Biochemical Basis for the Requirement of Kinase Activity for Cbl-dependent Ubiquitinylation and Degradation of a Target Tyrosine Kinase*

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Members of the Cbl family of ubiquitin ligases have emerged as crucial negative regulators of tyrosine kinase signaling. These proteins preferentially interact with and target activated tyrosine kinases for ubiquitinylation, thereby facilitating the lysosomal sorting of receptor tyrosine kinases or proteasomal degradation of nonreceptor tyrosine kinases. Recent work has indicated a crucial role of the target kinase activity in Cbl-dependent ubiquitinylation and degradation, but the biochemical basis for this requirement is not understood. Here, we have used the Src-family kinase Fyn, a well characterized Cbl target, to address this issue. Using defined Fyn mutants, we demonstrate that the kinase activity of Fyn is crucial for its Cbl-dependent ubiquitinylation and degradation, but a low level of ubiquitinylation and degradation of kinase-inactive Fyn mutants was consistently observed. Mutational induction of an open conformation enhanced the susceptibility of kinase-active Fyn to Cbl but was insufficient to promote the ubiquitinylation and degradation of kinase-inactive Fyn. Notably, the Cbl-dependent degradation of Fyn did not require the Fyn-mediated phosphorylation of Cbl. Finally, we show that the major determinant of the susceptibility of Fyn protein to Cbl-dependent ubiquitinylation and degradation is the extent to which it physically associates with Cbl; kinase activity of Fyn serves as a critical determinant to promote its association with Cbl, which we demonstrate is mediated by multiple protein-protein interactions. Our results strongly suggest that promotion of association with Cbl is the primary mechanism by which the kinase activity of the targets of Cbl contributes to their susceptibility to Cbl.

Src family kinases (SFKs)† are members of a large family of evolutionarily conserved protein-tyrosine kinases (PTKs), with crucial biological roles in tissue and organ development, cell differentiation, adhesion and migration, and immune responses (1, 2). Relatively subtle mutations can render SFKs dominantly oncogenic (2), and their deficiencies, either individually or in combination with additional family members, produce severe developmental and/or adult organ dysfunctions (3–5). Thus, understanding the mechanisms of their function and regulation is of great biological interest. As the first family of PTKs identified, SFKs have also served as an eminent model to understand the mechanisms of PTK regulation in general. SFKs exhibit a conserved domain structure: a membrane-anchoring N-terminal myristoylation signal, adjacent Src homology 3 (SH3) and SH2 domains, a kinase domain, and a tyrosine residue near the C-terminal tail whose phosphorylation by the C-terminal Src kinase is crucial to repress the enzymatic activity (1, 2). The crystal structures of the kinase and regulatory domains of several SFKs, together with extensive mutational analyses, have defined the molecular basis of SFK repression and suggested plausible mechanisms of activation (6–9). Intramolecular SH3 domain binding to a type II polyproline-like helix within the SH2-kinase linker region and the SH2 domain binding to a C-terminal phosphotyrosine residue force the kinase-active cleft into an inactive conformation and concurrently shield the SH2 and SH3 domains from intermolecular interactions with signaling intermediates (6, 7). Activation signals are thought to release the SH2 and SH3 domains from their intramolecular ligands, promoting the open, active conformation of the kinase domain and allowing the SH2 and SH3 domain-mediated assembly of signaling complexes (6, 7). Indeed, inactivating point mutations in the SFK SH3 or SH2 domains, deletion or substitution of the negative regulatory tyrosine, deletion of the C-terminal Src kinase gene (which mediates the phosphorylation of the C-terminal tyrosine), mutations in the SH2-kinase linker that abolish its binding to SH3 domain, or overexpression of high affinity SH3 domain ligands lead to constitutive activation of SFKs (2, 10–13). Conversely, mutational increase in the affinity of the C-terminal phosphotyrosine motif for the SH2 domain reduced the kinase activity of a SFK (14). Thus, it is evident that multiple mechanisms must exist inside a cell to precisely regulate the activity of SFKs.

It is not clear at present how the activated SFKs are regulated at the level of regulation at the level of tyrosine kinase; RTK, receptor tyrosine kinase; WT, wild type; KD, kinase-dead; SH2 and SH3, Src homology 2 and 3, respectively; IP, immunoprecipitate; IB, immunoblot; pAb, polyclonal antibody; HA, hemagglutinin; HSP, heat shock protein; GFP, green fluorescence protein; EGFR, epidermal growth factor receptor; Ub, ubiquitin; RIPA, radio-immune precipitation.

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† The abbreviations used are: SFK, Src family kinase; PTK, protein-tyrosine kinase; RTK, receptor tyrosine kinase; WT, wild type; KD, kinase-dead; SH2 and SH3, Src homology 2 and 3, respectively; IP, immunoprecipitate; IB, immunoblot; pAb, polyclonal antibody; HA, hemagglutinin; HSP, heat shock protein; GFP, green fluorescence protein; EGFR, epidermal growth factor receptor; Ub, ubiquitin; RIPA, radio-immune precipitation.

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turned to their basal repressed conformation. A fundamental mechanism to reverse the PTK activation is through the activity of phosphotyrosine phosphatases (15–17). However, given the clear evidence that SFKs require cellular chaperones, such as the members of HSP90 family, for posttranslational maturation (18, 19), it is likely that additional mechanisms for inactivation of activated SFKs and, by implication, other PTKs exist in cells. Recent studies, by our laboratory and others, indicate that the proto-oncoprotein Cbl provides one such mechanism for deactivation of SFKs (20–25). Cbl is a member of an evolutionarily conserved family of multidomain adaptor-like proteins that function as ubiquitin ligases toward activated PTKs (26–31). Cbl-dependent ubiquitylation of RTKs facilitates their sorting to the lysosomes, where they are degraded (30, 32, 33). Notably, transfection studies have shown that Cbl can target the activated pools of nonreceptor PTKs, such as Syk, ZAP-70, and SFKs, for degradation apparently via the proteasome (21, 22, 34–39). Thus, Cbl-regulated and ubiquitin-dependent degradation has emerged as a major mechanism for negative regulation of PTK signaling. Indeed, genetic ablation of murine Cbl led to hypercellularity and altered development of several radiation systems, whereas Cbl-b deletion led to hyperproliferation and hyperactivation of immune cells, resulting in autoimmunity, a phenotype even more prominently observed upon combined T cell-specific Cbl and Cbl-b deficiency (42–44). Notably, Cbl homologues in Caenorhabditis elegans and Dro sophila have been genetically defined as negative regulators of epidermal growth factor receptor (EGFR) signaling (45–48).

The molecular basis of how Cbl selectively functions as a ubiquitin ligase toward the active pools of PTKs has begun to be elucidated (24, 26–28). An evolutionarily conserved N-terminal tyrosine kinase binding (TKB) domain, composed of a four-helix bundle, an EF-hand, and an incomplete SH2 domain (49), specifically binds to negative regulatory phosphorylation sites induced by autophosphorylation; such motifs have been clearly delineated in Syk/ZAP-70 PTKs and a number of RTKs, generally conforming to an extended motif (N/D)XpYXXX(V/P)φ (where pY represents phosphotyrosine, and φ is a hydrophobic residue) (34, 50–56). A highly conserved RING finger domain, located between the TKB domain and the proline-rich region, interacts with ubiquitin-conjugating enzymes (22, 57, 58), juxtaposing the Cbl-associated, activated PTKs with a ubiquitin-conjugating enzyme, thereby leading to PTK ubiquitylation. Similarly, a proline-rich region in Cbl interacts with the SH3 domains of SFKs and other signaling proteins, whereas inducible tyrosine phosphorylation sites within the C-terminal region allow interaction with SH2 domain-containing proteins (24, 34, 58–60). Interaction with the TKB domain provides the sole mechanism for Cbl-mediated ubiquitylation of Syk/ZAP70 PTKs (34, 37, 38, 59). In the case of RTKs, such as EGFR, a secondary mechanism of Cbl recruitment is provided by adaptor molecules, such as Grb2; Grb2 SH3 domain binds to the proline-rich region of Cbl, whereas its SH2 domain binds to the autophosphorylated EGFR (54, 61, 62).

The nature of molecular interactions that mediate Cbl-dependent ubiquitylation of SFKs is more complex, and the relative roles of the various intermolecular interactions are less well defined. A prominent mechanism of Cbl-SFK association involves the SH3 domain binding to proline-rich sequences in Cbl (21, 23). Furthermore, the SH2 domains of SFKs can interact with phosphopeptide motifs in the C-terminal half of Cbl (63, 64). Finally, the Cbl TKB domain can directly interact with activated SFKs, apparently by binding to the phosphorylated activation loop (21, 65). Consistent with these complex intermolecular interactions, a TKB domain mutant of Cbl was fully capable of inducing the degradation of the SFK Fyn, and abrogation of Fyn SH3 domain binding to the proline-rich region of Cbl, in addition to a Cbl TKB mutation, was required to effectively block the effect of Cbl on Fyn (21). It is notable that the phosphorylation sites in Cbl that promote Cbl-SFK associations reside in its C-terminal region, which is dispensable for EGFR and Syk/ZAP-70 regulation (66, 67). Thus, there is a clear need to better define the relative importance of various mechanisms of Cbl-SFK association and their role in SFK-directed ubiquitin ligase activity of Cbl.

Given that Cbl is selectively recruited to active PTKs and is invariably a substrate of these kinases, the potential role of the kinase activity in controlling the Cbl-mediated ubiquitylation of PTKs has been of great interest. Indeed, the kinase-dead Src was markedly resistant to Cbl-dependent ubiquitylation and degradation (35), and inhibitors of the kinase activity reduced the Cbl-dependent ubiquitylation of Src (22). Similarly, the kinase-active EGFR (68) and SYK (39) mutants were resistant to Cbl-induced degradation. However, it is unclear whether the kinase activity of a target PTK is required to promote its association with Cbl, to induce a conformation that exposes the target lysine residues, or to regulate the activity of Cbl as a ubiquitin ligase. SFKs offer an excellent model to assess the role of the kinase activity versus the induced open conformation as determinants of the susceptibility of PTKs to Cbl-dependent ubiquitylation and degradation, since well-defined mutations in specific motifs can be introduced to promote an open conformation in SFKs (69, 70). Here, we have used this approach in the context of the SFK Fyn.

Fyn is a prototype SFK that plays important physiological roles in conjunction with other widely expressed family members, such as Src and Yes (2–4), as well as more selective roles in myelination, neuronal function, and T cell development (71–75). We have shown that coexpression with Cbl promotes the ubiquitylation and degradation of Fyn, resulting in the negative regulation of Fyn-dependent cellular activation, and that cells from Cbl+/− mice have elevated total and active Fyn protein levels (21, 23). In addition, Cbl-dependent Fyn degradation requires the ubiquitin ligase activity of Cbl and an intact cellular ubiquitylation machinery, as revealed by lack of Cbl-dependent Fyn degradation in Chinese hamster ovary cells with a temperature-sensitive defect in the ubiquitin-activating enzyme (25). By analyzing a series of defined mutants of Fyn, we show here that both the kinase activity and open conformation determine the susceptibility of a target PTK to Cbl, primarily by controlling the level of association with Cbl. Thus, the level of association with Cbl appears to be the major determinant of the selective ubiquitylation and degradation of an active PTK by Cbl.

MATERIALS AND METHODS

Expression Constructs and Site-directed Mutagenesis—The expression constructs encoding the hemagglutinin (HA)-tagged ubiquitin (HA-Ub; pMT vector), CD8-α chimera (CD8 extracellular and transmembrane domains fused to T cell receptor α; CD8α-Cα vector; pSRA.neo vector), HA-Cbl (pAlterMAX vector; Promega, Madison, WI), GFP-Cbl (pCDNA3 vector), and Fyn (in pAlterMAX) have been described previously (21, 34, 67, 76). Point mutants of Fyn and HA-Cbl (described under “Results” and in the figure legends) were generated using the QuikChange® site-directed mutagenesis kit (catalog no. 200518; Stratagene, La Jolla, CA) and the appropriate mutagenic primers (sequences available upon request). The sequences of all mutant constructs were verified.

Antibodies—The antibodies used in this study were as follows: monoclonal antibody anti-HA epitope tag (12CA5, IgG2b) (Covance Inc., Inc.,
Role of Kinase Activity in Cbl-mediated PTK Degradation

Fig. 1. The kinase activity of Fyn is critical for its susceptibility to Cbl-dependent ubiquitinylation and degradation as well as for Cbl-Fyn association. A, 293T cells were transfected using the calcium phosphate method with plasmids encoding the WT Fyn protein or its kinase-inactive mutant Fyn-K296R (Fyn-KD) (0.15 μg each) and a CD8 chimera (0.05 μg each) as an internal indicator of tyrosine phosphorylation activity. 48 h after transfection, cell lysates were prepared in RIPA buffer. 50-μg aliquots of cell lysate protein were resolved by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and subjected to anti-phosphotyrosine (top panel) or anti-Fyn (bottom panel) immunoblotting. B, 293T cells were transfected with plasmids encoding HA-ubiquitin (5 μg), WT or KD Fyn (0.15 μg), and GFP (-) or GFP-Cbl (+) (3 μg each). 48 h post-transfection, cell lysates were prepared in RIPA lysis buffer. Anti-Fyn antibody (15 μg) immunoprecipitation from 750-μg aliquots of lysate protein was subjected to anti-HA immunoblotting (top panel) to assess Fyn ubiquitinylation and reprobation with anti-Fyn to visualize immunoprecipitated Fyn (second panel). 50-μg aliquots of lysate protein were directly immunoblotted with an anti-GFP antibody to assess GFP-Cbl levels (bottom panel). C, duplicate sets of 293T cells were transfected with plasmid encoding WT or KD Fyn (0.15 μg) with or without HA-Cbl vector (1 μg). 48 h post-transfection, cell lysates were prepared in RIPA or Triton lysis buffer. 50-μg aliquots of RIPA lysate protein were serially immunoblotted with an anti-Fyn (top panel) and anti-HA antibodies (bottom panel). Anti-Fyn IPs from the 750-μg aliquot of Triton lysate protein were subjected to anti-HA immunoblotting to detect HA-Cbl co-IP (second panel), followed by anti-Fyn immunoblotting (third panel).

RESULTS AND DISCUSSION

Previous analyses with Src (22, 35), EGFR (68), and Syk (39) have shown that an intact kinase domain is required for the susceptibility of PTKs to Cbl-mediated ubiquitinylation and degradation. It is, however, unclear whether the kinase activity of the target PTKs is required for Cbl recruitment, to induce a kinase conformation that is more susceptible to ubiquitinylation, or to regulate Cbl function. If the kinase activity is primarily required to promote the association with Cbl, then abrogation of the kinase activity should concomitantly reduce the Cbl association. On the other hand, if the primary role of kinase activity is to promote the ubiquitinylation through the latter two mechanisms, then loss of kinase activity should not reduce the level of association. To address these possibilities in the context of SFKs, we have examined the structural determinants of the susceptibility of Fyn PTK to Cbl-dependent ubiquitinylation and degradation.

To assess whether the kinase activity of Fyn is required for its Cbl-dependent ubiquitinylation and degradation, we first compared the wild type (WT) Fyn protein with its kinase-dead (KD) mutant (Fyn-K296R) using transient transfection analyses in 293T cells. As demonstrated by the marked reduction in autophosphorylation as well as the absence of phosphorylation of a co-transfected CD8 chain chimera (Fig. 1A, lane 1 versus 293T cells. As demonstrated by the marked reduction in autophosphorylation as well as the absence of phosphorylation of a co-transfected CD8 chain chimera (Fig. 1A, lane 1 versus...
The mutant protein was completely inactive; the residual phosphorylation of Fyn-K296R is likely to be due to C-terminal phosphorylation, since it was unreactive with antiphospho-Src antibody, which recognizes the activation loop phosphorylation (not shown). To assess the effect of Cbl on Fyn ubiquitinylation, we utilized a GFP-Cbl construct together with HA-Ub. Anti-GFP immunoblotting of cell lysates demonstrated the expression of GFP-Cbl in the transfected cells (Fig. 1B, lower panel). Anti-HA immunoblotting of the anti-Fyn IPs revealed the expected (23) Cbl-dependent enhancement of the WT Fyn ubiquitinylation (Fig. 1B, upper panel, lanes 1 and 2).

In contrast, the KD mutant of Fyn showed a markedly reduced level of Cbl-dependent ubiquitinylation; however, an easily detectable level of residual ubiquitinylation was consistently observed (lanes 3 and 4). To assess the role of kinase activity in Cbl-dependent degradation of Fyn, we cotransfected the 293T cells with Fyn constructs together with HA-Cbl and assessed the Fyn protein level by anti-Fyn immunoblotting of cell lysates. In keeping with the reduced Cbl-dependent ubiquitinylation of the Fyn-KD mutant, the extent of its degradation was also severely reduced; however, a residual level of Cbl-dependent degradation was reproducibly observed (Fig. 1C, top panel, lanes 3 and 4 versus lanes 1 and 2).

Since the substantial but incomplete loss of Cbl-mediated ubiquitinylation and degradation of the kinase-inactive Fyn mutant, we asked whether the WT and KD Fyn proteins associate with Cbl at a comparable level. For this purpose, anti-Fyn IPs were carried out from lysates of 293T cells cotransfected with HA-tagged Cbl and Fyn (WT or KD), and these were immunoblotted with an anti-HA antibody. Although the levels of HA-Cbl protein expression were comparable (Fig. 1C, bottom panel, lane 2 versus lane 4) and KD Fyn mutant was expressed at slightly higher levels compared with WT Fyn (Fig. 1C, top panel, lane 2 versus lane 4), the level of HA-Cbl that coimmunoprecipitated with the Fyn-KD mutant was markedly lower compared with that with WT Fyn (Fig. 1C, second panel, lane 2 versus lane 4). This result suggested that the impaired association may be a major factor in the reduced susceptibility of the kinase-inactive Fyn mutant to Cbl-mediated ubiquitinylation and degradation. The residual association with Cbl, however, appeared sufficient to promote the Cbl-dependent ubiquitinylation and degradation of KD Fyn at a lower level, suggesting that the kinase activity is not absolutely essential for ubiquitinylation.

The inactive SFKs are folded into a compact structure with occluded SH2 and SH3 domains, and the active site tyrosine residue is in an unphosphorylated state (6–8), making these potential mechanisms of interaction unavailable for Cbl association. Thus, the requirement of the kinase activity for the susceptibility of Fyn to Cbl-dependent ubiquitinylation may reflect the open conformation of the active kinase, making the various motifs in Fyn available for interaction with Cbl. We utilized specific mutations in Fyn to further address this issue. One major mechanism for the interaction of WT Fyn and Cbl is via the Fyn SH3 domain binding to the proline-rich region of Cbl. Therefore, we engineered a Fyn SH2-kinase linker region mutant Fyn-P251A, analogous to the Src-P250 mutant (77), to release the SH3 domain from its intramolecular ligand. As anticipated (70, 77), the Fyn-P251A mutant exhibited a higher level of autophosphorylation on the activation loop tyrosine residue, reflective of its higher kinase activity (data not shown). This mutant showed a higher level of ubiquitinylation in the absence of Cbl cotransfection (Fig. 2A, upper panel, lanes 5 and 7) and was substantially more susceptible to Cbl-dependent degradation, as demonstrated by a markedly lower protein level in the anti-Fyn blot (Fig. 2B, top panel, lanes 1 and 2 versus lanes 5 and 6). This behavior is consistent with the predicted open conformation of this protein (70, 77). Notably, when a kinase-inactivating mutation was introduced in Fyn-P251A, the double mutant (Fyn-P251A-KD) showed a markedly reduced level of ubiquitinylation and degradation compared with that of the WT Fyn protein without the linker mutation (Fig. 2A, A and B, upper panels, lane 4 versus lane 8).

Furthermore, the Fyn-P251A-KD mutant was markedly more resistant to Cbl-mediated ubiquitinylation and degradation compared with the Fyn-P251A mutant with an intact kinase domain (Fig. 2, A and B, upper panels, lane 6 versus lane 8).
Thus, whereas the linker mutation in the context of an active kinase domain markedly enhanced the Cbl-dependent degradation of Fyn, the same mutation in the context of a kinase-dead Fyn protein had relatively marginal effect to promote Fyn degradation.

Given the reduced association the Fyn-KD mutant with Cbl (Fig. 1C, second panel, lane 2 versus lane 4), we assessed the level of Cbl association with the Fyn-P251A and Fyn-P251A-KD mutants compared with that with WT Fyn. Directly correlating with the level of Cbl-dependent ubiquitinylation and degradation, the Fyn-P251A mutant showed a substantially elevated level of association with Cbl (Fig. 2B, second panel, lane 6 versus lane 2), although this mutant was present at markedly reduced levels due to its Cbl-dependent degradation (Fig. 2B, upper panel, compare lane 1 with lane 2 and lane 5 with lane 6). In contrast, the level of association of the Fyn-P251A-KD mutant with Cbl was markedly lower and barely above that of the Fyn-KD mutant (Fig. 2B, second panel,
lane 4 versus lane 8). Thus, merely promoting an open conformation of Fyn through the mutation of its SH2-kinase linker, in the context of an inactive kinase domain, failed to promote Cbl-Fyn association or Cbl-dependent ubiquitylation and degradation.

Aside from the interaction of the SH3 domain with the SH2-linker region, the interaction between the SH2 domain and the C-terminal phosphotyrosine is the other crucial determinant of the repressed state of SFKs (7, 14, 78, 79). Therefore, we also examined the impact of relieving the Fyn protein of this second intramolecular interaction on its Cbl-dependent ubiquitylation and degradation. As previously noted (21), Fyn-Y528F mutant was substantially more susceptible to Cbl-dependent ubiquitylation and degradation (Fig. 3, A and B, upper panels, lane 2 versus lane 4). When the Fyn-Y528F protein was further mutated to be kinase-inactive (Fyn-Y528F-KD), there was a marked loss of the Cbl-dependent ubiquitylation and degradation; however, low levels of residual ubiquitylation and degradation were still observed (Fig. 3, A and B, lane 4 versus lane 6). Analysis of the Fyn-Cbl association revealed that the KD version of Fyn-Y528F mutant was markedly impaired in its association with Cbl (Fig. 3B, second panel, lane 4 versus lane 6).

Given the results that the kinase-dead Fyn mutants separately relieved of two crucial intramolecular interactions (SH3-linker and SH2-tail) did not show an increase in their susceptibility to Cbl, we also examined the Fyn mutant (Fyn-P251A-Y528F) in which both of these intramolecular interactions were disabled. Similar to the Fyn-P251A and Fyn-Y528F mutants, the kinase-active Fyn-P251A-Y528F mutant was more susceptible to Cbl-dependent ubiquitylation (Fig. 3A, upper panel, lane 8 versus lane 2) and degradation (Fig. 3B, upper panel, lanes 7 and 8 versus lanes 1 and 2) in comparison with the WT Fyn. When we compared the level of Cbl coimmunoprecipitation with the kinase-active versus the kinase-dead Fyn-P251A-Y528F mutants, we again observed a marked reduction in the association of Cbl with the KD version of this mutant (Fig. 3B, second panel, lane 8 versus lane 10). Thus, the level of association with Cbl correlated strongly with the susceptibility of the Fyn proteins to Cbl. Importantly, a Fyn protein completely relieved of its intramolecular interactions (predicted to be in a fully open conformation) did not become substantially more susceptible to Cbl-induced ubiquitylation and degradation if it lacked the kinase activity.

Since residual Cbl association as well as Cbl-dependent ubiquitylation and degradation were observed with all kinase-dead mutants examined above, it was likely that this association was mediated via the Fyn SH3 domain binding to the proline-rich region of Cbl. Indeed, when we introduced a point mutation (P134V) in the Fyn-P251A-Y528F-KD mutant, no association with Cbl was observed (Fig. 3B, second panel, lane 10 versus lane 12), and this mutant was completely resistant to Cbl-dependent ubiquitylation and degradation (Fig. 3A and B, upper panel, lanes 9 and 10 versus lanes 11 and 12). The behavior of this mutant further emphasizes the tight correlation between the level of association of the Fyn proteins with Cbl and their Cbl-dependent ubiquitylation and degradation.

Whereas the requirement for Cbl co-transfection strongly indicated that the ubiquitylation and degradation of the kinase-inactive Fyn proteins was mediated by Cbl, we wished to test it directly. Use of a ubiquitin ligase-inactive Cbl mutant, Cbl-70Z (38, 66, 67), demonstrated that the degradation of kinase-active as well as kinase-inactive Fyn mutants observed in the above experiments was strictly dependent on the ubiquitin ligase activity of Cbl. In each case, whereas a reduction in Fyn protein levels was seen in the presence of WT Cbl, no degradation was observed with Cbl-70Z (Fig. 3C, upper panel).

As expected from the lack of its association with Cbl, the level of Fyn-P134V-P251A-Y528F-KD mutant protein was comparable in the absence or presence of WT Cbl or Cbl-70Z mutant (Fig. 3C, top and second panels, lanes 16–18). The higher level of the association of Fyn proteins with the ubiquitin ligase-deficient Cbl-70Z mutant is likely to be due to inability of this mutant to target the associated Fyn proteins for ubiquitylation and degradation.

The above observations strongly suggested that the kinase activity of Fyn promotes its susceptibility to Cbl by facilitating the Cbl-Fyn association. The kinase-active Fyn protein can interact with Cbl through three potential binding mechanisms: the Fyn SH3 domain binding to the proline-rich region of Cbl (as seen above and previously shown to be an important mode of association), the Fyn SH2 domain binding to the induced phosphorylation sites within the C-terminal half of Cbl (21, 23, 63), and a potential interaction between the Cbl TKB domain and a phosphopeptide motif induced on the active kinase (21, 65). Therefore, we carried out a series of analyses to determine the relative importance of these modes of binding as determinants of the susceptibility of Fyn to Cbl.

Given the importance of the Cbl TKB domain-mediated interactions in the regulation of other PTKs targeted by Cbl, we wished to identify and selectively disrupt the TKB domain-binding motif on Fyn for further functional studies. Consistent with previous observations on Fyn and Src (21, 65), pull-down assays using glutathione S-transferase-Cbl-N (incorporating the Cbl TKB domain) showed that wild-type Fyn can bind to the Cbl TKB domain but not to its binding-defective mutant (Cbl-N-G306E) (not shown). The predicted Cbl TKB...
FIG. 5. The role of the interactions mediated by the Fyn SH2 domain in Cbl-dependent ubiquitylation and degradation of Fyn.
A, 293T cells were transfected with plasmids encoding HA-ubiquitin (5 μg), Fyn or its indicated mutants (0.15 μg), and GFP (–) (control) or GFP-Cbl (+) (3 μg each), and RIPA lysate was prepared after 48 h. Anti-Fyn IPs from 750-μg aliquots of the lysate protein were subjected to anti-HA immunoblotting (top panel) followed by anti-Fyn reprobing (second panel). 50-μg aliquots of cell lysate protein were immunoblotted with anti-GFP blots.

B. The role of the interactions mediated by the Fyn SH2 domain in Cbl-mediated PTK degradation. Role of Kinase Activity in Cbl-mediated PTK Degradation.
domain-binding sequence in Fyn, based on the consensus motif (N/D)XpYXXX(X/P)β (49, 53, 59), corresponds to the activation loop tyrosine phosphorylation site in Fyn (DNExpYTARQ). Indeed, the Fyn-Y417F mutant, similar to Src-Y416F mutant (65), failed to bind to Cbl TKD domain (not shown); however, since this mutant does not undergo autophosphorylation and full activation, it could not be employed in functional studies. Similar results were observed with mutations (Y417D or Y417E) engineered to partially mimic the negative charge of phosphorylation or alanine mutation of the aspartic residue (D415A) at position 2 N-terminal to phosphotyrosine (not shown), which was found critical in the TKB domain binding to target phosphopeptide motifs (50). Finally, none of a panel of mutations in position +4 relative to pY, a position critical for binding based on crystal structure of a phosphopeptide-bound Cbl TKB domain (49), could abrogate Cbl TKB domain binding to Fyn; in fact, some mutations (e.g. Q421A, Q421E, and Q421S) reproducibly enhanced binding (data not shown).

In view of our inability to design a point mutant of Fyn that retained the kinase activity but had lost the Cbl TKB domain binding, we resorted to an alternate strategy to determine the role of Cbl TKB domain-mediated interaction in Cbl-dependent ubiquitinylation and degradation of Fyn. As previously reported (21, 23), Cbl TKB domain mutation in the context of a full-length Cbl protein has relatively little impact on the degradation of Fyn (Fig. 4, upper panel, lane 2 versus lane 3). Notably, when the potential interactions mediated by the C-terminal half of Cbl (via the Cbl proline-rich region binding to Fyn SH3 and the binding of induced phosphorylation sites on Cbl to Fyn SH2 domain) were eliminated (in the Cbl(-1–436) truncation), less degradation of cotransfected Fyn was observed (Fig. 4, top panel, lane 4 versus lane 2). Notably, a TKB domain mutation of this truncated protein (Cbl(-1–436)-G306E) eliminated its ability to induce the degradation of Fyn (Fig. 4, top panel, lane 5 versus lanes 2 and 4), supporting the role of TKB domain-mediated interaction in Cbl-mediated degradation of Fyn. We also carried out the reciprocal analysis by mutationally eliminating the ability of the Fyn SH3 domain to bind to Cbl, a major mode of Cbl-Fyn interaction (21, 23) (see above). As expected from the increased kinase activity of the Fyn SH3 domain mutant (Fyn-P134V) (21), this Fyn mutant was substantially more susceptible to Cbl-dependent degradation (Fig. 4, top panel, compare lanes 1 and 2 with lanes 6 and 7). Notably, the TKB domain mutation of full-length Cbl led to a markedly reduced level of degradation of Fyn-P134V (Fig. 4, lanes 7 and 8 versus lanes 2 and 3). The residual degradation of this mutant by Cbl-G306E is likely to reflect its interactions with the SH2 domain of Fyn. Importantly, Chl(-1–436), which lacks the ability to interact with Fyn SH2 and SH3 domains, induced marked degradation of Fyn-P134V mutant (Fig. 4, top panel, compare lane 9 versus lane 6 and lane 4 versus lane 1); in this case, the Chl(-1–436)G306E mutant was essentially without an effect on Fyn protein levels (lane 10 versus lane 6). Comparison with a RING finger domain mutant of Cbl (Cbl-C3AHN) (67) demonstrated that the enhanced degradation of Fyn-P134V mutant by Cbl proteins was indeed dependent on the ubiquitin ligase activity (not shown). Collectively, the results with truncation mutants of Cbl, with or without an intact TKB domain, and wild type and SH3 domain mutants of Fyn demonstrate that the Fyn SH3 domain binding to the proline-rich region of Cbl and the Cbl TKB domain binding to autophosphorylated Fyn provide predominant mechanisms of association between Cbl and activated Fyn, with Fyn SH2 domain-mediated interaction with phosphorylated Cbl probably providing an additional mechanism.

To directly test the role of the Fyn SH2 domain suggested by the analyses presented above, we examined the Fyn proteins with inactivating point mutations in the SH3 (P134V, as above), the SH2 (W176K), and both the SH3 and SH2 domains either in the context of the wild type or a kinase-dead protein. Mutation of the SH2 domain alone did not significantly affect the susceptibility of the Fyn protein to ubiquitinylation (Fig. 5A, upper panel, lane 2 versus lane 5 or 6) or degradation (Fig. 5B, upper panel, lane 2 versus lane 5 or 6) by WT Cbl or its TKB domain mutant (G306E). As anticipated, the KD SH2 domain mutant of Fyn showed reduced ubiquitinylation and degradation (lanes 7–9). Thus, the SH2 domain appears to play a minor role under these conditions. In contrast, when the SH2 domain mutation was combined with the SH3 domain mutation, the active SH3 domain mutant showed reduced susceptibility to WT Cbl (lane 11 versus lane 17) as well as to Cbl-G306E (lane 12 versus lane 18). Furthermore, the SH3/SH2 double mutant was essentially fully resistant to ubiquitinylation and degradation by Cbl-G306E mutant, conditions under which all three potential interactions between Cbl and Fyn were interrupted (compare lane 18 with lanes 3, 6, 9, and 12). Co-IP analyses again showed a strong correlation between the extent of Fyn protein degradation and its association with Cbl proteins (Fig. 5B, second panel). Comparable results were observed with SH3, SH2, and double mutants of Y528F Fyn mutant (not shown). These analyses confirm the role of Fyn SH2 domain-mediated Fyn-Cbl interaction in Cbl-mediated ubiquitinylation and degradation of Fyn.

Studies presented here with Fyn, together with previous analyses of Src, Syk, and EGFR (35, 39, 68), clearly demonstrate that kinase activity of a target PTK is a major factor in determining its susceptibility to Cbl-dependent ubiquitinylation and degradation. However, the biochemical basis for the requirement of the kinase activity of Cbl targets has not been elucidated. Previous studies have suggested that one role of kinase activity is to enhance the ubiquitin ligase function of Cbl through phosphorylation of specific tyrosine residues in Cbl (65, 80). However, these studies did not address the potential modulation of Cbl-PTK association under their experimental conditions. Furthermore, one potential phosphorylation site that was initially suggested as such a regulatory site, the Cbl Tyr772 residue within the critical linker region between the TKB and RING finger domains (80), was subsequently shown by crystal structural analysis to be required for the integrity of the linker helix and its interaction with the RING finger domain (58). Our previous results with EGFR and Syk (66, 67) and the present studies with Fyn further demonstrate that a Cbl protein composed only of TKB and RING finger domains can effectively induce the degradation of an active target PTK. This truncated protein lacks the major tyrosine phosphorylation sites Tyr700, Tyr731, and Tyr774 (63) and is not significantly phosphorylated (67). Thus, it is unlikely that phosphorylation of Cbl on tyrosine residues underlies the requirement of the target PTK activity. Whereas some direct regulation of Cbl activity through phosphorylation or physical interaction with an anti-GFP antibody to visualize GFP-Cbl (bottom panel). B, duplicate sets of 293T cells were transfected with the indicated WT or mutant Fyn constructs (0.15 μg), with or without Chl (1 μg), and RIPA or Triton cell lysates were prepared after 48 h. 50-μg aliquots of RIPA lysate protein were serially immunoblotted with anti-Fyn (top panel), anti-HA (fourth panel), and with an-activated protein kinase (anti-MAPK, bottom panel; loading control) antibodies. Relative Fyn protein levels were determined by densitometry of scanned blots, assigning the lanes without Cbl a value of 1. Anti-Fyn IP's from 750-μg aliquots of the Triton lysate protein were immunoblotted with an anti-HA antibody (second panel), followed by anti-Fyn reprobing (third panel).
Given the close structural similarity of SFKs, the three modes of physical interaction identified between Fyn and Cbl are likely to hold true for other SFKs. Whether an individual molecule of Cbl and a target SFK concurrently engage in all three modes of physical association defined here will await direct structural studies; however, our studies make it clear that each interaction can independently contribute to Cbl-mediated ubiquitinylation of SFKs. Since the physical interactions of Cbl with its other PTK targets (e.g., Syk/ZAP70 and receptor tyrosine kinases) are also phosphorylation-dependent, it is likely that the essential role of the target kinase activity in these instances is also related to the promotion of physical association between Cbl and its targets. As such, the lessons learned through present studies may provide a general basis to understand the mechanism of the kinase dependence of the Cbl-mediated ubiquitinylation and degradation mediated by Cbl family ubiquitin ligases.

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