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Phosphorylation of Crk on tyrosine 251 in the RT loop of the SH3C domain promotes Abl kinase transactivation

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Abstract

Here, we report the identification and characterization of a novel tyrosine phosphorylation site in the carboxy-terminal Src Homology 3 (SH3) (SH3C) domain of the Crk adaptor protein. Y251 is located in the highly conserved RT loop structure of the SH3C, a region of Crk involved in the allosteric regulation of the Abl kinase. Exploiting kinase assays to show that Y251 is phosphorylated by Abl *in vitro*, we generated affinity-purified antisera against phosphorylated Y251 in Crk and showed that Abl induces phosphorylation at Y251 *in vivo*, and that the kinetics of phosphorylation at Y251 and the negative regulatory Y221 site *in vitro* are similar. Y251 on endogenous Crk was robustly phosphorylated in chronic myelogenous leukemia cell lines and in A431 and MDA-MB-468 cells stimulated with epidermal growth factor. Using streptavidin–biotin pull downs and unbiased high-throughput Src Homology 2 (SH2) profiling approaches, we found that a pY251 phosphopeptide binds specifically to a subset of SH2 domains, including Abl and Arg SH2, and that binding of pY251 to Abl SH2 induces transactivation of Abl 1b. Finally, the Y251F Crk mutant significantly abrogates Abl transactivation *in vitro* and *in vivo*. These studies point to a yet unrealized positive regulatory role resulting from tyrosine phosphorylation of Crk, and identify a novel mechanism by which an adaptor protein activates a non-receptor tyrosine kinase by SH2 domain displacement.

Keywords

Crk tyrosine phosphorylation; SH2 domains; Abl transactivation

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Conflict of interest

The authors declare no conflict of interest.

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Introduction

The Crk family of adaptor proteins (Crk II, Crk I and CrkL) comprises cytoplasmic Src Homology 2 (SH2) and Src Homology 3 (SH3) domain-containing proteins that assemble protein complexes and transmit signals downstream of tyrosine kinases (Mayer *et al.*, 1988; Matsuda *et al.*, 1990). Both Crk II and CrkL are composed of a single SH2 domain, followed by two tandem SH3 domains: SH3(N) and SH3(C) (Matsuda *et al.*, 1992; Reichman *et al.*, 1992). Crk II is also alternatively spliced to a minor product, Crk I, which is structurally and functionally more similar to the v-Crk oncogene encoded by the avian CT10 retrovirus (Feller, 2001; Birge *et al.*, 2009). Despite the fact that both Crk II and CrkL are ubiquitously expressed, their SH domains are highly homologous, they bind similar proteins, both are required for mouse development and exhibit distinct non-overlapping phenotypes in knockout mice (Guris *et al.*, 2001; Park *et al.*, 2006). Identification of isoform-specific signaling pathways for Crk versus CrkL remains elusive.

The adaptor function of Crk is mediated by the assemblage of selective signaling protein complexes that bind to the SH2 and SH3(N) domains in the context of sequence-specific consensus motifs. The Crk SH2 domain binds to selective targets in the context of a pTyr-X-X Pro (Birge *et al.*, 1993), whereas the SH3N domain binds to selective PPII peptides in the context of Pro-X-X-Pro-X- (Lys, Arg) (Knudsen *et al.*, 1994). Two of the best understood signaling pathways mediated by Crk arise from ternary complexes of $p130^{cas}$–Crk–Dock180 (Kiyokawa *et al.*, 1998), which is involved in the activation of Rac1 and actin cytoskeletal reorganization (Klemke *et al.*, 1998), and $p130^{cas}$–Crk–C3G (Matsuda *et al.*, 1994), which is involved in inside-to-outside integrin activation and cell adhesion (Tanaka *et al.*, 1994; Gotoh *et al.*, 1995).

On the other hand, Crk SH3C is an atypical SH3 domain that does not bind to conventional PPII motifs (Reichman *et al.*, 2005; Muralidharan *et al.*, 2006). It has been shown to exert a negative regulatory effect on the binding of ligands to the SH3N (Kobashigawa *et al.*, 2007; Sarkar *et al.*, 2007). Recently, we have described the structural basis for SH3C autoinhibition by virtue of the fact that Crk toggles between two conformations—a *cis*-inhibitory conformation stabilized by an intramolecular association of the two SH3 domains and a *trans*-uninhibited conformation that unhinges the closed conformation. These conformations are regulated by *cis* trans-isomerization at Pro238 (Sarkar *et al.*, 2011).

Negative regulation of Crk-mediated signaling is achieved by phosphorylation at Y221 (resulting in an intramolecular SH2–pY221 interaction) by the Abl, Arg tyrosine kinases that bind to the Crk SH3N domain (Feller *et al.*, 1994; Ren *et al.*, 1994). Such negative regulation is also involved in signaling from several receptor tyrosine kinases, including EGFR (Hashimoto *et al.*, 1998), platelet derived growth factor beta receptor (Sorokin *et al.*, 1998; Matsumoto *et al.*, 2000), TrkA (Ribon and Saltiel, 1996) and Ephrin B1/Eph (Nagashima *et al.*, 2002). However, in addition to its role in the disassembly of Crk protein complexes, association of Crk and Abl also induces transient Abl transactivation (Shishido *et al.*, 2001; Reichman *et al.*, 2005), which is mediated by the PNAY motif in the RT loop of the SH3C domain of Crk (Reichman *et al.*, 2005). Interestingly, Abl is activated downstream of EGFR, Her2/ErbB2 and Src, and constitutively activated in highly invasive breast cancer cell lines supporting a tumor-promoting role (Srinivasan and Plattner, 2006; Srinivasan *et al.*, 2008). However, the consequences of Abl activation by Crk remain to be understood and the precise mechanism of transactivation of Abl by Crk has still not been elucidated.

In this study, we have identified and characterized Y251 in the RT loop of the SH3C of Crk as a second phosphorylation site for Abl in addition to the previously identified negative regulatory site, Y221. Y251, when phosphorylated, binds to the Abl SH2 domain to

transactivate Abl 1b. These results lend insights into the mechanism of Abl activation by Crk—a novel mechanism of activation of a non-receptor tyrosine kinase by an adaptor protein. In addition, our data suggest that tyrosine phosphorylation of Crk can both positively and negatively regulate Abl activity and signaling pathways.

Results

Crk Y251 within the conserved PNAY motif in the SH3C is phosphorylated by Abl

The SH3C domain of Crk is an atypical SH3 domain in that it does not bind to conventional PPII-containing ligands (Muralidharan *et al.*, 2006). In a previous study to elucidate the biological function of this region of Crk, we substituted conserved amino acids on the surface of SH3C, which were predicted to provide a binding surface for target proteins (Reichman *et al.*, 2005). In doing so, we identified an important region that was required for the transactivation of mouse c-Abl type IV (homologous to human c-Abl 1b)—the highly conserved PNAY motif in the RT loop of SH3C (Reichman *et al.*, 2005).

To determine whether Y251 within the PNAY motif is phosphorylated by Abl, we co-incubated purified human Abl with bacterially expressed and purified glutathione-*S*-transferase (GST), GST-Crk or GST-Crk Y221F proteins in an *in vitro* kinase assay (Figure 1a). As indicated, substitution of the negative regulatory Y221 only partially reduced total tyrosine phosphorylation (by ~50%), suggesting the existence of other tyrosine phosphorylation sites on Crk. Furthermore, in the *in vitro* kinase assay described above, immunoprecipitation of Abl and analysis of the bound fraction revealed the presence of tyrosine-phosphorylated GST-Crk (Figure 1b), suggesting that a form of GST-Crk phosphorylated at one or more sites other than Y221 remained associated with Abl. To investigate whether tyrosine phosphorylation of Y221F Crk occurred in cell lines, we co-transfected CrkI or various mutants of Crk with mouse Abl type IV in 293T cells (Figure 1c). Consistent with the *in vitro* kinase assay in Figure 1a, total tyrosine phosphorylation (assayed by western blotting with a general anti-phosphotyrosine antibody) on the Crk Y221F mutant was again reduced by ~50% compared with wild-type Crk. As Y251 on human Crk (hCrk) was found to be phosphorylated in K562 cells using mass spectrometric analysis (http://Phosphosite.org, Cell Signaling Technology, Danvers, MA, USA), we co-expressed Y221F/Y251A or Y221F/P249A double mutants with Abl in 293T cells (Q275 on the surface of Crk SH3C was also mutated to alanine and the mutant was co-expressed with Abl). As shown in Figure 1d, tyrosine phosphorylation of the Y221F/Y251A double mutant was reduced over 50% compared with Y221F, suggesting that Y251 is phosphorylated when Crk is co-expressed with Abl.

Generation and characterization of phosphospecific antisera to human phospho (Y251)-Crk

To better examine tyrosine phosphorylation of Crk at Y251 *in vivo*, we generated polyclonal phosphospecific antisera against the human phospho (Y251) motif using phosphopeptides coupled to carrier proteins as immunogens and subsequently obtained affinity-purified anti-phospho (Y251) antibodies against hCrk. Co-expression of wild-type human or chicken Crk with human c-Abl 1b in 293T cells resulted in robust immunoreactivity of Crk with the anti-phospho-(Y251) Crk antibody. A hCrk Y251F mutant or a chicken Crk (cCrk) Y251A mutant when co-expressed with Abl showed no immunoreactivity (Figure 2a). Furthermore, immunoreactivity was completely blocked by the specific pY251 phosphopeptide (derived from the RT loop of hCrk SH3C with the sequence KRVPNApY^{251}DKTALALE) but not by a nonspecific pY221 phosphopeptide or by the unphosphorylated GST-Crk protein (Figure 2b), thereby confirming the specificity of the anti-phospho (Y251) antibody.

To investigate whether phosphorylation of hCrk at Y251 prevented or augmented phosphorylation at the negative regulatory site Y221 or *vice versa*, we co-expressed hCrk Y221F and Y251F with Abl 1b in 293T cells, followed by western blotting with anti-phospho (Y221) and anti-phospho (Y251) antibodies. We also co-expressed a Y239F Crk mutant with Abl as Y239, which, located at the boundary of hCrk SH3C, is a potential phosphorylation site. No mutant showed reduced or augmented phosphorylation at Y221 or Y251 (Figure 2c). We also incubated purified human Abl 1a (from insect cells) with bacterially expressed and purified GST-hCrk in the presence of ATP over a time course of 5 s to 10 min to examine kinetics (Figure 2d). Interestingly, both Y221 and Y251 were phosphorylated at the earliest time point examined (5 s).

Bcr-Abl induces phosphorylation of endogenous Crk at Y251 in CML cell lines, and EGF induces phosphorylation of endogenous Crk at Y251 in EGFR-expressing A431 and MDA-MB-468 cells

To investigate whether phosphorylation at Y251 could be detected in CML cell lines in which Crk is expressed endogenously, we analyzed five different cell lines, four of which (K562, KCL22, LAMA84 and MEG01) were derived from CML patients in blast crisis (Figure 3a). In all lines, phosphorylation of hCrk at Y251 was detectable in addition to phosphorylation at Y221 by western blotting with the anti-phospho (Y251) and the anti-phospho (Y221) antibodies, whereas no phosphorylation was detectable in unstimulated 293T cells. To test whether phospho (Y251) was labile to Bcr-Abl inhibition by Imatinib (Novartis, Boston, MA, USA), either K562 cells or 32D cells expressing wild type (WT) Bcr-Abl (imatinib-sensitive) or T315I Bcr-Abl (imatinib-insensitive) were treated with 5 μM imatinib for 2 h. As shown in Figure 3b, imatinib treatment resulted in reduced phosphorylation at Y251 inWT Bcr-Abl-expressing 32D cells but not in 32D cells expressing T315I Bcr-Abl, thus suggesting that Bcr-Abl induces phosphorylation of Crk at Y251 in CML cell lines. We also examined phosphorylation of Crk upon EGF stimulation in MDA-MB-468 (a human breast cancer cell line) and in A431 (a human vulvo-epithelial carcinoma cell line) cells. Cells were serum starved and stimulated with EGF for 1, 5 and 30 min. There was basal phosphorylation of Crk at Y251, which was enhanced upon EGF treatment at the earliest time point examined (1 min, Figure 3c, Supplementary Figure S1). We also observed phosphorylation of Crk at Y221 upon EGF stimulation. Interestingly, and in contrast to Bcr-Abl-expressing cells, pretreatment with Imatinib did not abrogate EGF-induced phosphorylation at Y251 suggesting that, in MDA-MB-468 and A431 cells, kinases other than Abl may impinge on phosphorylation of Crk at Y251 after EGF stimulation (Figure 3d). To test this further, we co-expressed WT Crk or the W170K mutant (that renders the Crk SH3N defective in binding to PPII motifs) with Abl or EGFR in 293T cells. As shown in Figure 3e, the W170K mutant exhibits greatly reduced phosphorylation at Y251 and Y221 when co-expressed with Abl (left panel) but not when co-expressed with EGFR and stimulated with EGF (right panel), suggesting that kinases other than Abl, downstream of EGF stimulation, can impinge on phosphorylation of Crk at Y251 by an SH3N-independent mechanism.

Phospho (Y251)-derived peptides from hCrk bind directly to the SH2 domain of Abl

To explore the biological function of phospho (Y251), we screened an SH2 domain library with a chemically synthesized N-terminally biotinylated 16-mer phosphopeptide with a centrally located phosphotyrosine residue (Biotin-LC-KRVPNApY^{251}DKTALALE, which will be referred to as pY251) complementary to the phospho (Y251) site in the RT loop. pY251 was diluted in a rosette-loading buffer and spotted onto gelatin-coated nitrocellulose membranes (Figure 4a) in register with the wells of a 96-well plate after which each well was incubated with a different GST-SH2 domain probe (labeled with GSH-horse radish peroxidase). The summary of the quantified data from replicate experiments showed that

SH2 domain binding was relatively modest and selective from the 96 SH2 domains tested in the assay (Figure 4b) with specificity towards Arg, ShcB, Brdg, Abl and SHIP2. We further examined binding of pY251 to the Abl SH2 domain in a streptavidin–biotin pull-down assay. Biotinylated pY251 or the unphosphorylated peptide Y251 was incubated with GST-Abl SH2 and pY221 with GST-Crk SH2 (as a positive control for the assay) subsequent to which streptavidin–agarose beads were added to achieve a pull down. The fraction bound to beads in each case was analyzed by western blotting with an anti-GST antibody. GST-Abl SH2 bound to pY251 and not to the unphosphorylated peptide Y251 or to beads (pY251 did not bind to GST, lane 6). Furthermore, the Abl SH2–pY251 interaction seemed to be weaker than the Crk SH2–pY221 interaction (Figure 4c, compare lanes 3 and 9). Finally, we examined the Abl SH2–pY251 interaction by isothermal titration calorimetry (Supplementary Figure S2). Consistent with the above results, a weak interaction between the phosphopeptide pY251 and Abl SH2 was detectable by isothermal titration calorimetry (K_d = 85.5 μM).

Phospho (Y251) is directly involved in the transactivation of Abl 1b

We next examined the contribution of phosphorylated Y251 of human Crk on transactivation of Abl by co-expressing wild-type or Y251F hCrk with Abl 1b in 293T cells. We also co-expressed Y221F (which has been previously shown to transactivate Abl (Shishido *et al.*, 2001)), Y239F, Y221F/Y239F and Y221F/Y251F hCrk mutants with Abl 1b. Abl activation was examined by western blotting with an anti-phospho (Y245) Abl antibody. Y251F and Y221F/Y251F mutants transactivated Abl to a lesser extent than did wild-type hCrk and the Y221F mutant, respectively (Figure 5a). Importantly, in five independent experiments, Y251F hCrk significantly abrogated Abl transactivation (Figure 5b). We next examined transactivation of Abl 1b by preincubating immunoprecipitated Abl with purified GST, GST-hCrk or GST-hCrk Y251F, followed by an *in vitro* kinase assay and western blotting with an anti-phospho (Y245) antibody (Figure 5c). The Y251F mutant showed a significantly diminished ability to transactivate Abl compared with wild-type Crk (the addition of which was sufficient to significantly activate Abl). Furthermore, GST-hCrk failed to transactivate the Abl SH2 domain mutant, R171L (Supplementary Figure S3), suggesting that phospho (Y251) on Crk was directly involved in Abl transactivation by SH2 domain displacement.

Finally, to examine transactivation of Abl by phospho (Y251) of hCrk, Abl 1b was overexpressed and immunoprecipitated from 293T cells. Immunoprecipitated Abl was preincubated with phosphopeptide pY251 derived from the RT loop of SH3C of hCrk or the corresponding unphosphorylated peptide subsequent to which an *in vitro* kinase assay was performed and autophosphorylation of Abl at Y245 and Y412 was examined by western blotting with anti-phospho (Y245) Abl and anti-phospho (Y412) Abl antibodies. As shown in Figure 5d, preincubation with pY251 resulted in enhanced autophosphorylation of Abl 1b at Y245 and Y412, which are indicative of Abl activation. Taken together, these results suggest that phosphorylated Y251 in the SH3C of hCrk binds to the SH2 domain of Abl and is likely to be directly involved in transactivation of Abl 1b by SH2 domain displacement.

Discussion

The ability of Crk to function as an adaptor protein is negatively regulated and terminated by phosphorylation on Y221, which results in an intramolecular SH2-pTyr clamp, thereby resulting in the disassembly of Crk-mediated signaling complexes (Feller *et al.*, 1994; Rosen *et al.*, 1995; Kobashigawa *et al.*, 2007). Here, we show that in addition to Y221, another tyrosine residue, namely Y251, located within the highly conserved RT loop of the SH3C domain of Crk is also concomitantly phosphorylated by the Abl and Bcr-Abl tyrosine kinases. Our present data amend our current understanding of the role that tyrosine

phosphorylation of Crk has in signal transduction, and clearly point to more elaborate and dynamic regulatory networks controlling the interaction between Crk and Abl.

Prompted by observations that Y221F Crk is phosphorylated by Abl using *in vitro* kinase assays, we set out to identify additional tyrosine phosphorylation sites on Crk. As the PNAY motif in the RT loop of SH3C was essential for Crk-mediated Abl transactivation (Reichman *et al*., 2005), we focused on Y251 which was a part of the PNAY sequence. In this study, we found that Y251 is phosphorylated by Abl, in Bcr-Abl-expressing CML cells and in A431 and MDA-MB-468 cells stimulated with EGF. In addition, we show that a 16-mer phosphopeptide flanking Y251 on Crk binds in *trans* to Abl SH2, and in doing so, stimulates the kinase activity of Abl. Consistent with this interpretation, co-expression of the Y251F Crk mutant with Abl 1b partially suppressed Abl activation, and also purified GST-hCrk Y251F had a significantly attenuated ability to transactivate Abl compared with GST-hCrk. In addition, GST-hCrk failed to transactivate the Abl SH2 domain mutant R171L, suggesting that SH2 displacement by phospho (Y251) comprises one important part of the mechanism for Abl transactivation by hCrk.

Despite the fact that pY251 binds selectively to the Abl SH2 domain, it is noteworthy that the sequence around phospho (Y251) in hCrk ([pY^{251}DKT]) does not conform to the experimentally determined consensus peptide-binding motif for the Abl SH2 domain (pY[E/T/M][N/E/D][P/V/L]) (Birge *et al*., 1993; Songyang *et al*., 1993), In addition, Abl SH2 was not the strongest binding partner of pY251 in the SH2 domain screen (four-fold lower binding than the Arg SH2 in the assay), and isothermal titration calorimetry revealed a low-affinity interaction between pY251 and Abl SH2. However, as Crk binds to Abl via the SH3N domain (Feller *et al*., 1994; Ren *et al*., 1994), phospho (Y251) on hCrk and Abl SH2 would be expected to be present at high local concentrations in Abl–Crk complexes that may override the apparent low affinity and drive binding of the phospho (Y251) motif to Abl SH2. Interestingly and consistent with this notion, we observe a modest reduction in the amount Abl that co-immunoprecipitates with hCrk Y251F compared with WT hCrk when each is co-expressed with Abl in 293T cells (Supplementary Figure S4), suggesting that the phospho (Y251)–Abl SH2 interaction, in addition to the Crk SH3N–Abl PXXP interaction, contributes to the stoichiometry of binding in Abl–Crk complexes.

Using *in vitro* kinase assays to reconstitute Crk and Abl *in vitro*, we observed that both Y221 and Y251 were phosphorylated at the earliest time point (5 s) examined. Therefore, it is not clear at the molecular level whether phosphorylation at Y251 (the transactivating phosphorylation) precedes phosphorylation at Y221 (the autoinhibition phosphorylation). Although it might be predicted that transactivation precedes inhibition, the present data suggest that both events occur quite rapidly and are likely in dynamic equilibrium, possibly in a manner to fine-tune Abl activation and its concomitant inhibition. Our data also imply that a pY221/pY251 dual phosphorylated species of Crk exist in cells. This predicts an interesting scenario in which Crk pY221/pY251 could dissociate from Abl by virtue of the negative regulation and pY251 binds in *trans* to other SH2/PTB-containing proteins. Indeed, in SH2 profiling screens, we found evidence that pY251 binds in *trans* to other SH2 domains selectively, which include ShcB, Brdg1 and Ship2. This interpretation would be consistent with previous reports that pY221 Crk retains biological activity (Abassi and Vuori, 2002), possibly by virtue of its ability to engage in new protein complexes after dissociation from Abl. Clearly, the identification of *in vivo* pY251 Crk containing complexes is an important future endeavor.

An equally important area will be the identification of additional tyrosine kinases, besides Abl, which can phosphorylate Y251. Using bioinformatic tools such as NetPhos (ExPaSy Proteomics Server, Swiss Institute of Bioinformatics, http://expasy.org), no kinases were

predicted to phosphorylate Y251, although in our study, we show that both EGFR and Abl can induce phosphorylation at Y251, at least when these kinases are overexpressed in cancer cells. Hence, our expectation is that Abl will not be the sole kinase capable of phosphorylating Y251 *in vivo*, but rather we anticipate that multiple kinases may converge on this motif, hence integrating multiple upstream pathways with Abl. Recent evidence suggests that Abl is activated in aggressive breast cancer cell lines (includingMDA-MB-468) and possibly promotes cell invasion (Srinivasan and Plattner, 2006; Srinivasan *et al.*, 2008). In light of our results that reveal phosphorylation of Crk at Y251 upon EGF stimulation of MDA-MB-468 cells and the fact that Abl is activated downstream of activated EGFR (Plattner *et al.*, 1999; Jones *et al.*, 2006), phospho (Y251) on Crk may be an important mediator of Abl activation downstream of EGFR. However, as activated EGFR also phosphorylates Crk at the negative regulatory tyrosine Y221 (Hashimoto *et al.*, 1998), the relative stoichiometry of phosphorylation at Y251 and Y221 might be critical as a high pY251/pY221 ratio on Crk typically induced by activated EGFR could favor Abl activation, and may resolve the issue of when Abl induces a tumor-suppressing signal (Noren *et al.*, 2006) versus a tumor-promoting signal (Srinivasan and Plattner, 2006). In addition, it also remains to be determined whether Y251 is phosphorylated in human cancers as Crk has been shown to be overexpressed in several human cancers and knockdown experiments suggest that at least one of its roles is to promote cell migration (Nishihara *et al.*, 2002; Miller *et al.*, 2003; Rodrigues *et al.*, 2005; Linghu *et al.*, 2006; Wang *et al.*, 2007; Fathers *et al.*, 2010). Notably, in Crk (−/−) mouse embryonic fibroblasts (MEFs) stably overexpressing enhanced yellow fluorescent protein (EYFP), EYFP-hCrk or EYFP-hCrk Y251F, WT hCrk and the Y251F mutant equally enhanced cell spreading on fibronectin, and additionally, there was no significant difference in cell migration towards high serum in a transwell migration assay between WT hCrk- and Y251F-expressing cells (data not shown), suggesting that the Y251F mutant does not behave as a dominant-negative protein. Phosphorylation of hCrk at Y251 could be a gain-of-function modification downstream of specific stimuli such as EGF, by means of which non-canonical signaling pathways are engaged by Crk.

Our present observations may also have relevance to explain functional differences between Abl activation by Crk versus CrkL. It is intriguing that the $PNAY^{251}$ motif in Crk SH3C is unique in all 266 known SH3 domains, and even diverges to a PCAY motif in CrkL. In addition to the PNAY in the RT loop of CrkSH3C, previous studies from Pawson's laboratory showed that the hCrk SH2 domain contains an extended loop (called the DE loop) that contains a PRPP motif, which binds in *trans* to the Abl SH3 domain (Anafi *et al.*, 1996). As such, double occupancy of the Abl SH2 domain with pY251 (in the RT loop of SH3C) and that of the Abl SH3 domain (by PRPP in the DE loop of the SH2 domain) may be required for full activation of Abl by hCrk. It is also noteworthy that such a proline-rich insert is lacking in CrkL, suggesting another important difference between Crk and CrkL (Figure 6), and it will be interesting to test the combined effects of pY251 and PRPP peptides in the aforementioned kinase assays to ascertain cooperation between these motifs in Crk.

Materials and methods

Reagents

High-performance liquid chromatography-purified biotin LC-phosphopeptides pY221 (Biotin-LC-GPEPGPpYAQPS VNTP) and pY251 (Biotin-LC-KRVPNApYDKTALALE) were purchased from Anaspec Inc. (San Jose, CA, USA). Anti-Crk and anti-Abl were purchased from Sigma (St Louis, MO, USA) and Calbiochem (Gibbstown, NJ, USA), respectively. Anti-phospho (Y245) Abl, anti-phospho (Y412) Abl and anti-phospho (Y221) Crk were purchased from Cell Signaling Technology. Anti-GST antibodies were obtained

from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Streptavidin– agarose beads were from Pierce Scientific (Rockford, IL, USA). Glutathione–sepharose beads were purchased from GE Healthcare (Piscataway, NJ, USA). Anti-Crk RF51 antisera have been described previously (Reichman *et al.*, 2005). Imatinib (trade name: Gleevec) was obtained from Novartis. Purified Abl (beginning at the second exon-encoded sequence) was provided by Dr Koleske, and purified Abl 1a (amino acid 47 to end) was provided by Dr Leszek Kotula (Xiong *et al.*, 2008; Dubielecka *et al.*, 2010). B210 cells were obtained from George Daley at Children's Hospital (Boston, MA, USA). K562 cells were purchased from ATCC (Manassas, VA, USA), whereas the KCL22, LAMA84 and MEG01 cell lines were provided by Dr Riccardo Alessandro (University di Palermo, Italy).

Cloning and mutagenesis

Plasmids for the expression of hCrk or cCrk have been described previously (Zvara *et al.*, 2001; Reichman *et al.*, 2005). Y251 and Y221 in hCrk are numbered Y252 and Y222, respectively, in cCrk. For clarity, we will refer to the tyrosine residue in the PNAY motif as Y251 and the negative regulatory tyrosine as Y221 throughout this paper. pEBB plasmids containing Crk mutant DNAs were generated using the PCR-based Quikchange mutagenesis system (Stratagene, La Jolla, CA, USA). The following hCrk mutants were generated in this study: Y221F, Y251F, Y221F/Y239F and Y221F/Y251F. The following chicken Crk mutants were generated: Y221F/Q275A, Y221F/P249A and Y221F/Y251A. The cCrk mutants W170K and Y221F have been described earlier (Escalante *et al.*, 2000). For *in vivo* studies, the murine Abl type IV or human c-Abl 1b (WT and R171L) (provided by Dr Giulio Superti-Furga) was used (Hantschel *et al.*, 2003).

GST-fusion proteins

GST, GST-cCrk, GST-cCrk Y221F, GST-hCrk Y251F and GST-Crk SH2 were expressed from pGEX vectors and purified as described previously (Reichman *et al.*, 2005). pGEX2T encoding GST-hCrk was provided by Dr Michiyuki Matsuda (University of Kyoto, Japan) (Matsuda *et al.*, 1992). pGEX encoding GST-Abl SH2 was provided by Dr Leszek Kotula.

In vitro kinase assays

In vitro kinase assays were carried out in one of the following methods. In the first method, purified Abl 1a was incubated with a 100 molar excess of GST or GST-Crk proteins in a kinase buffer (HNTG buffer containing 0.1% Triton X-100, 10mM $MgCl_2$, 100mM ATP and 5 μCi [γ^{32}P] ATP (3000 Ci/mmol)) (Tanis *et al.*, 2003; Reichman *et al.*, 2005). After 30 min mixing at RT, reactions were terminated by the addition of SDS– PAGE sample buffer. Reactions were examined by separating proteins by SDS–PAGE and exposing the gels directly to film or to a phosphoimager plate, and by quantification using a Typhoon Storm Phosphoimager (Amersham Biosciences Corp., Piscataway, NJ, USA). In a second method, purified Abl 1a (amino acid 47 to end) was incubated with a 100-fold molar excess of GST-hCrk in a kinase assay buffer (20mM Tris-Cl pH 7.5, 10mM $MgCl_2$, 1mM DTT (dithiothreitol)) in the presence of 0.1mM ATP for various times. Reactions were terminated by addition of SDS sample buffer. In a third method, immunoprecipitated Abl 1b was preincubated with 100 μM peptide (pY251 or Y251) or 8.75 μM GST-hCrk proteins in a kinase assay buffer. ATP was added to 0.2mM and reactions were incubated at 25 °C for 10 or 20 min. SDS sample buffer was added to terminate each reaction.

Cell culture and DNA transfection

293T cells were maintained in Dulbecco's modified Eagle's medium (Cellgro, Manassas, VA, USA) (4.5 g of glucose/l with L-glutamine) supplemented with 10% fetal calf serum. Interleukin-3-independent Bcr-Abl or T315I Bcr-Abl expressing 32D cells (Johnson *et al.*,

2009) (kindly provided by Kara Johnson and Brain Deninger, Oregon) were maintained in RPMI 1640 containing 10% fetal calf serum. Where indicated, Bcr-Abl-expressing cells were treated with 5μM imatinib (Novartis) for 2 h. Human CML lines K562, KCL22, LAMA84 andMEG01 were cultured in RPMI 1640 containing 10% fetal calf serum. 293T cells were transfected with 500 ng plasmid DNA using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's protocols.

Production of phosphospecific antibodies specific for phospho (Y251) in Crk

Phospho-(Y251) Crk antibody was generated by immunizing rabbits with a synthetic 14-mer phosphopeptide corresponding to residues surrounding Y251 of hCrk. Antibody was purified by positive and negative peptide-affinity chromatography. All phosphospecific antibodies were analyzed by phosphopeptide competitions using 10 μg/ml of peptides included in the western blots.

Western blotting

Western blotting was performed after SDS–PAGE and transferred to nitrocellulose (Bio-Rad, Hercules, CA, USA) or polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Densitometry was performed using the GeneTools software (Syngene, Frederick, MD, USA).

Immunoprecipitation

Cell lysates prepared in the HNTG buffer (20mM Hepes (pH 7.4), 150mM NaCl, 1% Triton X-100, 10% glycerol) or the Sigma buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, 1mM EDTA, 1% Triton X-100) supplemented with 1mM sodium orthovanadate, 1mM sodium molybdate, 1mM phenylmethylsulfonyl fluoride and aprotinin were immunoprecipitated with anti-Abl antibodies as described previously (Hantschel *et al.*, 2003).

SH2 domain arrays and screens

To select high-affinity SH2 domain-binding partners of pY251, the phosphopeptide was first dissolved (10 mg/ml) in a suitable solvent (distilled water) after which it was diluted with the rosette-loading buffer and spotted onto gelatin-coated BA79 nitrocellulose membranes. SH2-binding assays were performed using GSH-HRP-labeled GST-SH2 probes as described previously (Machida *et al.*, 2007).

Streptavidin–biotin pull down assays

GST-Abl SH2, GST or GST-Crk SH2 at 2.5 μM was incubated with pY251, Y251 or pY221, respectively, at 25 μM in phosphate-buffered saline containing 2mM DTT for 150 min at 4 °C. In all, 20 μl streptavidin–agarose beads and 25 μg bovine serum albumin were added to each peptide-fusion protein mix and incubated at 4 °C for 60 min. The beads were then spun down and washed three times with the HNTG buffer containing 0.1% Triton X-100 and 2mM DTT. Each sample was then boiled in SDS sample buffer.

Statistical analysis

The results of some experiments are the mean ± s.e.m. of three to five experiments. One-way ANOVA (analysis of variance) with Tukey's test was performed using GraphPad InStat Version 3 (GraphPad, La Jolla, CA, USA) or SPSS for Windows (SPSS Inc., New York, NY, USA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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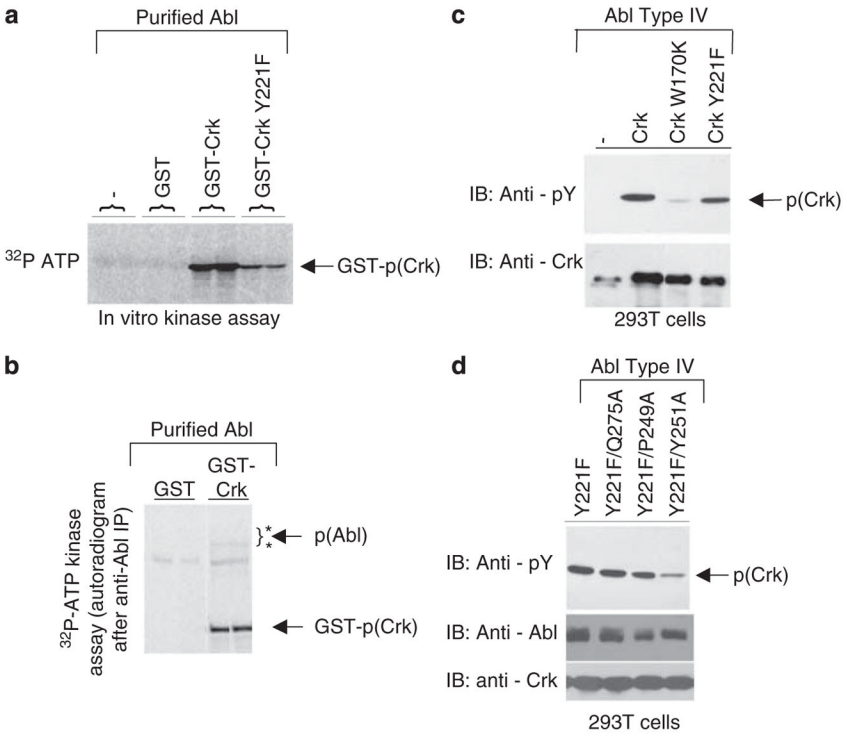

Figure 1.

Crk is tyrosine phosphorylated at sites other than Y221 by the Abl kinase. (**a**) Equivalent molar concentrations of GST, GST-cCrk or GST-cCrk Y221F were incubated with purified Abl (beginning at the second exon-encoded sequence) in an *in vitro* kinase assay after which reactions were analyzed by SDS–PAGE and autoradiography. (**b**) Immunoprecipitation of the reaction mix in panel **a** was accomplished with anti-Abl antibodies, and immune complexes were analyzed by SDS–PAGE and autoradiography. In the upper region, phosphorylated and hyperphosphorylated Abl are indicated by asterisks. (**c**) Lysates of 293T cells co-transfected with mouse Abl type IV and cCrk plasmids indicated at the top of the blot were immunoblotted with either anti-pY (top) or anti-Crk RF51 antisera (bottom). (**d**) 293T cells were co-transfected with mouse Abl type IV and the respective cCrk plasmids containing the indicated mutations in the PNAY motif, and immunoblotted as in panel **c**.

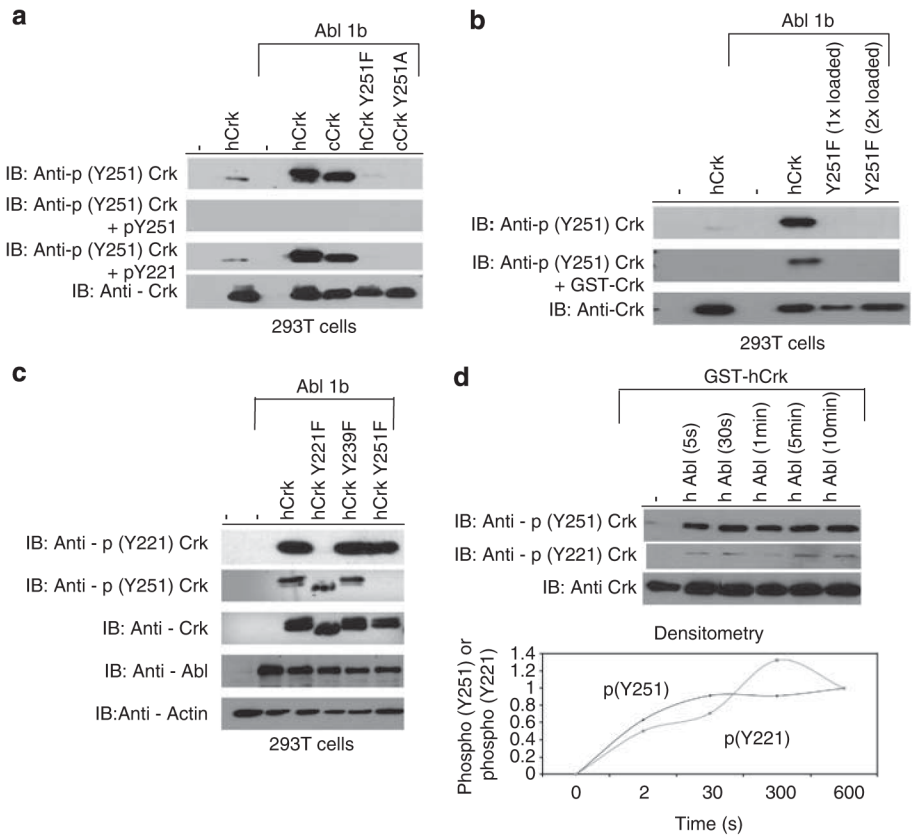

Figure 2.

Characterization of affinity-purified anti-phospho (Y251) Crk-specific antisera. (**a**) Lysates of 293T cells transfected with the indicated plasmid DNAs were immunoblotted with anti-phospho (Y251) Crk antisera (top panel) or anti-Crk antisera (lowermost panels). In the second and third panels, the anti-phospho (Y251) antibody was co-incubated with 10 μg/ml pY251 or 10 μg/ml pY221 phosphopeptides, respectively. (**b**) Same as panel **a**, but the anti-phospho (Y251) antisera were co-incubated with excess GST-Crk. Anti-Crk blot is shown in the lowest panel. (**c**) Lysates of 293T cells transfected with the indicated hCrk plasmids were immunoblotted with the indicated phospho-specific antibodies (upper panels) or anti-Abl and anti-Crk antibodies (lower panels). Anti-actin blot served as a loading control (lowest panel). (**d**) GST-hCrk and purified Abl 1a (amino acid 47 to end) were co-incubated at a molar ratio of 100:1 in an ATP-containing kinase buffer for up to 10 min at RT. At the indicated times, reactions were terminated and immunoblotted with phospho-specific antibodies as indicated. Anti-Crk blot is shown in the lowest panel. Quantification is shown below (the '1 min' time point was omitted). A full colour version of this figure is available at the *Oncogene* journal online.

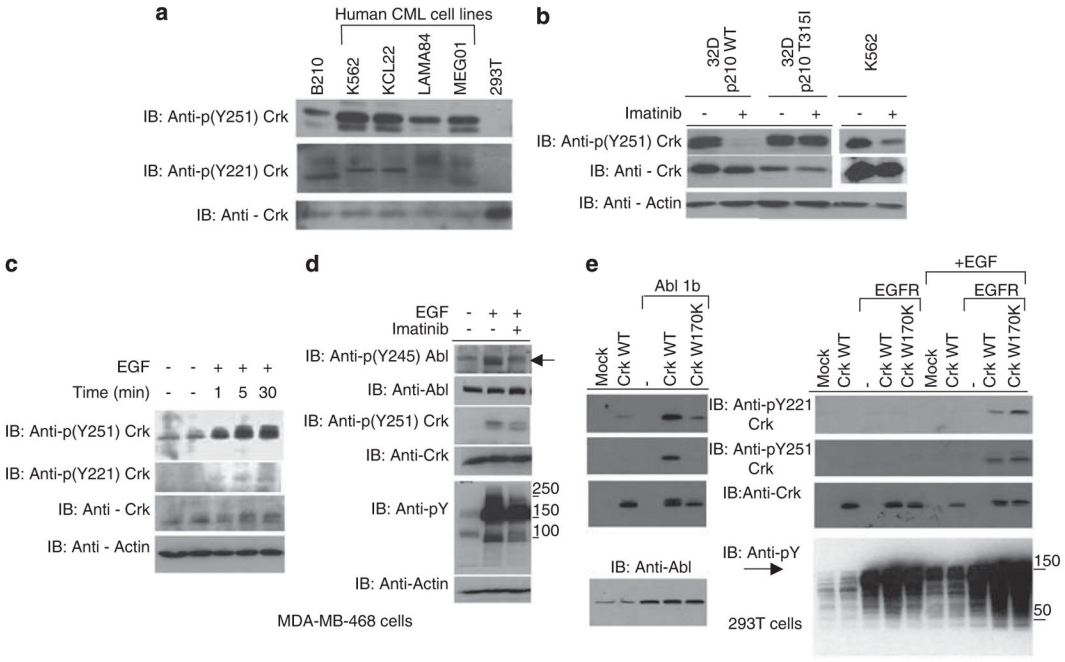

Figure 3.

Crk is tyrosine phosphorylated in CML cells and after stimulation of MDA-MB-468 cells with EGF. (**a**) Lysates of B210, K562, KCL22, LAMA84, MEG01 and 293T cell lines were immunoblotted with anti-phospho (Y251), anti-phospho (Y221) or anti-Crk antisera. B210 is derived from the mouse cell line Ba/F3 by stably transfecting p210 Bcr-Abl. (**b**) Lysates of WT Bcr-Abl- or T315I-Bcr-Abl-expressing 32D or K562 cells treated with imatinib (+) or DMSO (−) were immunoblotted with anti-phospho (Y251) or anti-Crk antisera. Anti-actin blot served as a loading control. (**c** and **d**) MDA-MB-468 cells were serum starved overnight and then stimulated with EGF for 1, 5 and 30 min as in panel **c** or pretreated with imatinib and stimulated with EGF for 10 min (right). Lysates were immunoblotted with anti-phospho (Y251) Crk, anti-phospho (Y221) Crk, anti-phospho (Y245) Abl, anti-phosphotyrosine, anti-Abl or anti-Crk antisera. Anti-actin blot served as a loading control. (**e**) 293T cells were transfected with the plasmids indicated. On the right, cells were serum starved overnight 48 h after transfection and then stimulated with EGF for 10 min. Lysates were immunoblotted with anti-phospho (Y221) Crk, anti-phospho (Y251) Crk, anti-phosphotyrosine, anti-Crk or anti-Abl antisera.

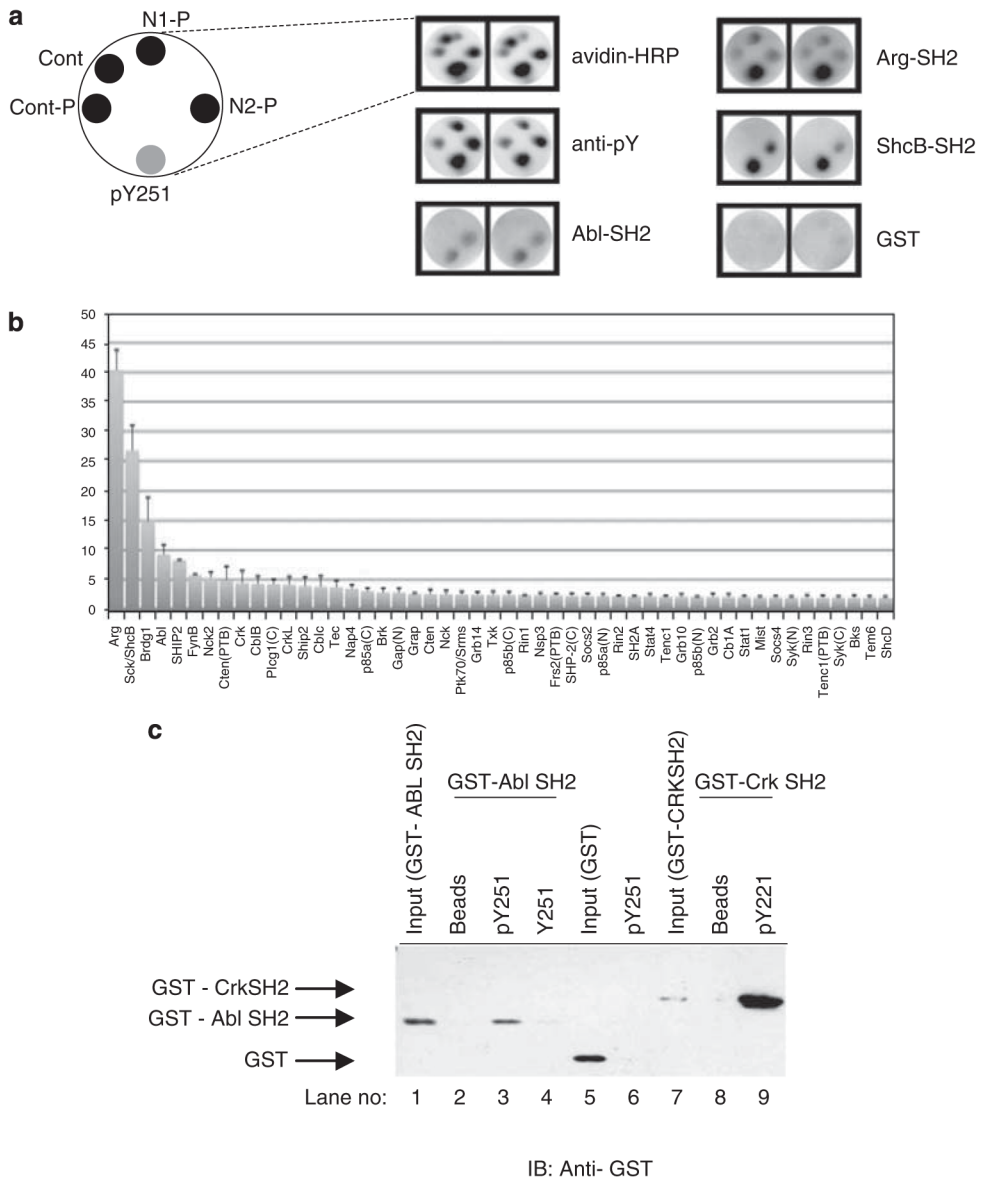

Figure 4.

SH2 profiling to determine binding partners of the phosphopeptide pY251. (**a**) The design of the rosette assay is shown, whereby biotinylated pY251 was spotted onto gelatin-coated nitrocellulose (N1-P, N2-P and Cont-P are nonspecific phosphopeptides, whereas ‘Cont’ is the unphosphorylated form of ‘Cont-P’). Spotting efficiency was verified using anti-pY antisera or avidin HRP. Wells incubated with Abl, Arg ShcB SH2 and GST are shown. (**b**) Summary of four independent experiments is shown. (**c**) Purified GST-Abl SH2, GST or GST-cCrk SH2 proteins were incubated with biotinylated pY251 or Y251 or pY221 peptides. A pull down was subsequently achieved using streptavidin–agarose beads, followed by analysis of the bound fraction by western blotting with anti-GST antibodies. Representative of three experiments is shown.

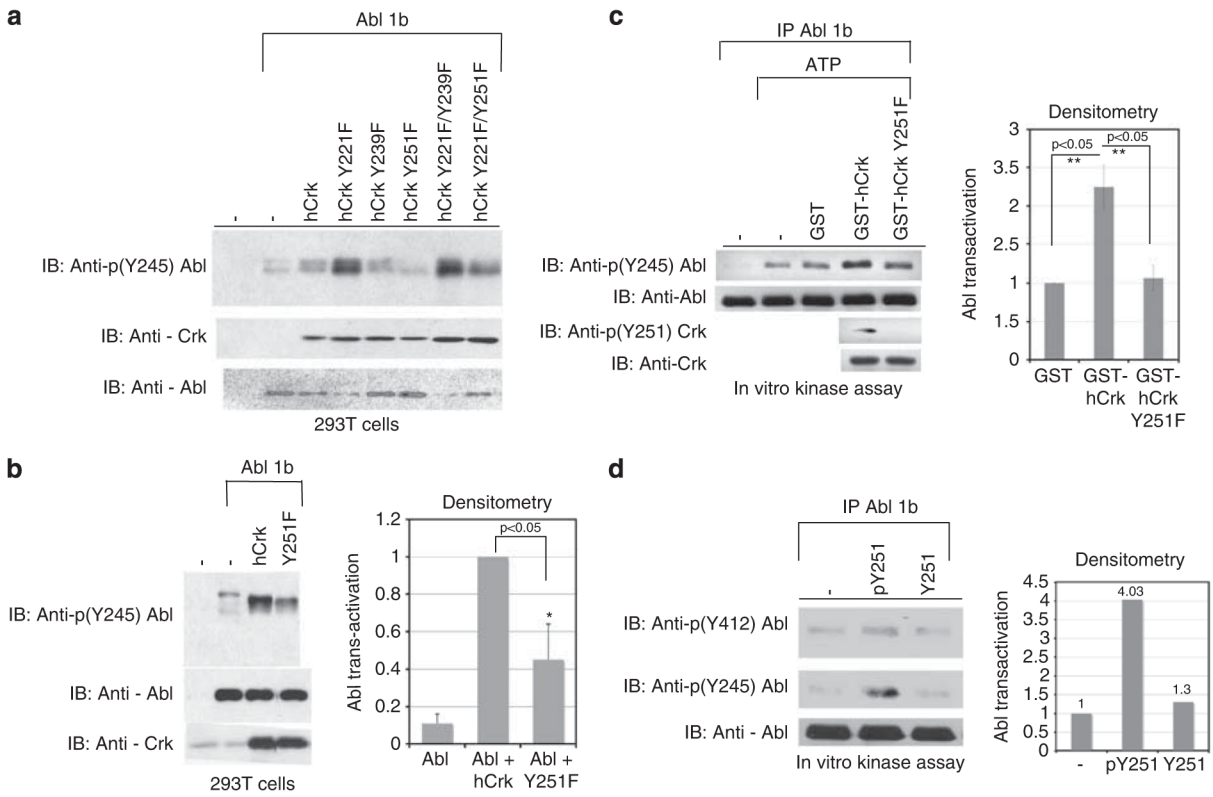

Figure 5.

Crk activates Abl 1b through phospho (Y251). (**a**) Lysates of 293T cells co-transfected with Abl 1b and the indicated hCrk mutants were immunoblotted with anti-phospho (Y245) (upper panel), anti-Crk (middle panel) or anti-Abl antibodies (lower panel). (**b**) A representative immunoblot showing the effect of Y251F substitution on Abl activation in 293T cells is shown. Quantification (±s.e. of five independent experiments) is shown. (**c**) Abl 1b was overexpressed in 293T cells, immunoprecipitated and then preincubated with the indicated proteins, followed by an *in vitro* kinase assay. Samples were immunoblotted with anti-phospho (Y245) (top panel), anti-Abl (second panel), anti-p (Y251) Crk or anti-Crk antibodies (lower panels). Representative of four independent experiments is shown with quantification. (**d**) Abl 1b was overexpressed in 293T cells, immunoprecipitated and then preincubated with pY251 or Y251 peptides, followed by an *in vitro* kinase assay. Samples were immunoblotted with anti-phospho (Y412) (upper panel), anti-phospho (Y245) (middle panel) and anti-Abl (lower panel) antibodies. Quantification of the middle panel is shown as an average of two independent experiments.

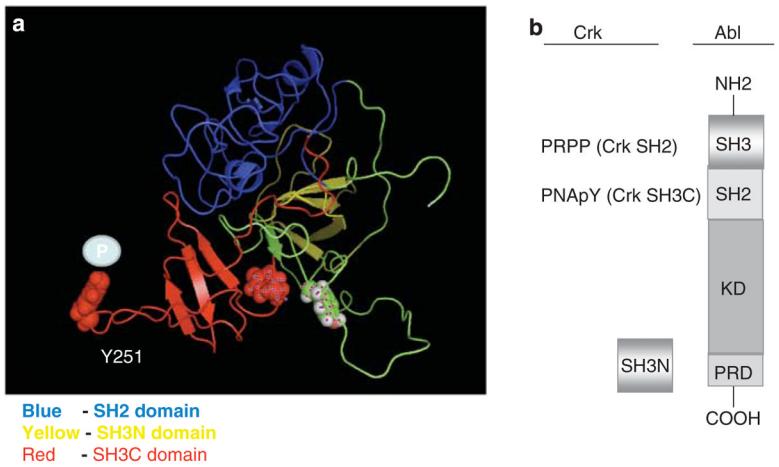

Figure 6.

Model for the transactivation of Abl 1b by Crk. (**a**) Location of Y251 in Crk based on the NMR-derived structure (PDB ID: 2EYZ). (**b**) Multiple Crk/Abl interactions and the proposed mechanism for Crk-mediated Abl transactivation is shown.