inactive insulin receptor and mimics a putative peptide substrate (Hubbard *et al*., 1994; Schindler *et al*., 2000). The side chain of Tyr412 faces inward and entertains hydrogen bonds with residues from the catalytic loop. The phenolic ring of Tyr412 appears to be positioned by a number of surrounding residues, mainly Pro421, Thr411, Met407 and Ser404 (Figure

6b). Except for Pro421, a highly conserved residue in the P+1 loop of tyrosine kinases thought to be involved in substrate binding, these residues were individually mutated to alanine, to test their involvement in regulation of c-Abl activity (Hanks *et al*., 1988; Knighton *et al*., 1991). In Src, we have previously found that the inactive conformation of the activation loop is stabilized by interactions with the αC-helix and with the β3/αC loop preceding it (Gonfloni *et al*., 2000). Judging from the Abl structure, Arg405 in the activation loop points towards the αC-helix and may interact with Glu298 in the β3/αC loop (Figure 6b). We mutated Arg405 to lysine, the only residue found in other tyrosine kinases, like Csk and the EGFR. Glu298 was mutated to alanine. The mutants were transfected transiently in HEK293 cells. All mutant proteins accumulated normally and were immunoprecipitated with comparable efficiency (Figure 7a). c-Abl is poorly tyrosine phosphorylated, reflecting its regulated state in the cell (Figure 7a, upper panel). In contrast, Abl-E298A, Abl-S404A, Abl-R405K, Abl-M407A and Abl-T411A showed increased tyrosine phosphorylation. Mutation of Glu298 and of Thr411 lead to the strongest phosphorylation levels, while the other mutants were comparable to Abl-E117K, a deregulated Abl allele described previously (Barilá and Superti-

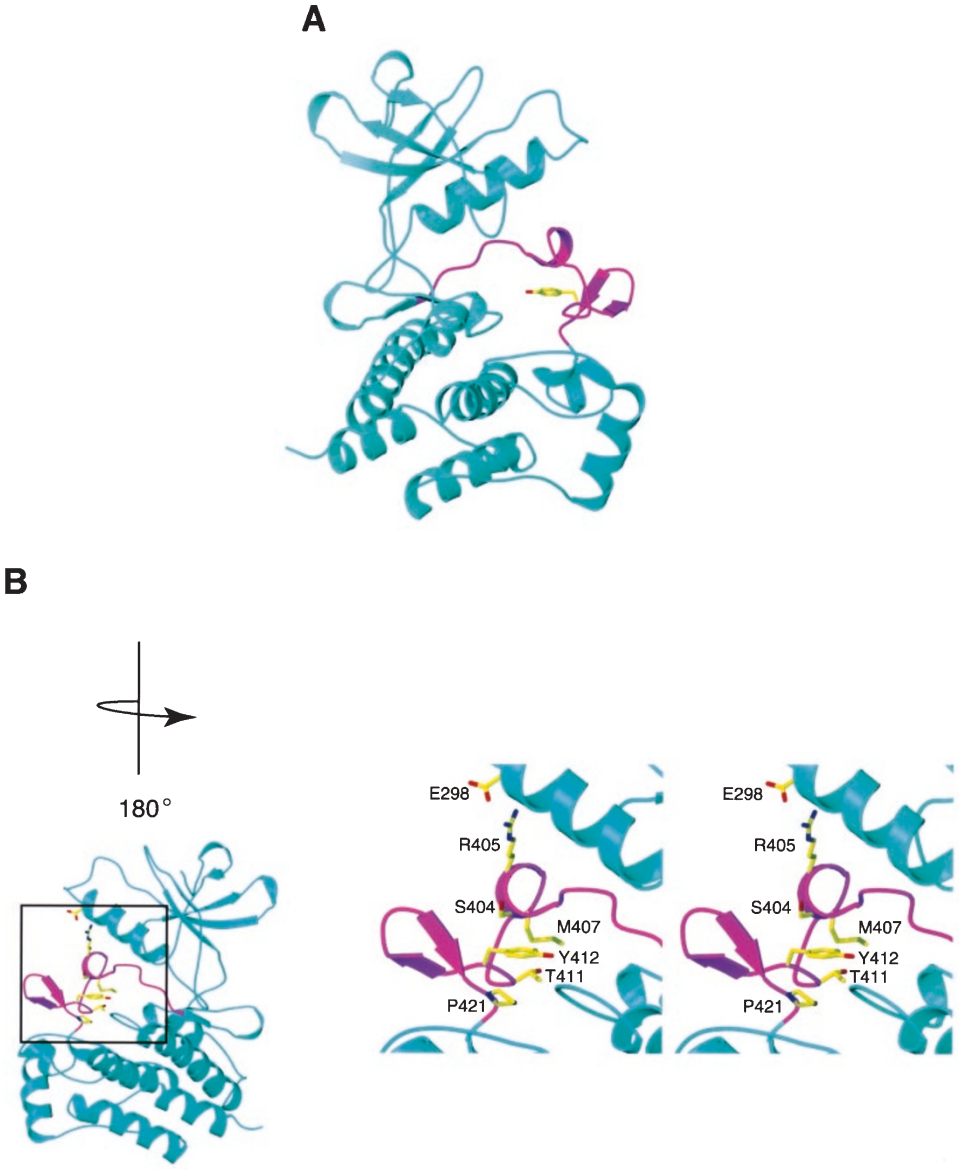

Figure 6 Crystal structure of inhibited c-Abl. (**a**) The crystal structure of the inhibited Abl catalytic domain is shown from the standard review (Schindler *et al*., 2000). The activation loop is in purple, Tyr412 is in yellow. (**b**) Closer stereo view of the region surrounding Tyr412. The structure was flipped 180° on the Y-axis compared to the standard view in (**a**), to allow vision of the amino-acids surrounding Tyr412. The activation loop is in purple, Tyr412 and the residues mutated in this study (except Pro421) are drawn as stick in yellow. The side chain of Met 407 is disordered in the crystal structure, and a possible rotamer conformation is shown in this model

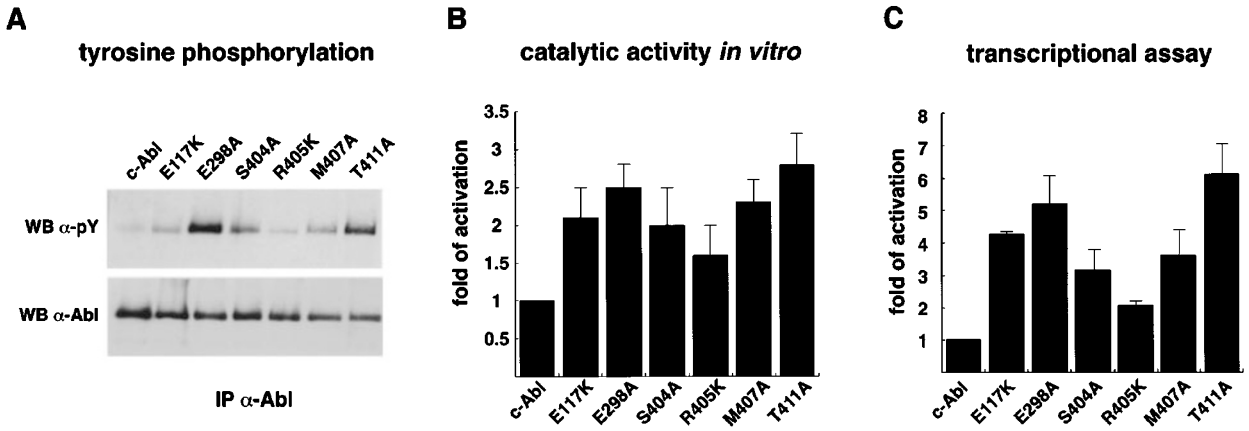

Figure 7 Activation loop mutants of c-Abl. (**a**) HEK293 cells were transfected as indicated. c-Abl was immunoprecipitated using anti-Abl antibodies. The immunocomplexes were analysed by immunoblot anti-Abl (lower panel) or anti-phosphotyrosine (upper panel). (**b**) Abl was immunoprecipitated and its catalytic activity was assayed as in Figure 1b. (**c**) HEK293 cells were transfected as indicated together with a luciferase reporter plasmid as described in Figure 3. The results show the mean of two independent experiments done in duplicate. The levels of c-Abl expression were controlled by Western blot (data not shown)

Furga, 1998). To test whether this increase in tyrosine phosphorylation in the cells was associated with an increase in kinase activity, we tested the ability of the immunoprecipitated proteins to phosphorylate GST-Crk *in vivo* (Figure 7b). All mutants displayed a higher activity than c-Abl, confirming their partially deregulated catalytic activity. Of all mutants, Abl-R405K was the weakest. While there seems to be an overall good correlation between the extent of tyrosine phosphorylation and catalytic activity, discrepancies may rely on the different sensitivity of the assays and on the fact that mutations may also affect catalytic activity.

Deregulated levels of Abl activity in the cell should lead to increased Abl-medicated signaling (Raitano *et al*., 1997; Zou and Calame, 1999). The new Abl mutants were potent stimulators of SRE-dependent transcription, suggesting that the increase in catalytic activity was coupled to an increased signaling capability (Figure 7c). Mutation of the residues either interacting with unphosphorylated Tyr412 in the crystal structure or likely to have a role in stabilizing the inactive conformation of the activation loop, resulted in increased tyrosine phosphorylation, catalytic activity and cellular signaling.

Discussion

The role of tyrosine phosphorylation in the regulation of c-Abl activity had not been elucidated previously. While the oncogenic forms of Abl were among the first proteins to be characterized as containing phosphotyrosine and Tyr412 in the activation loop was known to be important for specific functions, it was not known whether tyrosine phosphorylation played any role for the cellular form of the enzyme (Konopka *et al*., 1984; Pendergast *et al*., 1993a; Sefton *et al*., 1981; Witte *et al*., 1981). Earlier findings had indicated that DNA-associated c-Abl may be tyrosine phosphorylated in untreated cells, but it was not clear if the associated activity was rather the result of several purification steps required for detection nor what could be the relevant phosphorylation sites (Dikstein *et al*., 1996). Likewise, overexpressed c-Abl had been shown to become tyrosine phosphorylated after vanadate treatment, but no functional consequences were tested (Allen and Wiedemann, 1996).

Using conditions in which the activity of cellular tyrosine phosphatases is inhibited, we were able to detect tyrosine phosphorylation of cellular Abl in different cell types of human and murine origin. The identity of the Abl protein was inferred by the size, the immunoreactivity and most unambiguously by the absence in cells carrying the gene knock-out. A form bearing the Tyr412 mutation in the activation loop was not tyrosine phosphorylated. Most importantly, tyrosine phosphorylation of c-Abl correlated tightly with catalytic activity. Cellular Abl that became tyrosine-phosphorylated after pervanadate treatment had an almost 20-fold higher catalytic activity than the untreated.

It has been shown that PDGF activates c-Abl in a Src-dependent manner and that Src family kinases are able to phosphorylate c-Abl on Tyr412 *in vitro* (Plattner *et al*., 1999). We have now shown that active Src causes tyrosine phosphorylation and activation of endogenous c-Abl. Additionally, c-Abl mutated at Tyr412 in the activation loop is not activated after PDGF stimulation or by Src activity. It was recently found that Abl-Y412F can interfere with Src-dependent mitogenic stimuli, as if the ability of Src family kinases to stimulate mitogenesis would require c-Abl activity, a possibility confirmed using Abl-deficient fibroblasts (O Furstoss *et al*., in preparation). Active forms of c-Abl autophosphorylate on Tyr412 *in trans*. As a consequence, c-Abl becomes active and may in turn phosphorylate another c-Abl molecule, in a positive feedback mechanism.

In the crystal structure of the Abl catalytic domain bound to the STI-571 inhibitor, the activation loop

adopts a conformation that could inhibit substrate binding. Furthermore, activation loop residues required for catalysis are displaced and the side chain of Tyr412 interacts with other catalytic residues, suggesting further inhibition (Schindler *et al*., 2000). Do these interactions also occur in the full-length form of cellular Abl and are they involved in regulating the activity of the enzyme? We have mutated residues that in the crystal form the architectural frame that surrounds Tyr412 and residues that may be involved in stabilizing the unphosphorylated activation loop. We obtained active, tyrosine phosphorylated forms of c-Abl. Thus, interference with these structural constraints is sufficient to shift the enzyme into an active mode. This suggests that the inhibitory interactions inferred from the crystal structure of the Abl catalytic domain also occur in c-Abl.

Because Tyr412 points inward in the crystal structure, it may not be easily accessible for phosphorylation (Schindler *et al*., 2000). Our results have shown that Tyr412 is more easily phosphorylated in *trans* when the SH3 domain-mediated inhibition is not operational. This validates the crystal structure and suggests a link between conformation of the activation loop and inhibition of the enzyme. There may be several ways by which molecules like Src family kinases gain access to Tyr412 in the regulated conformation. Phosphorylation of c-Abl on another more accessible tyrosine may cause destabilization of the regulated conformation and allow Tyr412 to become more accessible. In cells, Src family kinases induce phosphorylation of c-Abl also on Tyr245 in the SH2-catalytic domain linker, as shown by mass spectrometry after purification (K Dorey and G Neubauer, unpublished results). Interestingly, mutation of Tyr245 impaired the ability of c-Abl to autoactivate *in vivo* (Brasher and Van Etten, 2000). We have found that mutation of Tyr245 to alanine activates c-Abl, suggesting a role of this residue in regulation (K Dorey, unpublished results). Alternative ways to gain access to Tyr412 may be through proteins binding c-Abl and relieving its conformational constraints, allowing release of Tyr412 from the inward-pointing position. At this stage we do not have enough elements to predict which of the possible activation routes is actually the most likely to occur physiologically and believe that all these mechanisms may be operational under one or another circumstance.

If c-Abl can activate itself catalytically, how would it return to the regulated state? Dephosphorylation of Tyr416 in Src is critical to achieve inhibition by phosphorylation of Tyr57, the C-terminal site (Gonfloni *et al*., 2000). Dephosphorylation of Tyr412 in c-Abl by a cellular tyrosine phosphatase may therefore be the most efficient way to shift the equilibrium of c-Abl back from an active conformation to the inhibited form. Indeed, it was recently found that PTP-PEST was required for downregulation of c-Abl after PDGF treatment suggesting that c-Abl regulation is controlled by its state of phosphorylation (Cong *et al*., 2000). Our data on activation of endogenous c-Abl by phosphatase inhibitors is in agreement with this hypothesis.

The role of Tyr412 has been investigated in this study by mutation to phenylalanine, which is not comparable to unphosphorylated tyrosine. Because phenylalanine can neither be phosphorylated nor undergo hydrogen bonds, it may not contribute to stabilization of the activation loop in the active or in the inactive conformation, creating a sort of 'in-between' situation (Schindler *et al*., 1999). We found no evidence for any partially activating effect of the Y412F mutation on c-Abl, suggesting that if it cannot be phosphorylated, failure to adopt a fully inhibited conformation is not sufficient to lead to an increase in activity.

The surprising ability to govern different aspects of c-Abl activity and function by a single residue and its state of phosphorylation suggests the existence of cellular regulatory pathways that act on Tyr412 and the activation loop. The description of the mechanisms by which these kinases, phosphatases (Cong *et al*., 2000) and binding proteins may act will clarify how c-Abl is linked to the various signaling pathways and may help elucidate the still obscure cellular function of c-Abl.

Materials and methods

DNA constructs

All constructs were cloned into the SV40 enhancer/promoter pSGT vector (Erpel *et al*., 1995). Constructs pSGT-Abl-ΔSH3, pSGT-Abl PP and pSGT-Abl-kin$^-$ were described (Erpel *et al*., 1995; Barilá and Superti-Furga, 1998). Point mutations were generated by PCR using QuickChange (Stratagene). All mutants were verified by sequencing. The construct pSGT-Abl-PP-32KIN corresponds to amino acids Asn 80 to Lys 531 of human c-Abl bearing the PP mutation. Fragments were amplified by PCR and cloned into pCDNA3 using *Bam*HI and *Eco*RI sites. The inserts were subcloned into pSGT using *Bam*HI and *Not*I sites.

Cell culture and transfection

HEK293 cells were cultured in DMEM supplemented with 10% FCS. Wild-type and Y412F mutant Abl were subcloned in MSCV-IRES-GFP vector and retroviruses produced in 293T cells. Abl,Arg−/− fibroblast were infected with the different viruses and positive cells sorted by multiple FACS selections essentially as described (Plattner *et al*., 1999). Abl,Arg−/−, Abl,Arg+/+ and the reconstituted cells were cultured in DMEM supplemented with 15% FCS.

HEK293 cells were transfected with the calcium phosphate method at 70% confluency in 10 cm dishes with the different expression constructs or empty vector for a total of 10–20 μg of DNA. Around 40 h after transfection, total cell lysates were obtained using 400 μl of lysis buffer (LB; described in Barilá and Superti-Furga, 1998).

Cells were treated with a solution at 1 mm pervanadate and 0.4 mm H202 final for 30 min. Reconstituted cells were stimulated with 12.5 ng/ml PDGF-BB (UBI).

Immunoblotting and immunoprecipitation

For HEK293 cells lysates, 50 μg of total protein extract were separated by SDS–PAGE, blotted onto nitrocellulose and

probed with specific antibodies. Abl was immunoprecipitated from 400 μg of total extract using 10 μl of 24-21 (Ab-3, Oncogene Sciences). Immunocomplexes were recovered using protein G-Sepharose beads (Amersham Pharmacia). To detect endogenous Abl phosphorylation, c-Abl was immunoprecipitated from 6 mg of total extract.

The following antibodies have been used: anti-Abl antibody 24-21 (Ab-3, Oncogene Sciences, anti-phosphotyrosine 4G10 (UBI), anti-HA 12CA5 (Boehringer Mannheim) as described in (Barilá and Superti-Furga, 1998) and anti-Src 2-17 (Microbiological Associates; Gonfloni *et al*., 2000). In all cases, horseradish peroxidase (HRP)-coupled secondary antibody and the ECL chemiluminescence detection method were used (Amersham Pharmacia).

Kinase assay

Immunoprecipitation of Abl was carried out as described above. The beads were washed twice in LB, twice in LB without NaCl and twice in kinase assay buffer (20 mM Tris pH 7.5, 10 mM $MgCl_2$, 1 mM DTT). A third of the immunoprecipitates were used for a Western blot, and the rest for a kinase assay. Reactions were performed in 20 μl of kinase assay buffer containing 2 μM GST-Crk as a substrate (Dorey *et al*., 1999), 1 μM ATP, 5 μCi of [γ-^{32}P]-ATP (5000 Ci/mmol, Amersham). Reactions were stopped at 10 and 20 min by adding 10 μl of SDS-containing sample buffer and boiled for 5 min. After SDS–PAGE, bands corresponding to the substrate were excised and incorporated radioactivity measured in a scintillation counter.

Luciferase 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) and the different constructions as indicated. After 24 h, cells were lysed in 300 μl of passive lysis buffer (Promega). Five μl of extracts were used for the luciferase assay, performed under standard condition (Ausubel *et al*., 1987).

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