Catalytic domains of tyrosine kinases determine the phosphorylation sites within c-Cbl

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Abstract Catalytic (SH1) domains of protein tyrosine kinases (PTKs) demonstrate specificity for peptide substrates. Whether SH1 domains differentiate between tyrosines in a physiological substrate has not been confirmed. Using purified proteins, we studied the ability of Syk, Fyn, and Abl to differentiate between tyrosines in a common PTK substrate, c-Cbl. We found that each kinase produced a distinct pattern of c-Cbl phosphorylation, which altered the phosphotyrosine-dependent interactions between c-Cbl and CrkL or phosphatidylinositol 3′-kinase (PI3-K). Our data support the concept that SH1 domains determine the final sites of phosphorylation once PTKs reach their target proteins.

Keywords: Protein tyrosine kinase; Substrate specificity; c-Cbl phosphorylation

1. Introduction

PTKs are fundamental components of the communication networks within cells, transmitting signals for growth, differentiation, adhesion, motility, and death. Alteration of PTK activity can lead to a variety of human diseases, including diabetes, cancer, and immune malfunction. The specificity of SH1 domains may regulate signaling outcomes involved in human disease. In multiple endocrine neoplasia type 2B, an SH1 domain mutation in the substrate-binding pocket of the RET receptor PTK was found to alter the peptide substrate preference of the kinase [1]. Clearly, more investigation into substrate specificity of SH1 domains is warranted.

The proto-oncogene c-Cbl is a common substrate of PTKs and acts as both a negative regulator of and a positive effector for PTKs [2,3]. Tyrosine phosphorylation of c-Cbl occurs rapidly and robustly in a wide variety of cell types and stimulating conditions [3]. c-Cbl is inducibly phosphorylated by receptor and non-receptor PTKs, but constitutively phosphorylated by oncogenic PTKs and in various cancer cell lines [3,4]. The carboxy (C)-terminus of c-Cbl contains multiple tyrosines that receive the bulk of phosphorylation relative to the rest of the protein [5–7]. Phosphorylation of three of those tyrosines, Y700, Y731, and Y774, allows the SH2 domains of Crk proteins, Vav, and the p85 subunit of phosphatidylinositol 3′-kinase (PI3-K) to bind c-Cbl. More recently, it has been shown that phosphorylation of Y700, Y731, and Y774 is necessary for the c-Cbl–CIN85 interaction, which mediates endocytosis and downregulation of the epidermal growth factor (EGF) and hepatocyte growth factor (c-Met) receptors [8–10].

The fact that the C-terminus of c-Cbl is the major site of phosphorylation in vivo suggests that either there are three-dimensional constraints that minimize phosphorylation upstream or that PTKs have an intrinsic ability to discriminate between the 22 tyrosines of c-Cbl. Within the C-terminus, Y700, Y731, and Y774 are heavily phosphorylated [5,6]. It has been posited that non-receptor PTKs phosphorylate these sites indiscriminately [3], suggesting a lack of selectivity for individual tyrosine motifs. In contrast, studies using peptide substrates have revealed that catalytic (SH1) domains of PTKs are capable of specificity [11]. It is unclear, however, whether the selectivity observed with peptides also applies to physiological substrates.

We used the protein c-Cbl to determine whether SH1 domains of PTKs are able to discriminate between individual tyrosines of a physiological substrate. c-Cbl is an ideal substrate because it is a common target for PTKs, it is a regulator of PTK signaling, and some in vivo sites of phosphorylation have been mapped. Using purified proteins, we show that the SH1 domains of Syk, Fyn, and Abl have different preferences for tyrosines within c-Cbl. Our data suggest that SH1 domains can provide an additional level of specificity in PTK signaling.

2. Materials and methods

2.1. In vitro kinase assays

The GST-Cbl proteins were expressed in *Escherichia coli* and purified over glutathione sepharose beads (Pharmacia). The fusion proteins were washed once in 0.5 M LiCl, twice in NP40 lysis buffer (1% NP40, 20 mM Tris, pH 8.0, 150 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM PMSF, 10 μg/ml Aprotinin, and 1 mM Na_3VO_4), and twice in kinase buffer (see below). Bead-bound fusion proteins were resuspended in kinase buffer. All kinase buffers included 20 mM Tris, pH 7.5, 50 mM NaCl, and 1 mM DTT. Syk also required 20 mM $MgCl_2$ and 5 mM $MnCl_2$. Fyn also required 10 mM $MgCl_2$ and 10 mM $MnCl_2$. Abl also required 10 mM $MgCl_2$. Preliminary kinase assays

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were conducted to determine the conditions necessary for maximum phosphorylation of substrate. Each kinase was used in excess to avoid limiting the final level of substrate phosphorylation. The reactions were carried out in 200 μM ATP for 1 h at room temperature. With the exception of Abl, the kinases were provided by N. Lydon (Kinetix Pharmaceuticals Inc.) as purified GST-SH1 fusions. The Abl SH1 domain was generated as previously described [12].

2.2. Gel overlay assays

Products of the in vitro kinase assays described above were separated by 10% SDS–PAGE and transferred to polyvinylidene difluoride membrane (PVDF Immobilon), (Millipore). The membrane was dried at room temperature 2 h – overnight and rehydrated by submersion in methanol and rinsing in water. The membrane was then rocked for 6–7 h at room temperature in a 10% non-fat milk – TBST (Tris buffered saline + 0.05% Tween 20) solution, washed three times (10 min each) in TBST at room temperature, rocked overnight at 4 °C in 2 μg/ml (CrkL-SH2 and p85 N + C-SH2) or 4 μg/ml (p85 N-SH2) of overlay protein in binding buffer (25 mM $NaPO_4$, pH 7.2, 150 mM NaCl, 2.5 mM EDTA, pH 8.0, 20 mM NaF, 1 mM Na_3VO_4, 1 mM DTT, 0.1% Tween 20, 1% non-fat milk), and washed five times (10 min each) in TBST at room temperature. Bound overlay protein was detected with either anti-Flag or anti-p85 antibodies and developed with Enhanced Chemiluminescence (Pierce) using a Roche Lumi-Imager. Quantification of signal intensity was accomplished with Roche LumiAnalyst software.

2.3. Immunoblotting

For the overlays, Flag-CrkL SH2 was detected with anti-Flag (Sigma, F3165). Both p85 N-SH2 and N + C-SH2 were detected with anti-p85 N-SH2 (Upstate Biotechnology, 05-217). PVDF membranes were stripped in 100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris–HCl for 40 min at 65 °C between immunoblots. Anti-c-Cbl (C15) and anti-GST (sc-138) were purchased from Santa Cruz Biotechnology. Anti-phosphotyrosine (4G10) was used as described [12]. Anti-mouse or rabbit IgG-HRP conjugate was purchased from Promega and used with Supersignal Chemiluminescent Substrate from Pierce to develop all immunoblots except 4G10. Anti-mouse IgG-AP conjugate was purchased from Promega and used with BCIP/NBT Color Development Substrate (Promega) to develop 4G10 immunoblots.

2.4. Plasmids

The GST-fusion constructs of Cbl were created by subcloning fragments of c-Cbl cDNA into pGEX bacterial expression vectors. NT-Cbl includes amino acids 1–220. RF-Cbl includes amino acids 144–450. PR-Cbl includes amino acids 451–541. CT-Cbl includes amino acids 542–906. The CrkL and p85 constructs were also expressed from pGEX vectors, but were cleaved from the GST tag with Factor Xa. The CrkL-SH2 was subcloned as previously described [13] and modified by the addition of a C-terminal Flag tag. The pGEX-p85-SH2 constructs were provided by T. Roberts (Dana Farber Cancer Institute).

3. Results

3.1. SH1 domains of PTKs exhibit distinct preferences for tyrosines within c-Cbl

In order to determine whether SH1 domains can discriminate between tyrosines of a c-Cbl, we subjected purified GST-fusion domains of c-Cbl to phosphorylation in vitro by the purified SH1 domains of Syk, Fyn, and Abl. Fig. 1A illustrates the structure of c-Cbl and maps the regions that were expressed as GST-fusion proteins. With the exception of one tyrosine, Y141, shared between NT-Cbl and RF-Cbl, each GST-fusion domain of c-Cbl contains non-overlapping tyrosines. In addition, every fusion protein contains one or more tyrosines, also noted in Fig. 1A. Full-length c-Cbl was not used in our studies because it was not possible to generate the protein with sufficient purity.

As shown in Fig. 1B–D and summarized in Table 1, all kinases tested readily phosphorylated the carboxy-terminus of c-Cbl (CT-Cbl), which includes the SH2-recognition sites Y700, Y731, and Y774. In agreement with data showing that the C-terminus of c-Cbl is the major site of tyrosine phosphorylation in vivo [5–7], we observed that CT-Cbl was the preferred substrate relative to other regions of c-Cbl. The fact that the in vitro phosphorylation patterns mimic c-Cbl phosphorylation in vivo suggests that the GST-Cbl fusion proteins fold into native conformations.

The 68 kDa GST-CT-Cbl protein migrates as a doublet in 10% polyacrylamide gels (uppermost bands in Figs. 1–5). GST-RF-Cbl also migrates as a doublet (Fig. 1). We microsequenced the larger of the two GST-CT-Cbl bands (data not shown) and confirmed the identity of GST-CT-Cbl. The microsequencing results revealed that the bacterial Hsp70 protein was also present. While copurification of Hsp70 with GST alone or the other GST-fusion proteins was not evaluated, other investigators have reported that copurification of chaperone proteins, such as GroEL, is problem encountered when purifying GST-fusion proteins from *E. coli* [14]. Also seen in Figs. 1–5 are smaller forms of the GST-fusion proteins, from bacterial lysates, which copurify with the full-length proteins and migrate as a smear ahead of the full-length proteins. The smaller forms suggest some level of degradation and explain the presence of doublet bands for GST-CT-Cbl and GST-RF-Cbl.

The SH1 domain of Abl only phosphorylated CT-Cbl (Fig. 1D). In contrast, Fyn phosphorylated RF-Cbl and CT-Cbl (Fig. 1C), while Syk phosphorylated all of the c-Cbl regions (Fig. 1B). In the case of PR1-Cbl, Syk phosphorylates tyrosine 455 as it is the only tyrosine present in PR1-Cbl. Phosphorylation of tyrosine 455 was confirmed by testing the tyrosine to phenylalanine mutant version of PR1-Cbl, in which there was no phosphorylation detectable (data not shown). Phosphorylation of the GST-Cbl substrates was independent of GST, as indicated by the failure of GST to be detected by phosphotyrosine immunoblotting (Fig. 1B–D). The differential phosphorylation of c-Cbl supports the concept that PTKs achieve some of their substrate specificity through their SH1 domains.

Because the Y700, Y731, and Y774 residues of c-Cbl have been reported to be the primary sites of phosphorylation of c-Cbl in vivo, we set out to determine whether SH1 domains of PTKs could discriminate between these individual tyrosines. Again, using in vitro kinase assays with purified proteins, we compared the phosphotyrosine levels of wild-type (WT) GST-CT-Cbl relative to single mutants (Y700F, Y731F, and Y774F) and a triple mutant (TM) in which Y700, Y731, and Y774 were mutated to phenylalanine, as illustrated in Fig. 2A.

Fig. 2B and E shows that the SH1 domain of Syk phosphorylated each of the single mutants comparably to WT, while phosphorylation of the TM was substantially reduced (Fig. 2B, upper panel, top two bands), suggesting that Syk phosphorylates all three tyrosines with similar ability. Autophosphorylated Syk is shown in each lane except the non-phosphorylated control, which was incubated without ATP. The degradation products of the CT-Cbl proteins are phosphorylated as well and comigrate with the Syk SH1 domain (upper panel, lower bands). The lower panel, which represents total CT-Cbl protein present, was used to normalize for quantification of phosphotyrosine levels (Fig. 2E).

In contrast to Syk, phosphorylation by Fyn was not equal between the single mutants (Fig. 2C and E). In the Fyn assays, phosphorylation of Y731F was reduced relative to WT, Y700F, and Y774F. In fact, Y731F and the TM appeared to

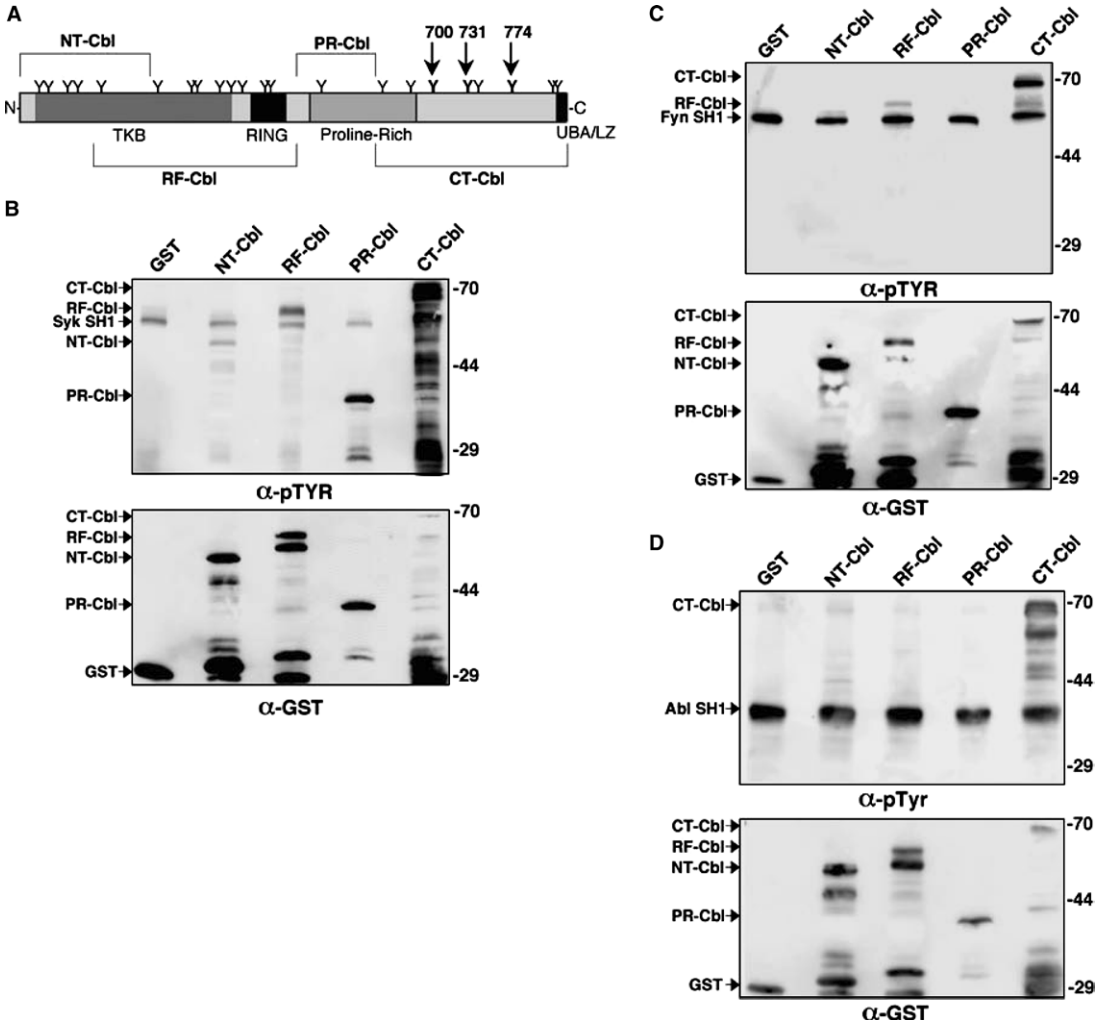

Fig. 1. The SH1 domains of PTKs differentially phosphorylate c-Cbl domains in vitro. (A) Structure of c-Cbl and schematic of the domains expressed as GST-fusions and used as substrates for in vitro kinase assays. The c-Cbl domains are designated as NT-Cbl, RF-Cbl, PR-Cbl, and CT-Cbl. Y, tyrosine; TKB, tyrosine kinase binding; RING, ring finger; UBA, ubiquitination site; LZ, leucine zipper; 700/731/774, SH2 docking sites. The migration sites of the GST-Cbl fusions and the autophosphorylated SH1 domains are indicated to the left. Top panel: phosphotyrosine immunoblot (αpTyr); bottom panel: GST immunoblot (αGST). Numbers to the right of the panels indicate molecular weight (kDa). (B) Syk SH1 assay. (C) Fyn SH1 assay. (D) Abl SH1 assay.

Table 1
Comparison of c-Cbl phosphotyrosine patterns generated by the SH1 domains of Syk, Fyn, and Abl

	NT-Cbl	RF-Cbl	PR-Cbl	CT-Cbl
Syk	+	+	+	++
Fyn		+		++
Abl				++

The ability of each kinase to phosphorylate a given region of c-Cbl (labeled at the top of the columns) is designated as +, where ++ is a higher degree of tyrosine phosphorylation. See Fig. 1A for a description of the c-Cbl fragments.

have similar levels of phosphorylation. These data suggest that Fyn prefers Y731 to other tyrosines in the C-terminus of c-Cbl. The Abl SH1 domain demonstrated yet another pattern of phosphorylation (Fig. 2D and E). We anticipated that Abl would phosphorylate Y700 and Y774 based on previous studies [1,5,15]. Mutation of Y700 significantly reduced the phosphorylation of CT-Cbl, similar to the levels of phosphorylation of the TM. Altering Y731 or Y774 had no obvious effect. The selection of Y700 by Abl is in agreement with recent data generated by mass spectrometry in which c-Cbl was found to be phosphorylated at Y700 in Bcr-Abl-expressing cells [16]. Thus, our assay is validated by two independent studies of Abl specificity for c-Cbl in vivo.

Fig. 2E summarizes the results of all in vitro kinase assays using the CT-Cbl proteins, all of which were repeated more than three times. We consistently observed that the SH1 domain of Syk is capable of phosphorylating Y700, Y731, and Y774 of c-Cbl. The Fyn SH1 domain prefers Y731, while the Abl SH1 domain prefers Y700. Thus, the three SH1 domains tested demonstrated distinct preferences for individual tyrosines that belong to SH2 recognition motifs of c-Cbl.

3.2. *Binding of the CrkL SH2 domain to c-Cbl indicates the sites of c-Cbl phosphorylation*

If SH1 domains of PTKs differentially phosphorylate c-Cbl, then binding of SH2 domains to their respective phosphotyrosine recognition motifs in c-Cbl should reflect the predicted

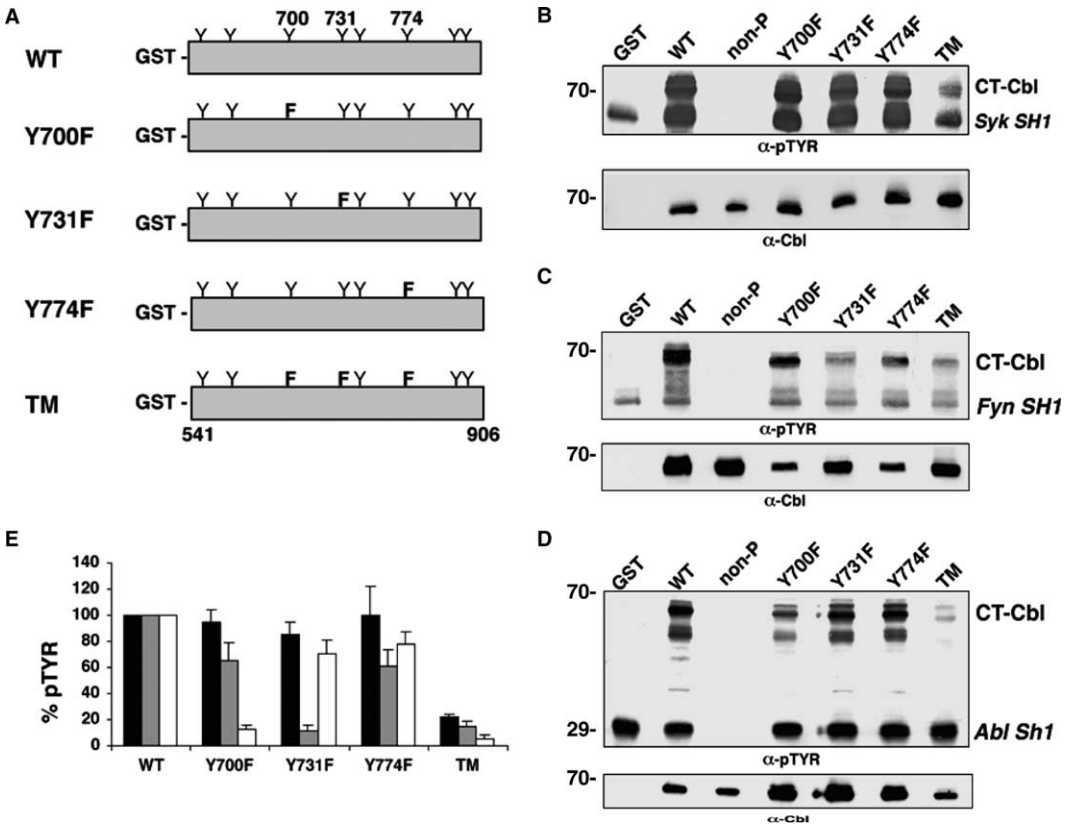

Fig. 2. The SH1 domains of PTKs discriminate between individual tyrosines in the C-terminus of c-Cbl. (A) Schematic of WT and tyrosine (Y) to phenylalanine (F) mutants of GST-CT-Cbl, which starts with amino acid 541 and ends with 906, as shown. TM, triple mutant; non-P, non-phosphorylated. For Syk (B), Fyn (C), and Abl (D) SH1 assays, top panel: phosphotyrosine immunoblot (α-pTyr) and bottom panel: c-Cbl immunoblot (α-Cbl). Numbers to the left of the panels (B–D) indicate molecular weight (kDa). (E) Average phosphorylation determined from three or more experiments, shown as % of WT. Black bars: Syk reactions, gray: Fyn, white: Abl. The phosphotyrosine levels were adjusted for loading differences. Error bars indicate standard error of the mean.

phosphorylation pattern of the kinase. This hypothesis was tested using gel-overlay assays. Purified GST-CT-Cbl and the four tyrosines to phenylalanine mutants were phosphorylated in vitro by Syk, Fyn, or Abl, separated by gel electrophoresis, transferred to PVDF membranes, and incubated with the purified SH2 domain of CrkL. When Syk was used as the kinase (Fig. 3A), the CrkL SH2 domain readily bound to phosphorylated WT CT-Cbl. Y700 and Y774 of c-Cbl belong to known recognition motifs for the CrkL SH2 domain and, as predicted, binding of the CrkL SH2 to Y700F and Y774F CT-Cbl was reduced relative to WT and Y731F. Binding of CrkL to the TM was completely abolished, reflecting the absence of both CrkL binding sites. The CrkL SH2 did not bind GST alone or the non-phosphorylated WT control, indicating that the interaction is dependent on phosphotyrosine. Fig. 3A also shows equal loading of the GST-CT-Cbl proteins (middle panel) and that the phosphotyrosine levels of each protein were as expected (bottom panel). The pattern of CrkL SH2 binding to the CT-Cbl proteins was consistent in three experiments, as illustrated graphically in Fig. 3D.

The binding pattern of the CrkL SH2 domain to the CT-Cbl proteins phosphorylated by Fyn was consistent with the phosphorylation pattern detected by immunoblotting. We had observed low levels of phosphorylation of Y700 and Y774 by Fyn relative to Y731 (Fig. 2C) and, therefore, expected some degree of CrkL binding to WT. Binding to WT (Fig. 3B and D) confirmed that Fyn has some activity toward Y700 and Y774. The activity of Fyn towards Y700 and Y774, however, was minimal. Mutation of either site alone was enough to abolish binding by CrkL, consistent with our previous observation that Fyn has more activity towards Y731 than Y700 or Y774. Surprisingly, binding to Y731F was consistently higher than WT, suggesting that Y700 and Y774 were more heavily phosphorylated when Y731 was absent, allowing more CrkL binding. It is possible that in our system, using excess kinase, Fyn directed more activity toward Y700 and Y774 because its preferred site, Y731, was absent.

When Abl was used to phosphorylate the CT-Cbl proteins, a third pattern of CrkL SH2 binding was generated (Fig. 3C). Because we had observed that Abl preferentially phosphorylates Y700, we anticipated that most of the CrkL SH2 binding would occur at Y700 of CT-Cbl. In support of this, the CrkL SH2 domain failed to bind Y700F CT-Cbl. Binding of the CrkL SH2 domain to Y774F was slightly reduced (Fig. 3C and D). The small reduction in binding of CrkL to Y774F suggests that there was enough phosphorylation of Y774 by Abl, albeit undetectable by phosphotyrosine immunoblotting (Figs. 2D and 3C), to mediate some of the interaction. Fig. 3D graphically represents all three Abl experiments and supports our conclusion that the Abl SH1 preferentially targets Y700 for phosphorylation.

In summary, we observed that when the Syk SH1 domain phosphorylates CT-Cbl, the CrkL SH2 domain readily binds both Y700 and Y774, but not other sites. Fyn was able to

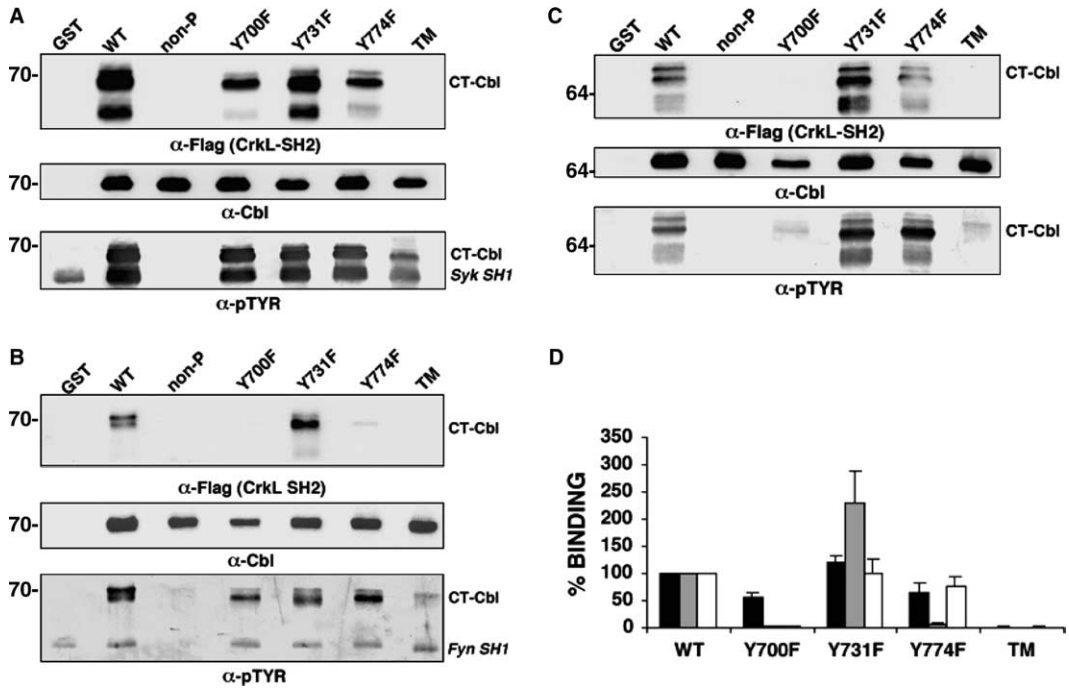

Fig. 3. Binding of the CrkL SH2 domain to phosphorylated CT-Cbl. CrkL-SH2 gel-overlay assays. GST alone or GST-CT-Cbl proteins were phosphorylated in vitro by the SH1 domain of Syk (A), Fyn (B), or Abl (C). Top panel: overlay, detected by anti-Flag (α-Flag) immunoblot. Middle panel: anti-Cbl (α-Cbl) immunoblot. Bottom panel: anti-phosphotyrosine (α-pTyr) immunoblot. Substrates include GST alone (no binding detected, data not shown) and the CT-Cbl proteins listed above top panel. Non-P, non-phosphorylated WT (no ATP in reaction). Numbers to the left of panel (A–C) indicate molecular weight (kDa). (D) Average binding determined from three or more experiments, shown as % of WT. Black bars: Syk reactions, gray: Fyn, white: Abl. Binding intensity was adjusted for loading differences. Error bars indicate standard error of the mean.

provide docking sites for CrkL at Y700 and Y774, but the interaction was weak, reflecting Fyn's selectivity for Y731 over the CrkL SH2 binding sites. When Y731 is not present, Fyn can shift its activity towards Y700 and Y774, as suggested by the increase in binding of the CrkL SH2 to Y731F. In contrast to Fyn and Syk, phosphorylation by the Abl SH1 limits CrkL SH2 binding primarily to Y700 of CT-Cbl.

3.3. Binding of the p85 (PI3-K) SH2 domains to c-Cbl indicates the sites of c-Cbl phosphorylation

Similar to the CrkL scenario, we reasoned that if the SH1 domains of Syk, Fyn, and Abl differentially phosphorylated c-Cbl, the binding of the p85 subunit of PI3-K to CT-Cbl should also reflect the phosphorylation patterns of the kinases. Y731 of c-Cbl belongs to a recognition motif (YxxM) for the p85 subunit of PI3-K [2]. We first tested p85N + C, containing both the amino and carboxy-terminal SH2 domains of p85, for direct interaction with CT-Cbl proteins. Fig. 4A and D shows that when the Syk SH1 domain phosphorylated the CT-Cbl proteins, p85N + C bound to WT, Y700F, and Y774F with similar affinity. Binding to the TM was abolished while some interaction occurred with Y731F, suggesting that p85N + C can bind to phosphoY700 or Y774 in addition to Y731.

The binding pattern of p85N + C to the CT-Cbl proteins phosphorylated by Fyn was identical to the binding pattern generated by Syk. Fig. 4B shows that the strength of the interaction, with each of the CT-Cbl proteins was equivalent with the exception of Y731F, which showed a substantial reduction in binding, and the TM, which failed to bind p85N + C altogether. The reduction in binding to Y731F was expected based on our observation that the Fyn SH1 preferentially phosphorylates Y731. The complete loss of interaction with the TM is consistent with our findings from the Syk assays in which p85N + C failed to interact with the TM. Again, the fact that multiple mutations cause additional loss of interaction supports the concept that p85N + C can associate with Y700 and/or Y774, in addition to Y731.

An alternative binding-site on CT-Cbl for p85N + C was confirmed when Abl was used to phosphorylate the CT-Cbl proteins. Because Abl primarily phosphorylates Y700, binding of p85N + C to Y700F should be absent if Y700 mediates the interaction. Consistent with this assumption, Fig. 4C and D shows that binding of p85N + C to Y700F was almost completely abrogated relative to WT. Binding to Y731F and Y774F was also reduced, although not dramatically. The binding pattern of p85N + C to the Abl-phosphorylated CT-Cbl proteins supports our conclusion that Abl preferentially phosphorylates Y700 of c-Cbl and that the SH2 domains of p85 can bind non-predicted sites in addition to Y731.

Y700 and Y774 belong to a YxxP motif and are not, therefore, predicted recognition sites for the p85 SH2 domains. Because p85 has two SH2 domains, we asked whether the ability to interact with phosphoY700 and Y774 could be ascribed to one particular SH2 domain of p85. Fig. 5A and D shows that p85-N had a reduced ability to bind Y700F and Y774F, when phosphorylated by Syk. Binding to Y731F was severely reduced. Based on these results and the observation that Syk phosphorylates all three tyrosines with similar efficiency, it appears that p85-N is capable of binding to phosphoY700 and Y774 in addition to Y731. We were unable to

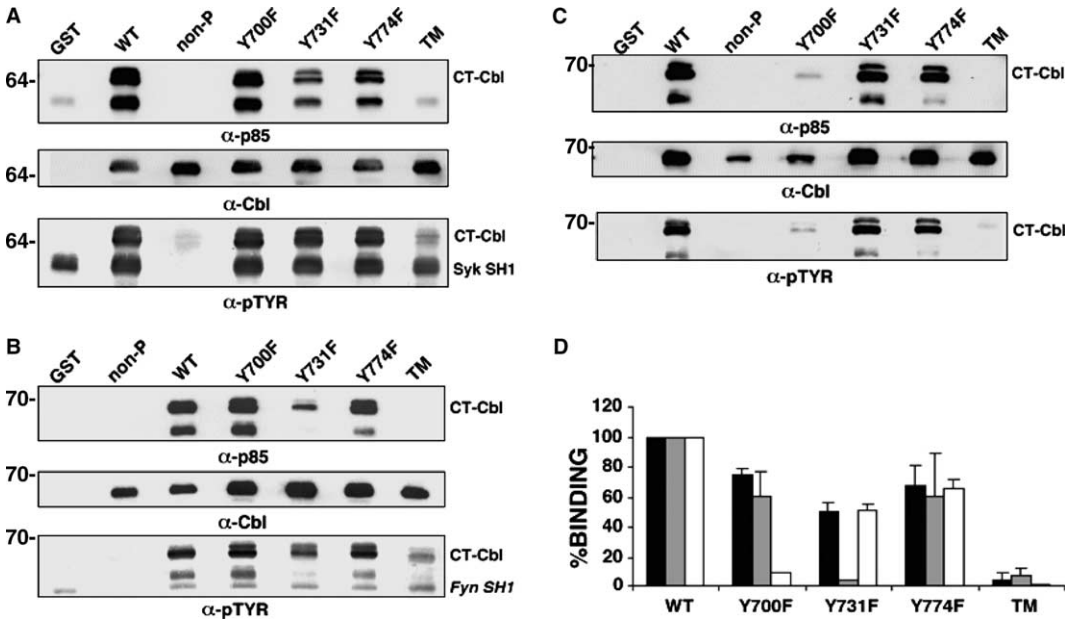

Fig. 4. Binding of p85-N + C to phosphorylated CT-Cbl. Gel overlays using both SH2 domains of the p85 subunit of PI3-K. GST alone or GST-CT-Cbl proteins were phosphorylated in vitro by the SH1 domain of Syk (A), Fyn (B), or Abl (C). Top panel: overlay, detected by anti-p85 immunoblot (α-p85). Middle panel: anti-Cbl immunoblot (α-Cbl). Bottom panel: anti-phosphotyrosine immunoblot (α-pTyr). Substrates include GST alone (no binding detected, data not shown) and the CT-Cbl proteins listed above top panel. Non-P, non-phosphorylated WT (no ATP in reaction). Numbers to the left of panels (A–C) indicate molecular weight (kDa). (D) Average binding determined from three or more experiments, shown as % of WT. Black bars: Syk reactions, gray: Fyn, white: Abl. Binding intensity was adjusted for loading differences. Error bars indicate standard error of the mean.

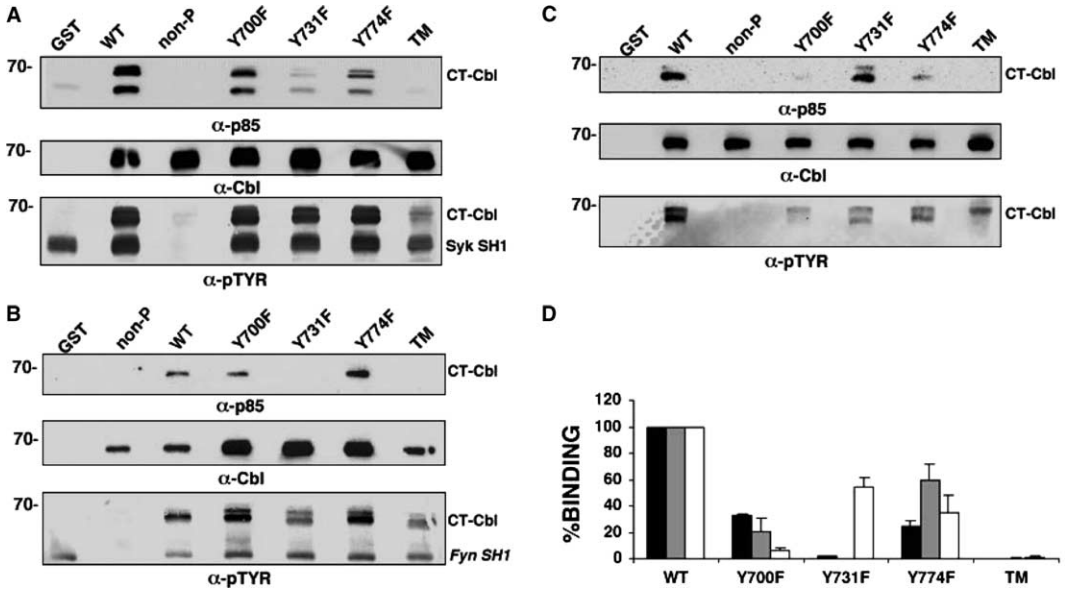

Fig. 5. Binding of p85-N to phosphorylated CT-Cbl. Gel-overlay assays with the amino-terminal SH2 domain of the p85 subunit of PI3-K. GST alone or GST-CT-Cbl proteins were phosphorylated in vitro by the SH1 domain of Syk (A), Fyn (B), or Abl (C). Top panel: overlay, detected by anti-p85 immunoblot (α-p85). Middle panel: anti-Cbl immunoblot (α-Cbl). Bottom panel: anti-phosphotyrosine immunoblot (α-pTyr). Substrates include GST alone (no binding detected, data not shown) and the CT-Cbl proteins listed above top panel. Non-P, non-phosphorylated WT (no ATP in reaction). Numbers to the left of panels (A–C) indicate molecular weight (kDa). (D) Average binding determined from three or more experiments, with the exception of Syk (represented by two experiments), shown as % of WT. Black bars: Syk reactions, gray: Fyn, white: Abl. Binding intensity was adjusted for loading differences. Error bars indicate standard error of the mean.

test the C-terminal SH2 domain of p85 because we failed to generate sufficient amounts of the purified protein.

When Fyn was used to phosphorylate the CT-Cbl proteins, a similar pattern was produced. The p85-N protein failed to bind Y731F while binding to Y700F and Y774F was reduced (Fig. 5B and D), providing further evidence that p85-N can bind at Y700 and Y774 in addition to Y731. Although the Fyn SH1 generates a phosphorylation pattern distinct from the Syk

SH1 (Fig. 2B vs. 2C), we learned from the CrkL SH2 overlays that Fyn phosphorylates Y700 and Y774 to some degree in our system, even though Y731 is the preferred site. Therefore, the reduction in binding of p85-N to Y700F and Y774F was not unexpected for the Fyn-mediated reactions.

Confirmation that p85-N can interact with phosphoY700 and Y774 of c-Cbl came from experiments with the Abl SH1 domain. When Abl was used to phosphorylate the CT-Cbl proteins, p85-N bound to WT, Y731F, and Y774F but failed to bind Y700F (Fig. 5C and D). Binding to Y774F was reduced but not as severely as Y700F. This binding pattern of p85-N indicates phosphorylation of Y700 and Y774 by the Abl SH1 domain, with Y700 being the favored site.

In summary, the SH2 domains of the p85 subunit of PI3 kinase directly interact in vitro with Y731 of c-Cbl when phosphorylated by Syk and Fyn. Syk also readily phosphorylates Y700 and Y774, providing additional docking sites for p85. When Abl drives the interaction between Cbl and p85, the binding not only occurs mostly at Y700 but also at Y774. The ability of p85 to interact with phosphoY700 and Y774 of c-Cbl is mediated, at least in part, by the amino-terminal SH2 domain of p85.

4. Discussion

The mechanism by which PTKs achieve specific signaling outcomes involves adaptor domains, which guide PTKs to their substrates [17]. The role of SH1 domains in selective signaling, however, has been questioned [17] despite evidence suggesting that PTKs selectively phosphorylate sites within their targets [5,15,16,18]. In Abl-expressing cells, for example, Y700 has been reported as a primary site of phosphorylation on ectopically expressed and endogenous c-Cbl [5,16], while Crk appears to be primarily phosphorylated at Y221 [18]. Both sites form the start of a YxxP motif that has been identified, using peptide substrates, as the consensus phosphorylation site for Abl [1,15]. Given that SH1 domains demonstrate preferences for peptide substrates, we reasoned that the specificity of phosphorylation observed in vivo resulted from the selection of particular tyrosines by the Abl SH1 domain. Broadening that concept, we hypothesized that SH1 domains, in general, confer specificity by selecting distinct tyrosines within their substrates.

We tested our hypothesis by in vitro kinase assay, using purified SH1 domains and c-Cbl domains, and then verified our findings by examining the pattern of phosphotyrosine mediated interactions between c-Cbl and downstream signaling intermediates that were generated by each PTK. We observed a preference for CT-Cbl, demonstrated by each SH1, consistent with the pattern of c-Cbl tyrosine phosphorylation found in vivo [5–7]. The fact that CT-Cbl was the preferred substrate by each kinase in our in vitro assays is significant for two reasons. First, these data recapitulate the in vivo pattern of c-Cbl phosphorylation, providing indirect evidence that the folding state of the GST-Cbl fusion proteins resembles that of the native c-Cbl protein. Second, it supports data suggesting that SH1 domains act selectively.

To further analyze the specificity of SH1 domains for c-Cbl tyrosines, we asked whether the SH1 domains of Syk, Fyn, and Abl could discriminate between Y700, Y731, and Y774 of c-Cbl. Y700 and Y774 are located within the amino acid sequences EDEYMTP and DDGYDVP, respectively. These sequences contain a YxxP motif, similar to the consensus phosphorylation motif of Abl, IYAxP, identified by peptide library screening [1,15]. The YxxP motif also appears in 11 out of the 21 substrates (not including c-Cbl) listed in the Phospho.ELM database (http://www.phospho.elm.eu.org/) [19] as Abl phosphorylation sites. Furthermore, Y700 and Y774 were found to be major sites of c-Cbl phosphorylation in Abl-transformed cells [5]. We anticipated, therefore, that Abl might demonstrate selectivity for Y700 and Y774 in our assays. We observed that the Abl SH1 domain can phosphorylate both residues but clearly prefers Y700. Interestingly, phosphotyrosine mapping of c-Cbl from Bcr-Abl-expressing cells, by mass spectrometry, detected phosphorylation of Y700 but not Y774 [16]. In essence, the preference we observed for Y700 by the Abl SH1 is in agreement with several independent reports of Abl specificity for c-Cbl in vivo. Again, the results of our assay reproduce c-Cbl phosphorylation in vivo, validating our assay and implying that SH1 domains are the ultimate determinants of phosphorylation sites within their substrates.

A consensus phosphorylation motif has not been reported for the Fyn SH1 domain. In addition, Phospho.ELM lists only eight substrates for Fyn other than c-Cbl. None of the other substrates contain sequences homologous to the c-Cbl sites phosphorylated by Fyn. It cannot be ruled out, however, that a common sequence may appear with a larger sample size of substrates to analyze. The preferred Fyn site that we identified in CT-Cbl, Y731, lies within the sequence SCTYEAM. The EAM sequence downstream of Y731 is similar to the consensus motif identified for Syk, which includes two negatively charged amino acids followed by DYE and two uncharged amino acids [20]. The sequences neighboring Y700 and Y774 (DTEYMTP and DDGYDVP, respectively) conform more than the Y731 sequences to the predicted Syk motif, with an adjacent D or E, negatively charged residues upstream, and uncharged residues downstream. Although the sequences are similar, they are not identical to the predicted phosphorylation motif for Syk. Likewise, many of the sequences listed in the Phospho.ELM database as Syk substrates deviate from the consensus motifs generated from peptide screens. Out of the 19 non-Cbl substrates listed, four contain YxxP motifs and one contains an YxxM motif. This variability in Syk substrates is consistent with our observation that the Syk SH1 domain is less selective than Abl or Fyn.

Feshchenko et al. [6] reported that coexpression of Syk or Fyn with c-Cbl in COS cells resulted in equivalent phosphorylation of Y700, Y731, and Y774. While our Syk data are concurrent with those of Feshchenko et al., our Fyn data are not. This discrepancy may well be due to the differences in techniques used. We used purified Fyn (SH1), providing absolute certainty that Fyn directly phosphorylates Y731. In cells, ectopic expression of full-length Fyn could have activated endogenous PTKs or Fyn may have associated with other PTKs and brought them to c-Cbl. Our use of purified proteins eliminated the potential for confounding effects from additional PTKs, a problem that exists when using immunoprecipitated proteins or in vivo coexpression studies.

The phosphorylation patterns observed with phosphotyrosine immunoblotting were confirmed in gel-overlay assays. The binding pattern of the CrkL SH2 domain, which recognizes phosphoY700 and phosphoY774, was in agreement with the predicted phosphorylation pattern of the PTK used. The

binding pattern of the SH2 domains of p85 also reflected the substrate preferences of the SH1 domains. Importantly, the GST-Cbl proteins were able to bind their respective SH2 partners in a specific manner, demonstrating again that these fusion proteins fold in a native manner. Interestingly, the Abl SH2 domain has been reported to selectively bind an YxxP-like motif [21], suggesting that there may be similarities in the substrate recognition properties of SH1 and SH2 domains.

In addition to confirming phosphorylation patterns, we observed that p85 is not limited to interacting with Y731 of c-Cbl. Simultaneous binding of both SH2 domains of p85 has been shown to result in optimal PI3-K activation [22]. Furthermore, c-Cbl has been reported to enhance PI3-K activation [23,24]. It is possible that c-Cbl engages both SH2 domains of p85 to facilitate activation of PI3-K.

Our findings imply that phosphorylation of c-Cbl by multiple PTKs may be necessary for the viability of downstream signaling pathways. For instance, the association of c-Cbl with CIN85 requires phosphorylation of Y700, Y731, and Y774 [8,10]. Differential phosphorylation of these tyrosines may be a point of regulation in the endophilin-CIN85-Cbl pathway, which facilitates EGF and c-Met receptor downregulation [9]. In Fyn and Abl signaling, phosphorylation of c-Cbl would be incomplete and require additional PTKs to be recruited. In T lymphocytes, Fyn and Syk are upstream activators of T cell receptor signaling [25] and associate with and phosphorylate c-Cbl [26,27] to enable the association of c-Cbl with CrkL and PI3-K [26]. Fyn may activate PI3-K by phosphorylating Y731 of c-Cbl but would have to rely on its ability to recruit Syk to c-Cbl [27] in order for the CrkL binding sites to be phosphorylated.

Similar to Fyn, Abl is known to associate with other PTKs [28]. In v-Abl transformed fibroblasts, phosphorylation of Y700, Y731, and Y774 appears to be necessary for c-Cbl to promote fibronectin deposition, adhesion and spreading [29,30]. While Y700, and possibly Y774, could be targets of Abl, phosphorylation of Y731 is likely to be carried out by a distinct PTK. Thus, differential phosphorylation of c-Cbl may allow for the integration of diverse upstream signals.

In summary, we have shown that SH1 domains of Abl, Syk, and Fyn differentially phosphorylate the protein c-Cbl. Fyn and Abl demonstrated striking preferences for particular sites, while Syk appeared to have less specificity. The site(s) of phosphorylation selected by each SH1 domain could be loosely predicted by the consensus motifs generated from phosphopeptide library analysis. More importantly, our data are in agreement with reports of c-Cbl phosphorylation patterns in vivo, validating our assay and providing indirect evidence that the c-Cbl substrates we used in vitro were folded in a native conformation. For example, the c-terminus of Cbl is the primary site of phosphorylation in vivo [5–7] and our in vitro data are identical. CT-Cbl was significantly more phosphorylated than all other domains. Second, our in vitro data for Abl reproduced the in vivo phosphorylation pattern of c-Cbl in Abl-expressing cells. And third, the CT-Cbl proteins acted like natively folded c-Cbl in our gel overlay assays by binding their respective SH2 partners in a phosphotyrosine specific manner. Because the SH1 domains were able to differentially phosphorylate natively folded c-Cbl fragments, we concluded that SH1 domains can be sufficient to confer specificity when phosphorylating a target protein. Our data support a model that SH1 domains determine sites of phosphorylation once a PTK has been targeted to its substrate by adaptor domain interactions. Selection of specific tyrosines within a substrate could conceivably limit the ability of a PTK to fully trigger downstream pathways, requiring additional kinases to contribute, but would ensure that a given pathway is consistently activated.

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