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Regulation of Cbl phosphorylation by the Abl tyrosine kinase and the Nck SH2/SH3 adaptor

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The Cbl proto-oncogene product is tyrosine phosphorylated in response to a wide variety of stimuli. Cbl and the Abl nonreceptor tyrosine kinase both bind to SH3 domains from the SH2/SH3 adaptor Nck, and are candidate effectors for Nck function. Numerous additional SH2- and SH3-domain-mediated interactions are also possible between Cbl, Abl, and Nck. We find that these three signaling proteins associate when overexpressed in mammalian cells and can regulate each other's activity. Co-expression of wt Cbl together with c-Abl, the activity of which is normally repressed *in vivo*, led to extensive Abl-dependent phosphorylation of Cbl. The major proline-rich region of Cbl was required for its phosphorylation by c-Abl, but not by a constitutively activated Abl mutant, suggesting Cbl activates c-Abl by engaging its SH3 domain. Efficient phosphorylation of Cbl and its stable association with Abl required the SH2 domain of Abl, suggesting that SH2-phosphotyrosine interactions prevent dissociation of active Abl from Cbl. We also show that overexpression of Nck could repress the phosphorylation of Cbl by Abl *in vivo*. Studies with Nck mutants suggested that the Nck SH2 domain is responsible for inhibiting the activity of Abl toward both Cbl and Nck itself, most likely by competing with the Abl SH2 for tyrosine-phosphorylated binding sites. *Oncogene* (2001) **20,** 4058–4069.

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Introduction

The interaction of signaling proteins plays a fundamental role in the transmission of signals regulating the growth and differentiation of complex eukaryotes. These interactions are often mediated by modular protein-interaction domains such as the SH2 domain, which binds tyrosine-phosphorylated proteins, and the SH3 domain, which binds proline-rich target peptides (Pawson and Scott, 1997). Protein interaction domains are found in enzymes that are regulated during signaling, such as protein kinases, and in adaptor proteins which do not contain catalytic domains but which mediate the association or relocalization of other proteins to which they bind.

The Abl non-receptor tyrosine kinase was originally reported as the transforming gene from Abelson murine leukemia virus (Goff *et al*., 1980). The 130 kDa proto-oncogene form of c-Abl is localized in both the nucleus and cytoplasm, and consists of an SH3, SH2, and tyrosine kinase catalytic domain, followed by a long C-terminal segment containing nuclear localization signals, nuclear export signal, a DNA-binding domain, actin-binding domains, and proline-rich regions implicated in SH3 domain binding (reviewed in Van Etten, 1999; see Figure 1). Abl is involved in human leukemias, where chromosomal translocation resulting in generation of a fusion protein with sequences from the *bcr* locus is associated with chronic myelogenous leukemia (Mes-Masson *et al*., 1986; Shtivelman *et al*., 1985). c-Abl knockout mice die soon after birth (Schwartzenberg *et al*., 1991; Tybulewicz *et al*., 1991), and the double knockout for both Abl and the highly related Arg gene results in early embryonic lethality, suggesting an important role for these kinases in early development (Koleske *et al*., 1998).

Normally c-Abl activity is tightly repressed, as overexpression does not lead to efficient transformation or increased tyrosine phosphorylation of cell proteins (Franz *et al*., 1989; Jackson and Baltimore, 1989). This negative regulation is mediated at least in part by the SH3 domain, as deleting, mutating, or altering the position of the SH3 is sufficient to activate the kinase and transforming activities of Abl (Franz *et al*., 1989;

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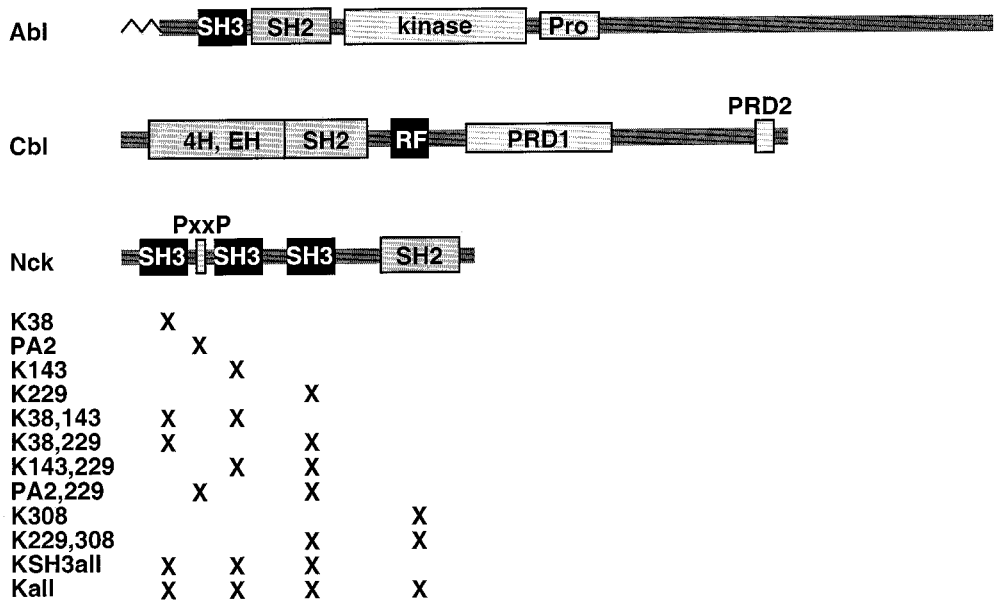

Figure 1 Structure of Abl, Cbl and Nck mutants used in this study. Each molecule is drawn approximately to scale. SH3, SH2 and kinase domains and proline-rich SH3-binding site (Pro) of Abl are indicated. Four-helix (4H), EH, SH2, ring finger (RF), and proline-rich domains (PRD1 and PRD2) of Cbl are indicated. SH3 and SH2 domains and putative SH3 binding site (PxxP) of Nck are indicated; below diagram, for each Nck mutant indicated the domains marked with an 'X' contain a point mutation designed to disrupt ligand binding

Jackson and Baltimore, 1989; Mayer and Baltimore, 1994; Van Etten *et al*., 1995). The precise mechanism whereby the SH3 domain regulates the kinase activity of Abl is still unknown, however. One model proposes the existence of a cellular SH3-binding inhibitor of Abl. There are several reports describing proteins that interact with the SH3 domain of Abl including Abi-1, Abi-2, AIP and PAG (Dai and Pendergast, 1995; Shi *et al*., 1995; Wen and Van Etten, 1997; Zhu and Shore, 1996). An intramolecular interaction between the Abl SH3 and the SH2-catalytic domain linker was also suggested (Barilá and Superti-Furga, 1998), consistent with a model in which Abl is regulated by intramolecular SH3-mediated interactions in a fashion similar to that seen in the Src family kinases (Moarefi *et al*., 1997; Sicheri *et al*., 1997; Xu *et al*., 1997).

The Cbl adaptor protein was originally described as the cellular form of the oncogene of the CAS NS-1 retrovirus (Blake *et al*., 1991; Langdon *et al*., 1989). Genetic studies in *C. elegans* and *Drosophila* suggest that Cbl is a negative regulator of receptor tyrosine kinases (Meisner *et al*., 1997; Yoon *et al*., 1995), and other studies have suggested that Cbl might have a similar negative effect on nonreceptor kinases (Ota and Samuelson, 1997). The mechanism of inhibition is likely to involve ubiquitination of Cbl-associated proteins, as Cbl has been found to be a component of a ubiquitin ligase complex (Joazeiro *et al*., 1999; Levkowitz *et al*., 1999; Waterman *et al*., 1999; Yokouchi *et al*., 1999). The 120-kDa wt Cbl protein contains an N-terminal atypical SH2 domain (Meng *et al*., 1999), multiple proline-rich potential SH3 domain binding sites, and multiple potential tyrosine phosphorylation sites (Andoniou *et al*., 1996), which provide binding sites for phosphotyrosine, SH3 domains, and SH2 domains respectively (Figure 1). Upon growth factor or immunological stimulation, Cbl is tyrosine phosphorylated very rapidly (Fukazawa *et al*., 1996; Galisteo *et al*., 1995; Meisner and Czech, 1995; Reedquist *et al*., 1996; Tanaka *et al*., 1995b). Cbl is found in complexes with critical signal transduction molecules (reviewed in Miyake *et al*., 1997), including Grb2, CrkL, Shc, Syk, PI-3 kinase, and Nck (Rivero-Lezcano *et al*., 1994). Cbl also binds to BCR-Abl and is tyrosine-phosphorylated in cells expressing BCR-Abl (Andoniou *et al*., 1994, 1996; Bhat *et al*., 1997; de Jong *et al*., 1995; Sattler *et al*., 1996), but the direct interaction with wild-type (wt) c-Abl has only recently been examined (Shishido *et al*., 2000).

Nck, a 47 kDa adaptor protein, consists of three SH3 domains and a C-terminal SH2 domain, as well as several potential tyrosine phosphorylation sites and a potential SH3 domain binding site (reviewed in McCarty, 1998; Figure 1). Nck is widely expressed in tissues and cell lines (Li *et al*., 1992; Park and Rhee, 1992). In mammalian cells Nck binds a wide variety of proteins via its SH3 domains, including Cbl (Izadi *et al*., 1998; Rivero-Lezcano *et al*., 1994) and Abl (Ren *et al*., 1994; Wunderlich *et al*., 1999), as well as a number of tyrosine-phosphorylated proteins via its SH2 domain (McCarty, 1998).

We have previously shown that overexpression of Nck in the dorsal side of developing *Xenopus* embryos results in anterior truncation, at least in part as a consequence of mesoderm ventralization (Tanaka *et al*., 1997). The ventralizing activity of Nck was

absolutely dependent on the first two SH3 domains (SH3-1 and SH3-2), and was increased by point mutation of the third SH3 domain (SH3-3) and decreased by mutation of the SH2 domain. We subsequently identified Cbl and an Abl relative, Arg, as candidate downstream effectors of Nck in *Xenopus* ventralization by screening of a *Xenopus* embryo expression library using the Nck SH3-1+2 region as a probe (Adler *et al*., 2000). Consistent with a role for these proteins in ventralization, we found that injection of a combination of RNAs (encoding a low amount of a ventralizing Nck mutant, plus Abl and Cbl) induced ventralization, while the pairwise combinations of two messages (Nck and Abl, Nck and Cbl, Cbl and Nck) did not (Adler *et al*., 2000). We also recently found that Nck SH3 domains could activate Abl in tissue culture cells (Smith *et al*., 1999). These data suggested that Nck, Abl, and Cbl might function together to regulate each other's activity *in vivo*, and led us to investigate the molecular details of their interactions. In this study, we expressed a panel of mutants of Abl, Cbl and Nck in human cells to analyse the domains involved in interactions between these proteins and the consequences of their association.

Results

Cbl engages c-Abl and is phosphorylated by it

To investigate the interaction of Abl and Cbl, wt and mutant proteins were overexpressed in human 293T cells and whole lysates or immunoprecipitates from transfected cells subjected to Western blotting analysis. As shown in Figure 2a, cotransfection of wt Cbl and wt c-Abl induced strong tyrosine phosphorylation of Cbl, which was not seen in the absence of Abl cotransfection. The phosphorylation of Cbl by c-Abl was quite specific, with little if any apparent phosphorylation of other proteins (including c-Abl itself); by comparison, an Abl mutant that has been oncogenically activated by deletion of its SH3 domain (ΔSH3 Abl) phosphorylated many proteins in the cell (Figure 2a). We used deletion mutants to determine the regions of Cbl required for phosphorylation by Abl. In contrast to wt Cbl, Cbl deletion mutants lacking the major proline-rich region (Cbl ΔPRD1 or Cbl ΔPRD(1+2); see Figure 1 for location of PRD 1 and 2) were not strongly phosphorylated on tyrosine when co-expressed with wt c-Abl (Figure 2b). This could be because these mutants lack phosphorylation sites or are in a conformation incompatible with phosphorylation, or they may be unable to activate the normally inactive c-Abl kinase. We found that Cbl mutants lacking the PRD1 region could be phosphorylated efficiently, however, by the activated Abl mutant (Figure 2b); this indicates that it is the ability to activate c-Abl, and not the ability to be phosphorylated by Abl, that is defective in these mutants. Furthermore, the lack of phosphorylation of the PRD1 mutants when co-expressed with c-Abl indicates that phosphorylation is not an inevitable consequence of overexpression of a protein together with c-Abl; instead, some proteins such as wt Cbl have specific attributes that allow their phosphorylation by the normally inactive c-Abl kinase.

Because the Abl SH3 domain has been implicated in the repression of c-Abl activity (Barilá and Superti-Furga, 1998; Franz *et al*., 1989; Jackson and Baltimore, 1989; Van Etten *et al*., 1995), and has been shown to bind to Cbl (Shishido *et al*., 2000), we examined whether the PRD1 region of Cbl specifically interacts with the Abl SH3. As shown in Figure 3a, the SH3 domain of Abl bound directly to full-length Cbl, but not to deletion mutants lacking PRD-1, when assayed in 'far-Western' filter-binding experiments. These results are consistent with a model in which the PRD-1 region plays a role in activating c-Abl by engaging its SH3 domain.

We next tested mutants of c-Abl for their ability to phosphorylate Cbl and associate with it. An SH2 domain deletion mutant (ΔSH2 Abl) which is defective in cell transformation (Mayer and Baltimore, 1994) phosphorylated both wt Cbl and the ΔPRD(1+2) mutant only weakly (Figure 2c). As expected, expression of a kinase-dead Abl mutant (K290M) did not lead to significant phosphorylation of wt or mutant Cbl, although very weak phosphorylation of wt was seen in some experiments (see Discussion). The stable association of Abl with Cbl was also investigated by immunoprecipitation. Wt c-Abl, the SH3-deleted activated mutant, and the kinase-dead mutant all coprecipitated with both wt Cbl and Cbl ΔPRD(1+2), while in contrast the SH2-deleted mutant bound neither wt Cbl nor ΔPRD(1+2) (Figure 2c). Therefore in all cases, the Abl SH2 domain was critical for maintaining a stable association with Cbl, most likely by binding to tyrosine phosphorylated sites on Cbl. Consistent with this, we could demonstrate direct binding of the Abl SH2 to tyrosine-phosphorylated Cbl by far-Western analysis (Figure 3b). The inability of the SH2-deleted Abl mutant to stably associate with Cbl is likely to be responsible for its inability to phosphorylate Cbl efficiently.

Domains of Nck involved in binding to Abl and Cbl

Previous data has shown that both Abl and Cbl bind to the SH3 domains of Nck (Adler *et al*., 2000; Wunderlich *et al*., 1999). To further identify which domains of Nck are important for interaction with Abl and Cbl *in vivo*, plasmids encoding various mutant Nck constructs (see Figure 1 for mutants) were co-transfected into 293T cells along with wt Cbl, wt Abl, or both, and cell lysates were subjected to immunoprecipitation using anti-Nck Ab followed by immunoblotting. Interaction between Abl and Nck was decreased by mutation of the second SH3 domain of Nck (mutants K143; K38,143; K143,229; KSH3all) or the SH2 domain (mutants K308; K229,308), while mutation of SH3-1 or SH3-3 had little if any effect. The dependence on the Nck SH2 domain was eliminated by co-transfection of Cbl (Figure 4a, panels

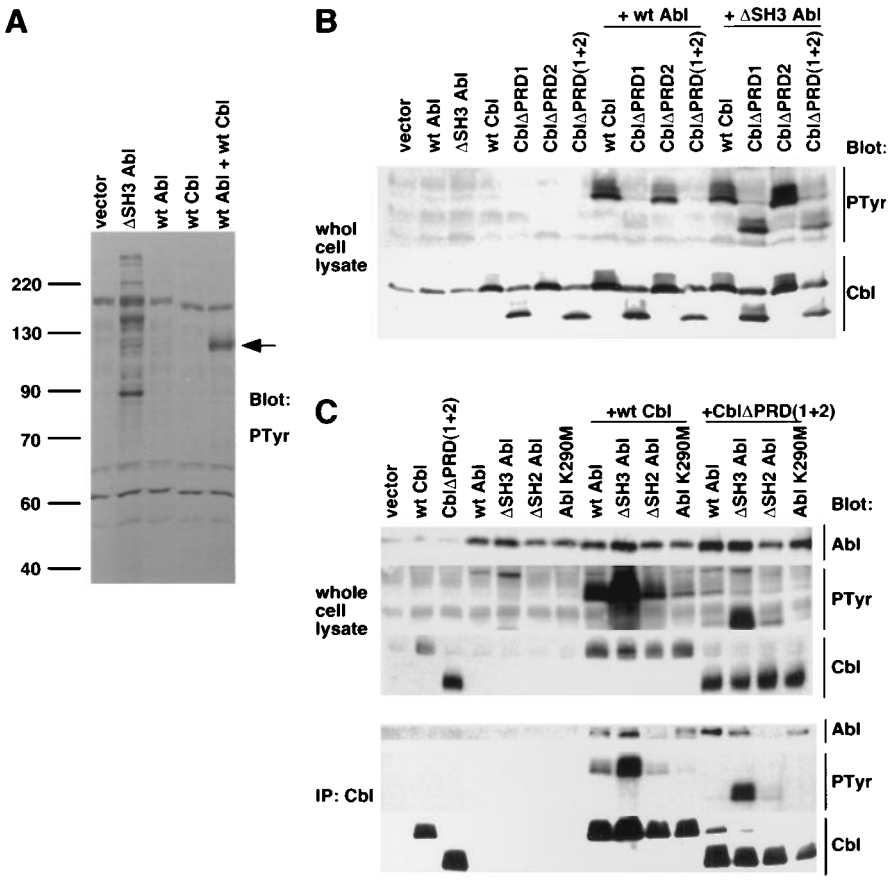

Figure 2 Phosphorylation of Cbl by Abl and binding of Abl to Cbl. (**a**) 293T cells were transfected with plasmid expressing activated Abl (ΔSH3 Abl), wt Abl, wt Cbl, or wt Cbl plus wt Abl as indicated. Whole cell lysates were subjected to immunoblotting with antiphosphotyrosine antibody (PTyr). Arrow indicates position of phosphorylated Cbl. Migration and approximate molecular mass of prestained markers are indicated on the left (in kDa). (**b**) Plasmids encoding Cbl mutants indicated were transfected into 293T cells with or without wt c-Abl or activated ΔSH3 Abl mutant. Whole cell lysates were subjected to immunoblotting for PTyr or Cbl. (**c**) Plasmids encoding Abl mutants and/or Cbl mutants indicated were transfected into 293T cells and whole cell lysates or anti-Cbl immunoprecipitates (IP:Cbl) were subjected to immunoblotting with Ab for Abl, PTyr, or Cbl as indicated. Note that because the Cbl antibody used for blotting recognizes a C-terminal epitope, it binds more efficiently to endogenous Cbl than to overexpressed Cbl (which is C-terminally tagged); therefore the actual expression level of transfected Cbl is underestimated in the anti-Cbl blots

1 and 2), perhaps as a consequence of Nck hyperphosphorylation. Interaction between Cbl and Nck was diminished by mutation of each SH3 domain and the SH2 domain of Nck (Figure 4a, panel 3). Among the three SH3 domains, mutation of SH3-2 (e.g. mutant K143) had the strongest effect on the amount of Cbl co-precipitated. Cotransfection of Abl did not significantly affect the domain dependence of the interaction between Cbl and Nck (Figure 4a panel 4), despite the dramatic increase in tyrosine phosphorylation of Cbl in the presence of Abl (Figure 2).

Phosphorylation levels of Cbl co-precipitated with anti-Nck Ab were also examined (Figure 4a, panel 5). There was little difference in the extent of tyrosine phosphorylation of Cbl associated with the various Nck mutants (Figure 4a, compare panels 4 and 5) although in the case of SH2 domain mutants (K308 and K229,308) the phosphorylation of Nck-associated Cbl was slightly elevated. A much more dramatic difference was observed in the tyrosine phosphorylation of Nck itself. When Cbl was cotransfected along with Abl and Nck, the tyrosine phosphorylation of Nck SH2 domain mutants (K308 and K229,308) was strongly and reproducibly enhanced by approximately fivefold relative to wt Nck (Figure 4a, panel 7). This result suggests that the intact Nck SH2 domain can antagonize the activity of Abl toward Nck. In the absence of Cbl cotransfection only modest tyrosine phosphorylation of Nck proteins was observed, most evident in the case of wt and the PA2 and K308 mutants (Figure 4a, panel 6).

Effect of Nck on Abl-dependent Cbl phosphorylation

Because Nck could interact with both Abl and Cbl, we more closely examined whether overexpression of wt or mutant Nck could affect the ability of Abl to phosphorylate Cbl. We expected that Nck might facilitate Cbl phosphorylation, as we had previously shown that the Crk adaptor could facilitate phosphorylation of the substrate $p130^{Cas}$ by Abl (Mayer *et al*., 1995; Parrini and Mayer, 1999). We found, however,

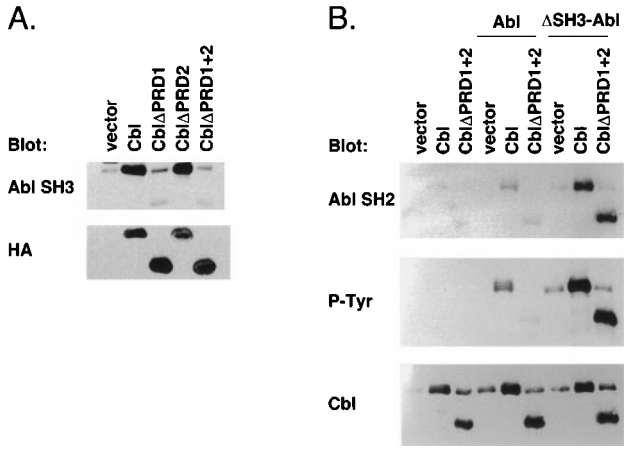

Figure 3 Binding of Abl SH3 and SH2 domains to Cbl. (**a**) Abl SH3 binds the PRD1 region of Cbl. Lysates of 293T cells transfected with wt, ΔPRD1, ΔPRD2, and ΔPRD(1+2) mutants of Cbl (HA-tagged) were immunoprecipitated with Cbl antibody, separated by SDS–PAGE, and transferred to filters. Filters were probed with GST-Abl SH3 domain fusion protein (top) or anti-HA antibody to detect transfected Cbl (bottom). (**b**) Abl SH2 binds to phosphorylated Cbl. 293T cells were transfected with wt or ΔPRD(1+2) Cbl with or without wt Abl or ΔSH3 Abl. Lysates were immunoprecipitated with anti-Cbl Ab and filters were probed with GST-Abl SH2, anti-PTyr, or anti-Cbl as above

that co-expression of various Nck mutants either had little effect or inhibited Cbl phosphorylation; in no case was Cbl phosphorylation enhanced (Figure 5a). SH3 domain mutants (e.g. KSH3all) strongly inhibited Cbl phosphorylation, while by contrast the inhibition by SH2 domain mutants (e.g. K308) and wt Nck was weaker. To more carefully gauge the relative ability of each Nck mutant to inhibit Cbl phosphorylation, the amount of plasmid encoding Nck mutants was varied while the amounts of Abl and Cbl were held constant (Figure 5b,c and data not shown). The consistent trend was that SH3 mutants such as KSH3all were more potent inhibitors than wt Nck, while SH2 mutants such as K308 were weakest. These data suggest that the Nck SH2 domain can inhibit phosphorylation of Cbl by Abl, but that this inhibition is to some extent counteracted by the Nck SH3 domains; only when the SH3 domains are mutated is the full inhibitory activity of the Nck SH2 domain unmasked.

One possible explanation for this inhibition is competition between Nck and Abl for binding sites on Cbl, so we next examined the effect of increasing amounts of Nck and Nck mutants on the complexes between Abl, Cbl, and Nck. As expected, both Abl and Cbl were coprecipitated with anti-Nck Ab (Figure 6a, IP:Nck). Both Abl and Nck were co-precipitated with anti-Cbl Ab (Figure 6a, IP:Cbl), suggesting that Abl and Cbl are associated directly or in a trimolecular complex with Nck. Nck, but not Cbl, could also be co-precipitated with anti-Abl Ab (Figure 6a, IP:Abl). Because Abl could be easily detected in anti-Cbl immunoprecipitates, however, it is most likely that the apparent lack of Cbl co-precipitation is due to steric hindrance with this anti-Abl Ab. When the amount of DNA encoding wt Nck was varied over a range of 0, 0.01, 0.02, 0.05 and 0.1 μg/transfection, coprecipitation of the three proteins increased roughly in proportion to the amount of Nck transfected with the exception of a modest (70%) reduction in the amount of Abl coprecipitating with Cbl when intermediate amounts (0.02 μg) of Nck wt DNA were co-transfected. The amount of Abl that coprecipitated with Cbl returned to 100% upon co-transfection of even greater amounts (0.1 μg) of wt Nck plasmid. At every dose of wt Nck, Cbl was phosphorylated to approximately the same extent (Figure 6a, 'PTyr(Cbl)' panels).

Because Nck SH3 mutants are particularly potent inhibitors of Cbl phosphorylation (Figure 5), we further examined the effect of SH3 domain mutation on complex formation. When Nck K143, an SH3-2 mutant, was cotransfected in various amounts, no decrease in the amount of Abl co-precipitated with anti-Cbl Ab was observed despite a dramatic decrease in Cbl phosphorylation (Figure 6b). When Nck KSH3all (which lacks any functional SH3 domain) was used, the amount of Abl co-precipitated with anti-Cbl Ab was only modestly decreased at the highest dose of Nck KSH3all (Figure 6c), while again phosphorylation of Cbl was strongly inhibited. In sum these coprecipitation results indicate that although Nck might have some modest effects on the extent of association between Abl and Cbl, the inhibition of Cbl phosphorylation cannot be explained by simple competition for binding: Nck SH3 mutants can strongly inhibit the ability of Abl to phosphorylate Cbl even under conditions where Abl and Cbl can still be shown to stably interact.

Because the Nck KSH3all mutant strongly inhibits phosphorylation of Cbl by Abl, the inhibitory activity must reside in the SH2 domain of Nck. To characterize the binding activity of the SH2 domains of Abl and Nck, anti-Nck immunoprecipitates were transferred to filters and probed with the Abl or Nck SH2 domains. As shown in Figure 7, both SH2 domains bound strongly to phosphorylated Cbl, but weakly if at all to unphosphorylated Cbl (from cells not overexpressing Abl). In this and other experiments (not shown), the pattern of binding partners for the Abl and Nck SH2 domains appeared qualitatively similar. These results suggest that the Abl and Nck SH2 domains might compete for binding to the same phosphorylated sites on Cbl. Consistent with this, an excess of unlabeled Nck SH2 domain could almost completely block the binding of labeled Abl SH2 domain to phosphorylated Cbl in filter-binding assays (Figure 7b).

Discussion

In this study we have investigated the mechanism and consequences of interaction between the adaptors Nck and Cbl and the tyrosine kinase Abl. This work grew out of studies in *Xenopus* that suggested both Cbl and

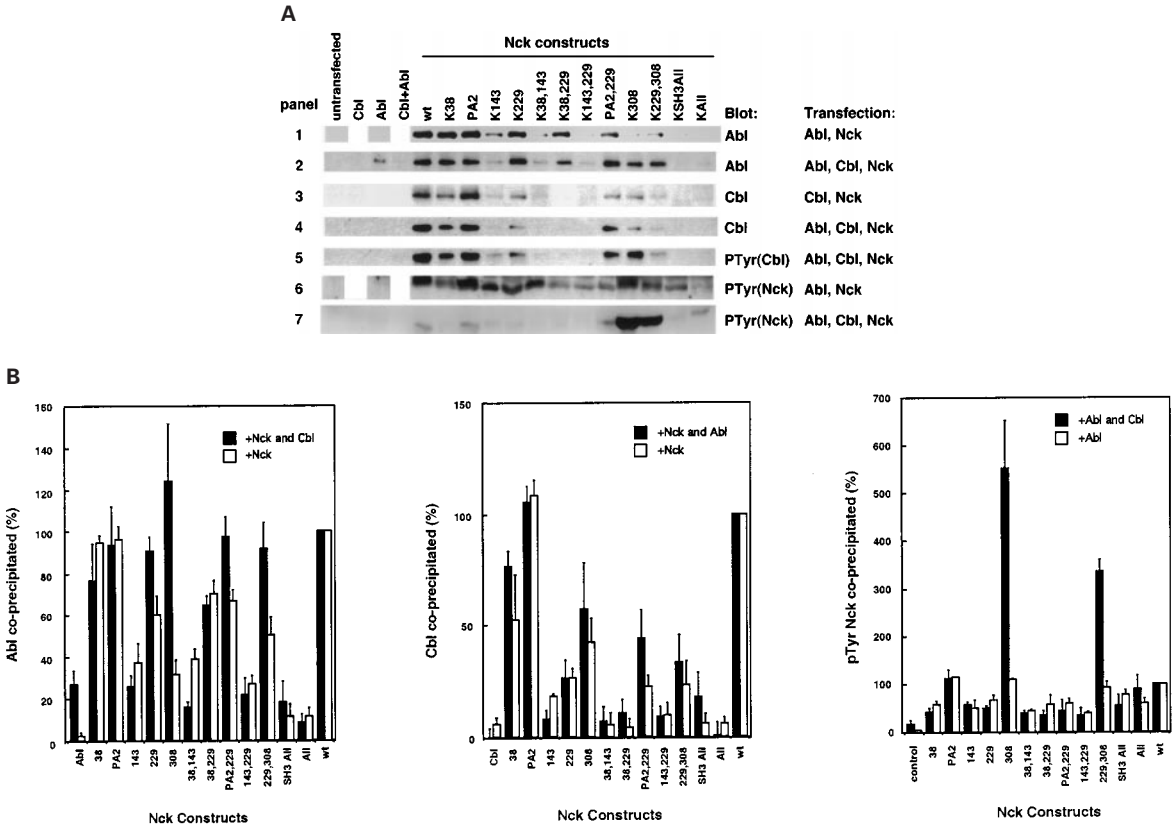

Figure 4 Association of Abl and Cbl with Nck, and tyrosine phosphorylation of Cbl and Nck. (**a**) Plasmids encoding wt c-Abl, Cbl, and Nck mutants were transfected into 293T cells in combinations indicated (column labeled 'Transfection') and cell lysates immunoprecipitated with polyclonal anti-Nck Ab. Precipitates were subjected to immunoblotting analysis with probes indicated (column labeled 'Blot'). Levels of Nck were similar for each lane where Nck was overexpressed (not shown). Panel 6 was exposed to film for a longer time than panel 7 to detect very low levels of Nck phosphorylation. Data is representative of at least two independent transfection experiments; to assess the influence of experimental variation between experiments, bands were quantified, normalized to Nck amounts, and the mean and standard error were calculated (**b**)

Abl bound the SH3 domains of Nck, and together could synergize with Nck in a mesoderm patterning phenotype when overexpressed during early development (Adler *et al*., 2000). The interaction of these three proteins, each of which has been implicated in regulation of mitogenic signaling, serves as a model for other complex interactions among signaling proteins. We have examined their interactions of Abl, Cbl, and Nck by overexpressing wt and mutant proteins in tissue culture cells; while this is admittedly an imperfect system, it allowed us to analyse a wide panel of mutants thereby providing valuable information about potential modes of regulation of the endogenous proteins. We find that Nck, Abl, and Cbl bind to each other via multiple SH3- and SH2-domain-mediated interactions, and that each protein is capable of modulating the activity and/or phosphorylation of the others in the complex. Most notably, c-Abl is activated by the proline-rich domain of Cbl leading to extensive tyrosine phosphorylation of Cbl, while on the other hand the ability of c-Abl to phosphorylate Cbl or Nck can be inhibited by Nck in an SH2-dependent fashion.

Interaction between Abl and Cbl

Although a number of publications have reported that Bcr-Abl interacts with and phosphorylates Cbl, interactions between wt c-Abl and Cbl have been less well characterized. We demonstrate here that Cbl could specifically engage c-Abl and thereby induce its own phosphorylation. We show that deletion mutants of Cbl lacking the major proline-rich region were not phosphorylated by wt c-Abl, but were efficiently phosphorylated by an activated mutant of Abl. An SH2 domain deletion mutant of Abl did not co-precipitate efficiently with Cbl or Cbl ΔPRD(1+2); in contrast, a kinase-dead mutant of c-Abl was co-precipitated with both the wt and mutant Cbl proteins. These data suggest two distinct interactions between Abl and Cbl. First, the proline-rich region of Cbl can bind directly to the SH3 domain of wt Abl, releasing Abl from negative regulation by its own SH3 domain and thereby allowing phosphorylation of Cbl. This SH3-mediated interaction is important for the activation step but not for subsequent phosphorylation, as the constitutively active Abl efficiently phosphorylated

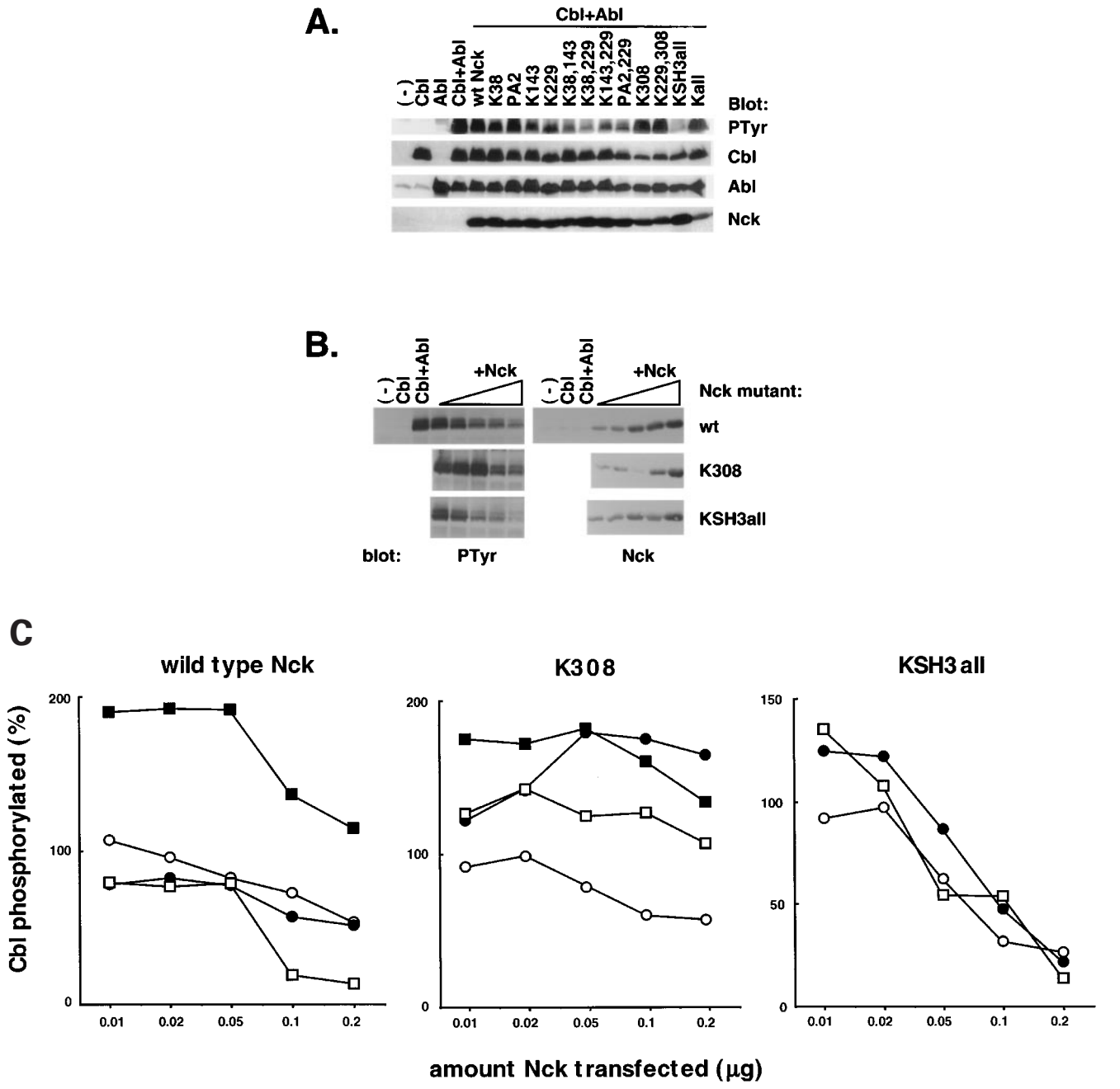

Figure 5 Nck inhibits the phosphorylation of Cbl by Abl. Plasmids encoding Abl, Cbl and Nck mutants indicated were transfected into 293T cells and the phosphorylation level of Cbl was examined by immunoblotting. (**a**) After transfection of 0.1 μg of Nck mutant DNA with 0.5 μg of Cbl DNA as described in Materials and methods, whole cell lysates were subjected to immunoblotting with Ab for PTyr, Cbl, Abl, or Nck as indicated. (**b**) The amount of Nck DNA used for transfection was varied over the range of 0.01 to 0.2 μg and whole cell lysates subjected to immunoblotting for PTyr (left) or Nck (right). Cbl protein level was essentially constant in each lane (not shown). (**c**) Data from the experiment shown in panel **b** and two (KSH3all) or three (wt, K308) similar experiments were quantitated and presented graphically. For each experiment, phosphorylation of Cbl in cells transfected with Abl alone was set as 100%

Cbl even though it lacks an SH3 domain. A second interaction involving the SH2 domain of Abl is required for stable complex formation with and extensive phosphorylation of Cbl. Consistent with this model, we show that the Abl SH3 domain and SH2 domain bind directly to the PRD-1 region of Cbl and tyrosine-phosphorylated Cbl respectively.

It has long been known that the SH3 domain of Abl negatively regulates Abl kinase activity (Franz *et al*., 1989; Jackson and Baltimore, 1989). Our data strongly suggest that the proline-rich region of Cbl engages the SH3 domain of Abl and releases this negative regulation. A similar mechanism of Abl activation by proline-rich motifs in proteins was suggested in the

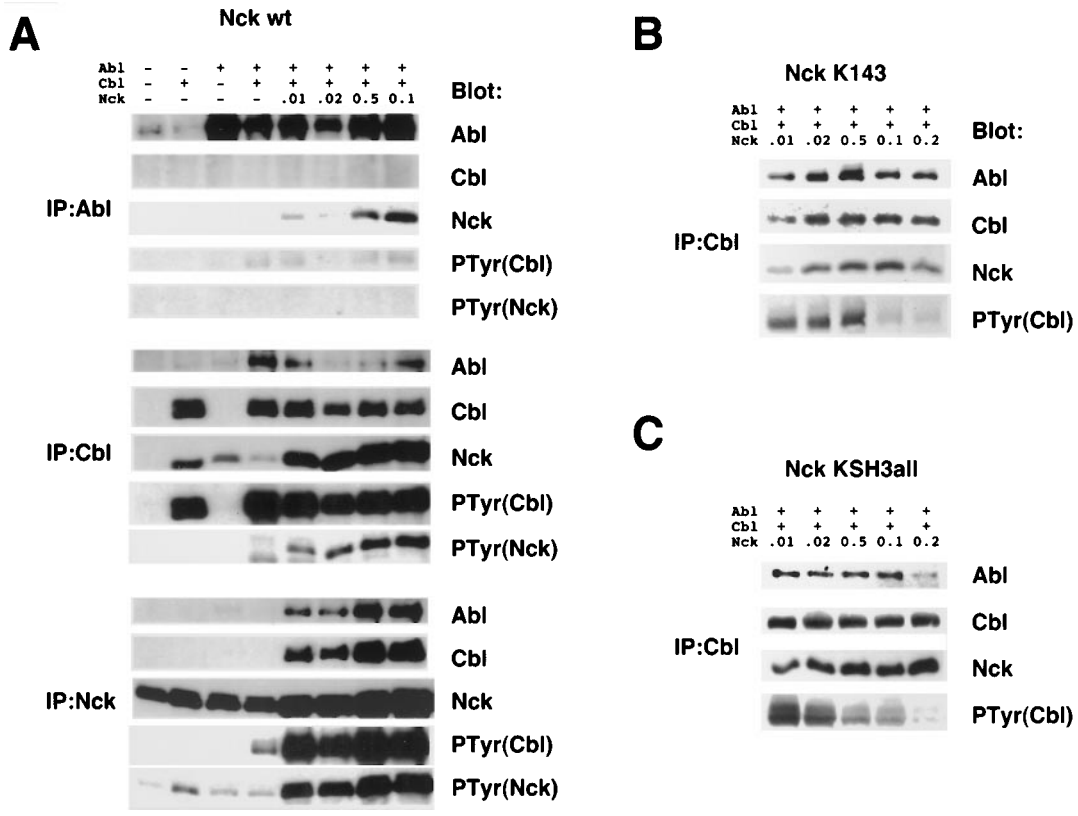

Figure 6 Association of Abl, Cbl and Nck in cotransfected cells. Lysates prepared from 293T cells transfected with plasmids encoding Abl, Cbl and Nck mutants indicated (**a**: wt Nck; **b**: Nck K143; **c**: Nck KSH3all) were immunoprecipitated using anti-Abl, anti-Cbl, or anti-Nck Ab, and precipitates subjected to immunoblotting and probed for Abl, Cbl, Nck or PTyr as indicated. The amount of Nck DNA transfected was varied over a range as indicated (amounts in μg/35 mm well)

cases of the ATM, ST5 gene products and RFXI (Agami and Shaul, 1998; Majidi *et al*., 1998; Shafman *et al*., 1997), and recently Shishido and colleagues have independently found that overexpressed Cbl can be phosphorylated by overexpressed c-Abl (Shishido *et al*., 2000). The interaction between the Abl SH3 domain and the Cbl proline-rich domain, while sufficient to activate c-Abl, cannot mediate stable association of the two proteins because an SH2-deleted Abl mutant did not coprecipitate with Cbl. Given the relatively weak affinity between SH3 domains and their peptide ligands (dissociation constants in the range of 10^{-5} M) (Yu *et al*., 1994) this is not surprising.

We and others have proposed that the SH2 domain of Abl promotes processive phosphorylation of substrates by mediating stable association between kinase and substrate (Duyster *et al*., 1995; Mayer and Baltimore, 1994; Mayer *et al*., 1995). The results presented here are consistent with this model, as the SH2-deleted Abl mutant not only lost the ability to bind Cbl stably, but also was incapable of phosphorylating it effectively. Somewhat surprisingly, a kinase-dead mutant of Abl retained the ability to bind tightly to Cbl. Although the phosphorylation level of Cbl in cells expressing the Abl K290M mutant was quite low, it is possible that at least one site is constitutively phosphorylated in a fraction of Cbl, and this is sufficient to mediate association with the SH2 domain of Abl. Alternatively, there may be site(s) in Cbl that bind the Abl SH2 in a phosphotyrosine-independent manner, as previously suggested for binding of the Abl SH2 to Shc and Bcr (Muller *et al*., 1992; Raffel *et al*., 1996); however our inability to detect robust binding of the Abl SH2 to unphosphorylated Cbl in filter-binding assays makes this unlikely (Figure 3b). It is also possible that the interaction is indirect, via a Cbl-associated protein. Shishido and colleagues proposed that binding of Abl induces a conformational change in Cbl that allows binding of Src via its SH3 domain (Shishido *et al*., 2000); association of endogenous Src could explain the weak phosphorylation of Cbl occasionally induced by kinase-inactive Abl (Figure 2c). Further experiments will be required to clarify this point.

The interaction reported here between Abl and Cbl has important general implications for the relationship between nonreceptor tyrosine kinases and their substrates. We show that specific phosphorylation of a proline-rich substrate by Abl can be induced in the cell by high concentration of the two proteins, in the absence of generalized activation of the kinase. Therefore, in the presence of Abl, increased local

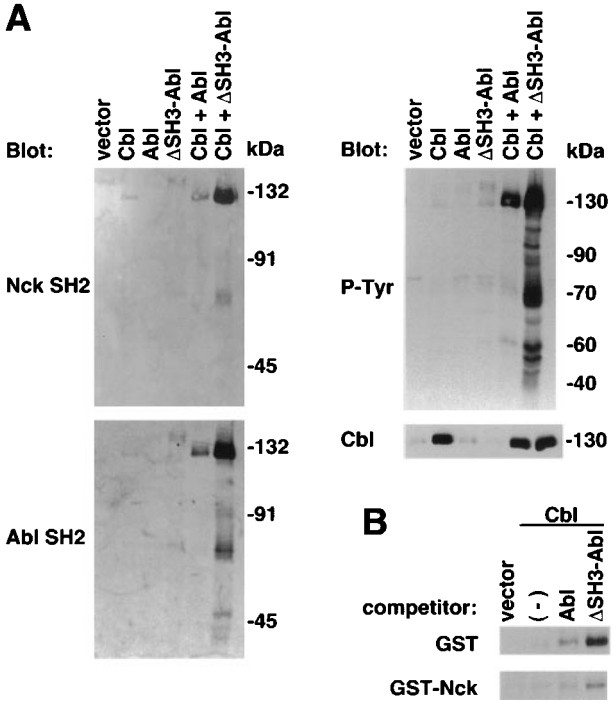

Figure 7 Abl and Nck SH2 domains bind directly to phosphorylated Cbl. (**a**) 293T cells were transfected with wt Nck, with or without wt Cbl, wt Abl, or ΔSH3 Abl as marked. Lysates were immunoprecipitated with anti-Nck Ab and probed with GST-Abl SH2, GST-Nck SH2, anti-PTyr, or anti-Cbl as marked. Migration and approximate molecular mass (in kDa) of prestained markers are indicated on the right. (**b**) Lysates of 293T cells transfected with wt Cbl with or without wt or ΔSH3 Abl were immunoprecipitated with anti-Cbl Ab and filters probed with biotinylated GST-Abl SH2 in the presence of 100-fold excess unlabeled GST or GST-Nck SH2 as competitor. Region of filter containing Cbl is shown

concentration of SH3-binding substrates such as Cbl (driven by aggregation or relocalization) could be sufficient to induce their phosphorylation. Furthermore, 'activation' of Abl in such a situation would be specific for and limited to that substrate: high-affinity interaction between the SH2 domain of the kinase and the phosphorylated substrate would impede release of the kinase, and any Abl that did escape from the complex would presumably once again be subject to repression by its own SH3 domain. In our experiments where c-Cbl was highly phosphorylated by overexpressed c-Abl, few if any other proteins in the total 293T cell lysate showed significantly increased phosphorylation (Figure 2a). Such a mechanism, in which the substrate plays an active role in inducing its own phosphorylation by an otherwise inactive kinase, has obvious advantages where indiscriminate kinase activity can lead to disregulated signaling. A similar mechanism may be involved in phosphorylation by the Src kinase of $p130^{Cas}$ and the Cas-like protein Sin, which can engage both the SH3 and SH2 domains of Src (Alexandropoulos and Baltimore, 1996; Burnham *et al*., 2000; Xing *et al*., 2000).

Interaction between Abl and Nck, and between Cbl and Nck

There are many potential SH2- and SH3-domain-mediated interactions between Abl, Cbl, and Nck, so it is important to determine which interactions play an important role *in vivo*. We show that when Nck and Abl were transfected, stable binding of Nck to Abl required both the SH3-2 and SH2 domains of Nck. When Cbl was also overexpressed, however, the interaction between Nck and Abl no longer depended on the Nck SH2 domain. Because Nck SH2 mutants became more highly phosphorylated than wt Nck under these conditions, stable binding in this case might be mediated by interaction of the Abl SH2 domain with tyrosine phosphorylated sites on Nck. In contrast, all three SH3 domains and the SH2 domain of Nck played a role in the stable association of Nck with Cbl. Of these domains, mutation of the SH3-2 had the most drastic effect on Cbl binding. These domain requirements were essentially unaffected by co-transfection of Abl.

Abl has several potential binding sites for Nck, including sites of tyrosine phosphorylation and several proline rich motifs in the C-terminus (Feller *et al*., 1994; Ren *et al*., 1994). In other work, we have shown that the major binding site in mouse Abl for Nck SH3 domains is located at residues 631–636 (Adler *et al*., 2000). Mutation of the 'PA2 site' of Nck, which is a conserved potential SH3 binding site (T Miyoshi-Akiyama and BJ Mayer (2001), unpublished observation), did not significantly affect the interaction between Abl and Nck or the activity of Abl, suggesting that the SH3 domain of Abl does not bind to this site on Nck. The dependence of the association on the Nck SH2 domain is somewhat puzzling, as c-Abl is thought to be essentially unphosphorylated on tyrosine unless activated (Franz *et al*., 1989; Pendergast *et al*., 1991; Ponticelli *et al*., 1982). It is possible that the Abl which associates stably with Nck is that small fraction which is tyrosine phosphorylated under these conditions of overexpression; alternatively, the Nck SH2 might bind indirectly through Abl-associated proteins.

We have previously shown that two distinct segments of Cbl contribute to Nck SH3 domain binding: a long proline-rich region between residues 483–688 previously implicated in binding other SH3 domains, and a single PxxP site at position 820–826 (Adler *et al*., 2000). The Nck SH2 is also important for binding, and we show by far-Western blotting that it can bind directly to tyrosine phosphorylated Cbl. In the case of the Crkl adaptor, binding to Cbl is entirely dependent on phosphorylation of tyrosines located near the C-terminus of Cbl by Bcr-Abl or v-Abl (Andoniou *et al*., 1996). By contrast, our coimmunoprecipitation experiments show that the Nck SH2 associates with Cbl even when it is not highly phosphorylated (Figure 6c and data not shown), though it is possible that this interaction is indirect. It has also been reported that interaction between Nck and Cbl in myeloid cells following Fc receptor

stimulation is dependent on Cbl phosphorylation, and *in vitro* binding experiments using GST-Nck constructs suggested it is mediated by the SH3-2 domain of Nck (Izadi *et al*., 1998). Our coimmunoprecipitation data are broadly consistent with this conclusion, although in our system every SH3 domain of Nck makes some contribution to binding.

Role of Nck in interaction between Abl and Cbl

Because Nck, Cbl, and Abl all interact with each other and are implicated in a mesoderm patterning defect in *Xenopus* (Adler *et al*., 2000), it was of interest to understand what role Nck might have in modulating the phosphorylation of Cbl by Abl. To our surprise we found that Nck actually inhibits the tyrosine phosphorylation of Cbl by Abl. The modest inhibitory effect of wt Nck was enhanced by mutation of the SH3 domains and was almost entirely dependent on an intact SH2 domain. Mutation of the Nck SH2 domain also led to a dramatic increase in the tyrosine phosphorylation of Nck itself in the presence of Cbl and Abl. These data suggest that the SH2 domain of Nck is responsible for the repressing the ability of Abl to phosphorylate both Cbl and Nck, and that the SH3 domains of Nck can at least partially counteract this inhibitory activity.

One model to explain such an inhibitory activity is that the SH2 domain of Nck competes with the Abl SH2 for the same binding site(s) on Cbl. Consistent with this, we show here that Abl SH2 function is required for Abl to stably associate with Cbl and efficiently phosphorylate it (Figure 2c), and also that the Abl and Nck SH2 domains both bind directly to phosphorylated Cbl and can compete for binding to the same sites (Figure 7). An alternative possibility is that the Nck SH2 binds to tyrosine-phosphorylated Abl and thereby inhibits its activity directly. However, using purified baculovirus-produced Abl we have been unable to demonstrate inhibition by either Nck SH2 domains or full-length Nck in *in vitro* kinase experiments (BJ Mayer, unpublished observation). Shishido and colleagues have recently reported that the SH3-SH2 region of kinase-inactive (KD) Src can also inhibit phosphorylation of Cbl by Abl, though in this case both the SH2 and SH3 regions of Src were required (Shishido *et al*., 2000). They proposed that KD Src inhibits Abl by a steric mechanism; our observation that inhibition by Nck is strictly dependent on the SH2 domain and independent of the SH3 domains is we feel more consistent with our SH2 competition model. We also cannot rule out the possibility that the Nck SH2 affects the activity of endogenous phosphatases.

The observation that mutation of the Nck SH2 leads to a dramatic increase in the phosphorylation of Nck by activated Abl is intriguing. This suggests that one function of the Nck SH2 might be to bind intramolecularly to monophosphorylated Nck, preventing binding of the Abl SH2 and thereby preventing its processive phosphorylation by Abl. It has previously been shown that the SH2 domain of the Crk adaptor binds in cis to its major site of phosphorylation by Abl (Rosen *et al*., 1995), conceptually similar to the Nck SH2-phosphotyrosine interaction suggested here. Filter binding experiments show that binding of the Nck SH2 to phosphorylated Nck is weak at best (Figure 7a and data not shown), but when the SH2 and the phosphorylated tyrosine are on the same molecule their interaction would be favored by high local concentration. For example, it is known that the SH2 domain of Src binds stably in cis to a C-terminal site for which it has relatively low affinity (Payne *et al*., 1993). We further found that the increased phosphorylation of SH2-mutated Nck is almost entirely dependent on overexpression of Cbl (Figure 4). We assume this is because Cbl serves to activate c-Abl, and Nck is phosphorylated as a component of a complex containing Abl, Cbl, and Nck in which the activated Abl can phosphorylate either Cbl or Nck.

What is the role of the Nck SH3 domains in a Nck-Cbl-Abl complex? Clearly they can mediate interactions between Nck and Abl, and Nck and Cbl, but these interactions are presumably of relatively low affinity and insufficient to confer stable binding. Their role is likely to be in initiating interactions, in further stabilizing SH2-mediated interactions, or in orienting molecules within the complex. SH3 domain mutations actually enhance the ability of Nck to inhibit Abl-mediated phosphorylation of Cbl; this rules out the possibility that Nck inhibits Cbl phosphorylation by competing with the Abl SH3 for binding to Cbl. One possibility is that the Nck SH3 domains, by binding to Abl, facilitate the initial phosphorylation of Nck by Abl. As suggested above, such phosphorylation might in turn provide a binding site in cis for the Nck SH2, simultaneously relieving inhibition of Cbl phosphorylation by the Nck SH2 and blocking the binding of the Abl SH2 to Nck (thereby preventing the processive phosphorylation of Nck). In this scenario, we would expect that recruitment of wt Nck to a Cbl/Abl complex would normally lead to its functional inactivation via intramolecular SH2-phosphotyrosine binding.

Materials and methods

Cell culture and transfection

293T cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum plus antibiotics. Cells were transfected by calcium phosphate coprecipitation as described (Tanaka *et al*., 1995a). For 35-mm wells, 0.1 μg/well plasmid DNA for Nck mutants, 0.3 μg/well for Abl mutants, 0.5 μg/well for Cbl mutants were used as standard conditions for transfection experiments unless otherwise indicated. One to 2 days post-transfection, cells were lysed with 400 μl (35-mm well) or 1 ml (10-cm dish) lysis buffer containing 25 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10% glycerol with protease inhibitors and phosphatase inhibitors.

DNA constructs

All Nck and Cbl mutants used for transfection experiments were constructed in the pEBB vector (Tanaka *et al*., 1995a). Abl constructs have been described previously (Mayer and Baltimore, 1994); c-Abl and ΔSH3 Abl were constructed in pBPN (a slightly modified version of pBABE-puro; Morgenstern and Land, 1990), ΔSH2 Abl was constructed in pGDN (Mayer and Baltimore, 1994) and K290M was constructed in pPLcIV (Jackson and Baltimore, 1989). Constructs expressing wt human Nck and its mutants have been described previously (Tanaka *et al*., 1995a, 1997). The Nck 'PA' mutant was generated by PCR and contains a point mutation that changes proline 84 to alanine, eliminating the potential SH3 binding site conserved in human, *Xenopus*, and mouse Nck (see Genbank accession AF084183 for corrected mouse Nck sequence). Mutants of HA-tagged human Cbl are more completely described elsewhere (Adler *et al*., 2000). Briefly, ΔPRD1 has a deletion encompassing amino acids 483–688, ΔPRD2 has a deletion encompassing amino acids 820–826, and Δ PRD1+2 has both deletions.

Immunoprecipitation and immunoblotting

One hundred and fifty μl of cell lysates were incubated with antibody (Ab) (1 μg for anti-Abl [K-12, Santa Cruz Biotechnology] or anti-Cbl [C-15, Santa Cruz] polyclonal Ab and 2 μl for crude rabbit anti-Nck serum) for 2 h at 4°C. Five μl of Immunopure immobilized Protein G (Pierce) was then added and incubated for a further 1.5 h. The beads were washed four times with 1 ml of Tris-buffered saline containing 0.05% Tween-20 (TBST) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were transferred electrophoretically to nitrocellulose or PVDF membranes and subjected to immunoblotting. Anti-Abl monoclonal Ab (8E9, PharMingen, San Diego, CA, USA; or Ab3, Oncogene Research Products, Cambridge, MA, USA), anti-Cbl monoclonal Ab (A-9, Santa Cruz), anti-phosphotyrosine monoclonal Ab (4G10, Upstate Biotechnology), anti-Nck monoclonal Ab (Transduction Laboratories) were used for detection.

For Figures 4 and 5, after detection of the stained protein bands by chemiluminescence (ECL, Amersham Pharmacia Biotech, Piscataway, NJ, USA), films were scanned or imaged with a digital camera to digitize the data and analysed with the computer program NIH image. The data from multiple transfection experiments were quantified and mean value with standard error was calculated.

Far-Western blotting

Lysates were prepared and immunoprecipitated as above, separated by SDS–PAGE, and proteins were transferred to nitrocellulose membranes. For Figures 3b and 7a, filters were blocked overnight in 5% non-fat dry milk in TBST. Blots were then incubated with 2 μg/ml of purified glutathione S-transferase (GST) fusion protein in the presence of 3 μM reduced glutathione (Sigma) and 3% bovine serum albumin (BSA) for 1 h. Blots were further incubated with 1/50 dilution monoclonal anti-GST hybridoma supernatant (Tanaka *et al*., 1994) for 1 h and detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Pierce). For Figures 3a and 7b, membranes were blocked overnight in 5% non-fat dry milk in TBST and then in 3 μM reduced glutathione in TBST with 0.5% BSA for 1 h. Blots were then incubated with 2 μg/ml of a biotinylated purified GST fusion protein in the presence of reduced glutathione (3 μM) and 3% BSA for 1 h. Blots were detected with HRP-conjugated streptavidin (GIBCO–BRL). For Figure 7b, unbiotinylated GST or GST-Nck SH2 competitor was added at 100-fold excess over biotinylated GST-Abl SH2 (200 μg/ml and 2 μg/ml respectively). When GST-Abl SH3 was used (Figure 3a) all steps described above were carried out at 4°C, in all other cases incubations were at room temperature. GST fusion proteins were prepared and biotinylated as described (Mayer *et al*., 1991). The Nck SH2 construct contains amino acids 275–377 of human Nck.

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