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A Conserved DpYR Motif in the Juxtamembrane Domain of the Met Receptor Family Forms an Atypical c-Cbl/Cbl-b Tyrosine Kinase Binding Domain Binding Site Required for Suppression of Oncogenic Activation*

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The activation and phosphorylation of Met, the receptor tyrosine kinase (RTK) for hepatocyte growth factor, initiates the recruitment of multiple signaling proteins, one of which is c-Cbl, a ubiquitin-protein ligase. c-Cbl promotes ubiquitination and enhances the down-modulation of the Met receptor and other RTKs, targeting them for lysosomal sorting and subsequent degradation. The ubiquitination of Met by c-Cbl requires the direct interaction of the c-Cbl tyrosine kinase binding (TKB) domain with tyrosine 1003 in the Met juxtamembrane domain. Although a consensus for c-Cbl TKB domain binding has been established ((D/N)*X*pY*XX*(D/E0Φ), this motif is not present in Met, suggesting that other c-Cbl TKB domain binding motifs may exist. By alanine-scanning mutagenesis, we have identified a DpYR motif including Tyr^{1003} as being important for the direct recruitment of the c-Cbl TKB domain and for ubiquitination of the Met receptor. The substitution of Tyr^{1003} with phenylalanine or substitution of either aspartate or arginine residues with alanine impairs c-Cbl-recruitment and ubiquitination of Met and results in the oncogenic activation of the Met receptor. We demonstrate that the TKB domain of Cbl-b, but not Cbl-3, binds to the Met receptor and requires an intact DpYR motif. Modeling studies suggest the presence of a salt bridge between the aspartate and arginine residues that would position $pTyr^{1003}$ for binding to the c-Cbl TKB domain. The DpYR motif is conserved in other members of the Met RTK family but is not present in previously identified c-Cbl-binding proteins, identifying DpYR as a new binding motif for c-Cbl and Cbl-b.

The Cbl family of proteins have been identified recently as ubiquitin-protein ligases that are involved in the ubiquitination and subsequent down-regulation of receptor tyrosine kinases (RTKs)[1] (1, 2). There are three mammalian Cbl proteins: c-Cbl; Cbl-b; and Cbl-3. The c-Cbl and Cbl-b genes are expressed ubiquitously with the highest levels of expression in hematopoietic tissues (3, 4). In contrast, Cbl-3 mRNA is expressed mainly in organs rich in epithelium such as pancreas, liver, small intestine, colon, and placenta and is expressed at low levels in hematopoietic tissues (5, 6). Despite the lower levels of c-Cbl and Cbl-b proteins in epithelial tissues, the c-Cbl-deficient mice, in addition to having hematopoietic defects, develop mammary hyperplasias (7), signifying that c-Cbl is important for growth regulation of mammary epithelia.

The conserved amino-terminal domain of Cbl proteins is composed of a tyrosine kinase binding (TKB) domain and a RING finger domain, which are both required for ubiquitin-protein ligase activity. The TKB domain mediates the recruitment of Cbl proteins to the tyrosine-phosphorylated substrate, whereas the RING finger domain associates with the ubiquitin-conjugating enzyme (UbcH7) (8). The presence of several binding sites for SH2 and SH3 domain-containing proteins within their carboxyl-terminal end confers to c-Cbl and Cbl-b the ability to function as adaptor proteins.

The Cbl TKB domain interacts with phosphotyrosine residues located in protein tyrosine kinases such as ZAP-70, Syk, and Src and with residues located in RTKs such as EGFR, Met, and colony-stimulating factor-1 (CSF-1) receptor (1, 9, 10). It is composed of a four-helix (4H) bundle, an EF-hand calcium binding domain, and a variant SH2 domain that together are able to bind to phosphotyrosine residues (11). The crystal structure of the c-Cbl TKB domain complexed to its binding site on ZAP-70 kinase reveals that a medium-sized hydrophobic residue (Φ) at position $pTyr^{+4}$ and an acid residue (Asp/Glu) at position $pTyr^{+3}$ would constitute the primary and specific determining interactions (11). In addition, an aspartate residue at position $pTyr^{-2}$ forms a hydrogen bond. This is in accordance with a degenerate phosphopeptide library screen using the c-Cbl TKB domain as a bait, which revealed a preference for an aspartate or an asparagine residue at position $pTyr^{-2}$ (12). The (D/N)*X*pY*XX*(D/E)Φ sequence is found not only in the ZAP-70 kinase but also in other c-Cbl-binding proteins including the Syk kinase, the EGFR, and Sprouty adaptor proteins (12–16).

We have shown recently that the hepatocyte growth factor receptor, Met, is also regulated negatively by c-Cbl (10). The Met receptor is expressed primarily in epithelial and endothelial cells, and its activation leads to the loss of cell-cell adhesion and enhances cell migration and proliferation (17). The chronic

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[1] The abbreviations used are: RTK, receptor tyrosine kinase; p, phospho; 4H, four-helix bundle; CSF, colony-stimulating factor; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; GST, glutathione *S*-transferase; SH2, Src homology 2; TKB, tyrosine kinase binding; WT, wild type; DMEM, Dulbecco's modified Eagle's medium.

activation of the Met receptor is associated with the genesis and the progression of multiple types of tumors including carcinomas, melanomas, and sarcomas (18). The stimulation of the Met receptor with its ligand, hepatocyte growth factor, induces tyrosine phosphorylation and polyubiquitination and, ultimately, the degradation of the receptor (19–21). We have reported previously that the c-Cbl ubiquitin-protein ligase is recruited to the Met receptor by two distinct mechanisms. The carboxyl-terminal region of c-Cbl can be recruited indirectly to Tyr^{1356} in the Met receptor via the Grb2 adaptor protein, whereas the TKB domain of c-Cbl associates directly to the juxtamembrane Tyr^{1003} in the Met receptor (10). The latter interaction is required for full ubiquitination of the Met receptor. A Met receptor lacking the c-Cbl TKB domain binding site (Y1003F) has a prolonged half-life and is oncogenic in cell culture, identifying c-Cbl and ubiquitination as important negative regulators for this receptor (10). Here, we have performed alanine-scanning mutagenesis of amino acids surrounding Tyr^{1003} and have identified a DpYR motif as being essential for the recruitment of the c-Cbl/Cbl-b TKB domain to the Met receptor as well as receptor ubiquitination and down-regulation. Based on the c-Cbl·ZAP-70 complex crystal structure, we also propose a structural mechanism for the association of the Cbl TKB domain to the DpYR motif in the Met receptor.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Antibodies raised in rabbit against a carboxyl-terminal peptide of human Met were used (22). Met $pTyr^{1003}$ and $pTyr^{1234-1235}$ phosphorylation site-specific antibodies were purchased from BIOSOURCE (Nivelles, Belgium). HA antibody was from BABCO (Richmond, CA). Anti-pTyr (4G10) was from Upstate Biotechnology (Lake Placid, NY), and anti-c-Cbl (sc-170) and anti-ubiquitin (P4D1) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The HA-ubiquitin expression plasmid is described by Treier *et al.* (23).

Cell Culture, DNA Transfections, and Transformation Assays—Human embryonic kidney 293T cells were transfected using the calcium phosphate method. For transformation assays in Rat-1 fibroblasts, 4×10^5 cells were seeded in 60-mm plates in DMEM containing 10% FBS and transfected the next day with 2 μg of DNA using the calcium phosphate method. After 12 h, cells were washed twice with phosphate-buffered saline and maintained in DMEM containing 10% FBS for 2 days. The cells then were maintained in DMEM containing 5% FBS with the medium being changed every 3–4 days until the appearance of foci. For the generation of Rat-1 stable cell lines, the foci were obtained in the presence of 25 ng/ml CSF-1, picked, and expended in DMEM containing 10% FBS.

Site-directed Mutagenesis—Site-directed mutagenesis was performed using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions using CSF-Met (24) in pMX-139 as a template. Asn^{998} was converted to Ala using the 5′-GAAATGGTTTCAGCTGAATCTGTAGAC-3′ primer. Glu^{999} was converted to Ala using the 5′-GGTTTCAAATGCATCTGTAGACTACCG-3′ primer. Ser^{1000} was converted to Ala using the 5′-GTTTCAAATGAAGCAGTCGACTACCGAGCTAC-3′ primer. Val^{1001} was converted to Ala using the 5′-GTTTCAAATGAATCTGCAGACTACCGAGC-3′ primer. Asp^{1002} was converted to Ala using the 5′-GTTTCAAATGAATCTGTCGCCTACCGAGCTAC-3′ primer. Arg^{1004} was converted to Ala using the 5′-GAATCTGTAGACTACGCGGCTACTTTTCCAG-3′ primer. Thr^{1006} was converted to Ala using the 5′-CTGTAGACTACCGAGCAGCTTTTCCAGAAGATCAG-3′ primer. Phe^{1007} was converted to Ala using the 5′-CTACCGAGCTACTGCGCCAGAAGATCAG-3′ primer, and Pro^{1008} was converted to Ala using the 5′-CTACCGAGCTACTTTTGCAGAAGATCAGTTTC-3′ primer.

In Vitro Binding Assays—The amino-terminal portion of c-Cbl and Cbl-3 fused to GST were provided by Dr. Hamid Band (25) and Dr. Vincent Ollendorff (26), respectively. The coupling of GST fusion proteins to glutathione-Sepharose beads (Amersham Biosciences) was performed at 4 °C for 1 h. The complexes were washed three times with TGH lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 10% glycerol) containing 1 mM phenylmethylsulfonyl fluoride and 1 mM sodium vanadate and then incubated with cell lysate for 2 h at 4 °C, washed four times with TGH lysis buffer, and resuspended in Laemmli sample buffer.

Immunoprecipitations and Western Blotting—293T cells were serum-starved in 0.1% FBS overnight and harvested in TGH lysis buffer. Lysates were incubated with the indicated antibody overnight at 4 °C with gentle rotation. Proteins collected on either protein A- or protein G-Sepharose were washed three times in TGH lysis buffer, resolved by SDS-PAGE, and transferred to a nitrocellulose membrane as described previously (27). Proteins were visualized with an ECL detection kit (Amersham Biosciences). To detect CSF-Met receptor ubiquitination in Rat-1 cell lines, cells were stimulated for 5 min with 500 μg/ml CSF-1 and lysed immediately in radioimmune precipitation assay buffer (0.05% SDS, 50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate) containing 25 mM *N*-ethylmaleimide (Sigma), 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM sodium vanadate.

Far-Western Blotting—For far-Western analysis, the CSF-Met receptor was immunoprecipitated from 1 mg of 293T whole cell lysates, resolved on an 8% SDS-page gel, and transferred to a nitrocellulose membrane. The membrane was blocked overnight in 20 mM Tris, pH 7.5, 150 mM NaCl, and 1 mM Na_3VO_4 containing 10% nonfat milk and 0.1% Tween 2, and then probed for 1 h at room temperature with 40 μg/ml of purified GST-Cbl-N proteins previously coupled to 1 μg of horseradish peroxidase-glutathione (g-6400, Sigma-Aldrich) (30 min, room temperature) in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 2.5 mM EDTA, 0.1% Tween 20). After four washes of 5 min each with TBST, the bound proteins were detected with an ECL detection kit (28).

RESULTS

Mapping of the c-Cbl TKB Domain Binding Site in Met—By far-Western analysis and peptide competition experiments, we have shown that the c-Cbl TKB domain binds directly to the phosphorylated Tyr^{1003} in the Met receptor (10). The sequence surrounding Tyr^{1003} differs from the identified c-Cbl TKB domain binding site in the ZAP-70, Syk kinase, and EGFR (Fig. 1*A*). To establish the molecular requirements for the association of the c-Cbl TKB domain to Tyr^{1003} in the Met receptor, we performed an alanine-scanning mutagenesis of the amino acid residues surrounding Tyr^{1003} (Asn^{998}-Pro^{1008}) (Fig. 1*B*). The mutant Met receptors were expressed to similar levels and, with the exception of Met Y1003F, were phosphorylated on Tyr^{1003} to similar levels as the WT Met receptor as detected using anti-phosphotyrosine 1003 serum. In addition, the mutant receptors all were phosphorylated comparably on the conserved twin tyrosine residues ($Tyr^{1234-1235}$) located in the activation loop (Fig. 1*C*). These tyrosines are required for full activation of the Met kinase (29). This indicates that the activation of the Met kinase and subsequent tyrosine phosphorylation of Tyr^{1003} are not altered detectably in the alanine substitution mutants.

A DpYR Motif Is Required for Recruitment of the c-Cbl TKB Domain—To define the c-Cbl TKB domain binding site, the ability of each mutant to co-immunoprecipitate with an HA-tagged c-Cbl TKB domain protein was assessed following transient co-expression. As shown previously (10), the substitution of tyrosine 1003 in the Met receptor with a non-phosphorylatable phenylalanine residue abrogates the co-immunoprecipitation of Met with the c-Cbl TKB domain protein (Fig. 2*A*). In addition, the substitution of both Asp^{1002} and Arg^{1004} with alanine residues significantly reduces the ability of the c-Cbl TKB domain protein to co-immunoprecipitate with these mutant Met receptors (Fig. 2*A*), even if Tyr^{1003} is present and phosphorylated to similar levels as the WT Met receptor (Fig. 1*C*). All of the other Met alanine substitution mutants tested co-immunoprecipitate with the c-Cbl TKB domain protein to levels similar to the WT Met receptor (Fig. 2*A*). This indicates that Asp^{1002} and Arg^{1004} are required independently for co-immunoprecipitation with the c-Cbl TKB domain.

To establish whether there is a requirement for residues Asp and Arg for the direct binding of the c-Cbl TKB domain, we performed a far-Western analysis using a GST-c-Cbl TKB do-

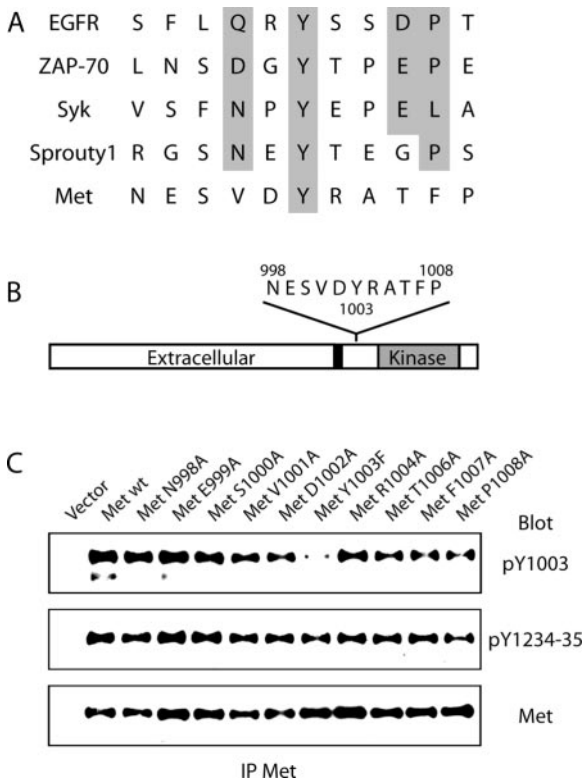

FIG. 1. **The c-Cbl TKB binding site in Met is distinct from the identified c-Cbl TKB domain binding site in the ZAP-70, Syk, and EGFR tyrosine kinases.** *A*, the alignment of the c-Cbl TKB direct binding site identified in human EGFR, ZAP-70, Syk, Sprouty1, and Met proteins. *B*, schematic representation of the Met receptor. The c-Cbl TKB direct binding site (Tyr^{1003}) is located in the juxtamembrane domain of the Met receptor. The amino acids from Asn^{998}-Phe^{1008} residues have been substituted individually for alanine residues. *C*, 293T cells were transfected transiently with plasmids expressing CSF-Met WT (*wt*) or mutants. Cell lysates were subjected to immunoprecipitation (*IP*) with Met antibodies followed by immunoblotting with phosphorylation site-specific antibodies raised against the juxtamembrane Tyr^{1003} residue and against the kinase domain Tyr^{1234} and Tyr^{1235} residues. Nitrocellulose membranes were stripped and reprobed with Met antibodies.

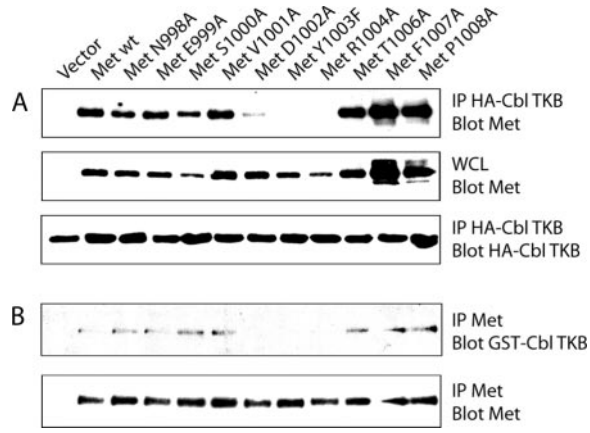

FIG. 2. **Asp^{1002}, Tyr^{1003}, and Arg^{1004} residues are required for the direct binding of the c-Cbl TKB domain to the Met receptor.** *A*, protein lysates from 293T cells transiently co-transfected with plasmids expressing HA-Cbl-TKB and CSF-Met WT (*wt*) or mutants were subjected to immunoprecipitation with HA antibodies. Membranes were immunoblotted with Met antibodies, stripped, and reprobed with HA antibodies. Whole cell lysates were immunoblotted with Met antibodies. *B*, CSF-Met receptors were immunoprecipitated from 293T cells transfected with CSF-Met WT and mutants, resolved by SDS-PAGE, and transferred to a nitrocellulose membrane that was immunoblotted then with purified GST-Cbl-TKB proteins conjugated to horseradish peroxidase-glutathione. The membrane was stripped and reprobed with Met antibodies.

main fusion protein. Probing with GST-c-Cbl TKB conjugated to glutathione-horseradish peroxidase revealed the direct binding of the GST-c-Cbl TKB fusion protein to WT Met receptor and to Met alanine-scanning mutants with the exception of the D1002A, Y1003F, and R1004A Met receptor mutants (Fig. 2*B*). This finding indicates that, in addition to Tyr^{1003}, the Asp and Arg residues also are required independently for the direct association of the Met receptor with the c-Cbl TKB domain, and together, these data demonstrate that the DpYR residues of the Met receptor form an alternative binding motif for the c-Cbl TKB domain.

The DpYR Motif Is Required for the Recruitment of the Cbl-b TKB Domain—The TKB domain is well conserved throughout the Cbl protein family (2). Therefore, we examined the ability of the Cbl-b and Cbl-3 TKB domains to interact with the Met receptor. We performed *in vitro* binding assays using GST-TKB fusion proteins. When compared with the GST-c-Cbl TKB fusion protein, the GST-Cbl-b TKB fusion protein bound to levels similar to the Met receptor (Fig. 3*A*). Moreover, as observed for the c-Cbl TKB domain, the substitution of the aspartate or the arginine residue impairs the association of the Cbl-b TKB domain with the Met receptor (Fig. 3*C*). We did not detect the association of the GST-Cbl-3 TKB domain to the Met receptor under the same conditions (Fig. 3*A*), even though similar levels of GST fusion proteins were used (Fig. 3*B*).

An Intact DpYR Motif Is Required for Met Receptor Ubiquitination and Suppression of Transformation—We have demonstrated previously that the uncoupling of the c-Cbl TKB domain from the Met receptor by the Y1003F mutation strongly impairs the ubiquitination of the receptor and converts the receptor into a transforming protein (10). Therefore, we examined whether the D1002A and R1004A mutations, which uncouple the c-Cbl TKB domain from the Met receptor, affect receptor ubiquitination and transforming activity. When co-expressed with c-Cbl, the D1002A and R1004A receptor mutants, similar to the Met Y1003F receptor mutant, are ubiquitinated to levels significantly less than the WT Met receptor and other alanine-scanning mutant receptors (Fig. 4*A*). The biological activity of the alanine-scanning mutant Met receptors was tested by examining their ability to induce the foci of transformed cells on confluent monolayers of Rat-1 fibroblasts. Consistent with their inability to recruit the c-Cbl TKB domain and their reduced ubiquitination, both the D1002A and R1004A mutants are able to transform Rat-1 fibroblast cells (Fig. 4, *B* and *C*), although the transforming activity of the Asp^{1002} receptor mutant was consistently less than that of the Y1003F or R1004A receptor mutants. In three independent experiments, the WT Met receptor and other alanine-scanning mutant receptors failed to induce foci (Fig. 4*C*). This finding demonstrates that, in addition to Tyr^{1003}, the Asp^{1002} and Arg^{1004} residues are essential to suppress the transforming activity of the Met receptor.

To further examine the role of the DpYR motif in Met receptor regulation, we established Rat-1 fibroblast cell lines expressing the WT Met receptor as well as V1001A, D1002A, Y1003F, and R1004A Met receptor mutants. Multiple (2–5) stable cell lines that express each mutant were isolated. The steady-state protein levels of the D1002A, Y1003F, and R1004A Met receptor mutants were consistently higher than those of WT Met and Met V1001A receptors, and we were unable to generate cell lines with equal protein levels (Fig. 5*A*). Moreover, the base-line and ligand-induced phosphorylation of each mutant receptor reflects their protein levels and is elevated in D1002A, Y1003F, and R1004A receptor mutants (Fig. 5*A*). Ligand stimulation provokes robust

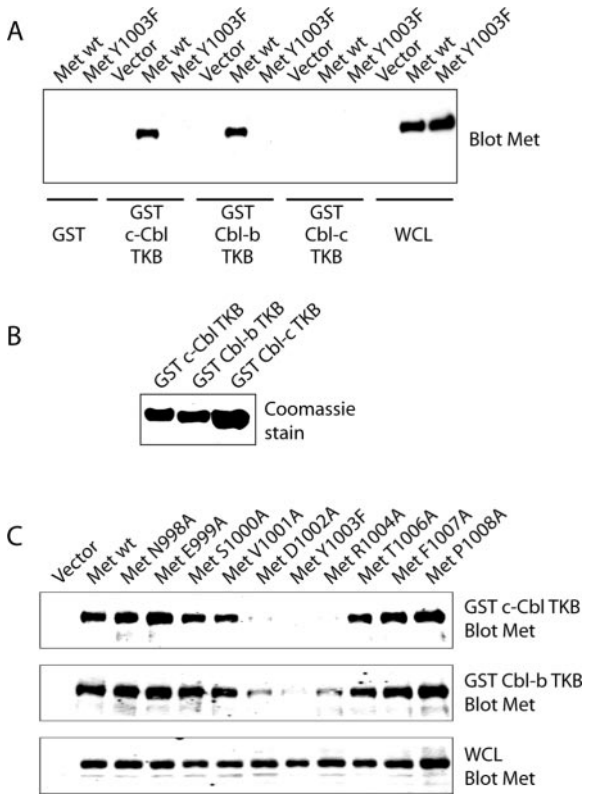

FIG. 3. **An intact DpYR motif in the Met receptor is required for its association with the Cbl-b TKB domain.** *A*, protein lysates from 293T cells transiently transfected with vector alone or plasmids expressing CSF-Met WT (*wt*) or Y1003F were subjected to *in vitro* binding assays with GST, GST-c-Cbl-TKB, GST-Cbl-b-TKB, or GST-Cbl-3-TKB. The membrane was immunoblotted with Met antibodies. *B*, the amount of GST-Cbl-TKB fusion proteins used for the *in vitro* binding assays was determined by Coomassie Blue staining. *C*, 293T cells were transfected transiently with plasmids expressing CSF-Met WT or mutants. Cell lysates were subjected to *in vitro* binding assays with GST-c-Cbl-TKB and GST-Cbl-b-TKB followed by immunoblotting with Met antibodies. Whole cell lysates were resolved separately and immunoblotted with Met antibodies.

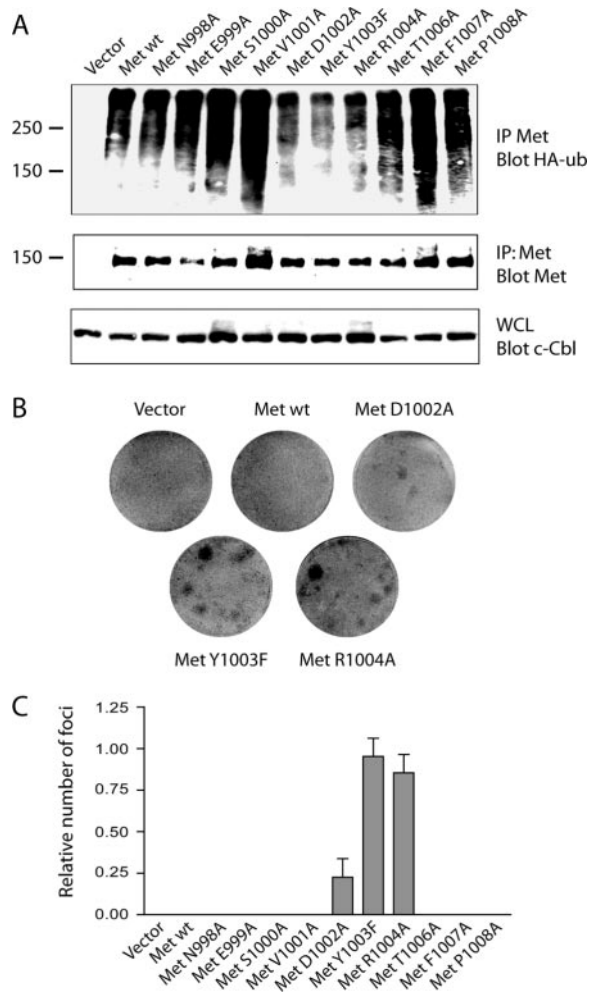

FIG. 4. **Mutation of the DpYR motif prevents Cbl-mediated Met receptor ubiquitination and promotes receptor-oncogenic activation.** *A*, lysates from 293T cells co-transfected with HA-ubiquitin, c-Cbl, and CSF-Met WT (*wt*) or mutants were subjected to immunoprecipitation (*IP*) with Met antibodies and immunoblotted with HA antibodies. The membrane then was stripped and reprobed with Met antibodies. Whole cell lysates (*WCL*) were immunoblotted with c-Cbl antibodies. *B*, Rat-1 fibroblast cells were transfected with either CSF-Met WT or mutants (2 μg of DNA/60-mm Petri dish) and grown in DMEM containing 5% FBS in the absence of ligand until there was an appearance of the foci (~2 weeks). *C*, the graph represents the relative number of foci per dish from four different experiments.

ubiquitination of Met WT and Met V1001A, whereas ligand-induced ubiquitination of Met D1002A, Y1003F, and R1004A receptors was low or not detectable (Fig. 5, *A* and *B*). The low levels of ubiquitination in response to stimulation correlate with the higher transforming activity of the Y1003F and R1004A Met receptor mutants when compared with the Met D1002A receptor mutant (Figs. 4 and 5).

A Molecular Model Predicts That Asp^{1002} and Arg^{1004} Form a Salt Bridge Required for the Projection of pTyr^{1003} into the c-Cbl TKB Domain Binding Pocket—To determine how the substitution of either Asp^{1002} or Arg^{1004} with alanine in the DpYR motif could affect the binding of the Met receptor with c-Cbl, a model of the Met peptide S1000-P1008 bound to the TKB domain of c-Cbl was constructed based on the structure of the c-Cbl·ZAP-70 peptide complex (Fig. 6) (11). The modeled structure of the Met peptide S1000-P1008 predicts that Asp^{1002} and Arg^{1004} in the DpYR motif of the Met receptor form a salt bridge. This would stabilize the peptide in the conformation most favorable to expose pTyr^{1003} toward the phosphotyrosine binding pocket of the c-Cbl TKB domain. Furthermore, the main chain carbonyl and amino groups of Asp^{1002} and Arg^{1004} form hydrogen bonds with the TKB domain of c-Cbl to provide additional support for binding pTyr^{1003} to the phosphotyrosine binding pocket. The specific association of the Met receptor to the TKB domain of c-Cbl could be reinforced by the binding of Phe^{1007} positioned at pTyr^{+4} to the hydrophobic pocket (Fig. 6).

DISCUSSION

The Cbl family of proteins has been reported to interact through their TKB domains with a variety of proteins. The TKB domain of c-Cbl can associate directly with the juxtamembrane tyrosine 1003 on the Met RTK, and this interaction is required for Met receptor ubiquitination and down-modulation (10). The crystal structure of the c-Cbl TKB domain, complexed to its binding site on the ZAP-70 kinase, has revealed a consensus binding site (D/N)*X*pY*XX*(D/E)Φ that is found in three other c-Cbl TKB domain-binding proteins: the Syk kinase; EGFR; and Sprouty (Fig. 1) (12–16). However, the c-Cbl TKB domain binding site in the Met receptor does not conform to this consensus sequence, suggesting that other c-Cbl TKB domain binding motifs may exist.

Mutagenesis studies of the Cbl TKB binding site on the Met receptor have revealed a Cbl TKB binding core motif, DpYR (Fig. 2). This motif binds c-Cbl as well as the Cbl family member Cbl-b but not Cbl-3 (Fig. 3). The decreased association of c-Cbl and Cbl-b TKB domain with the D1002A and R1004A Met receptor mutants does not reflect the decreased phospho-

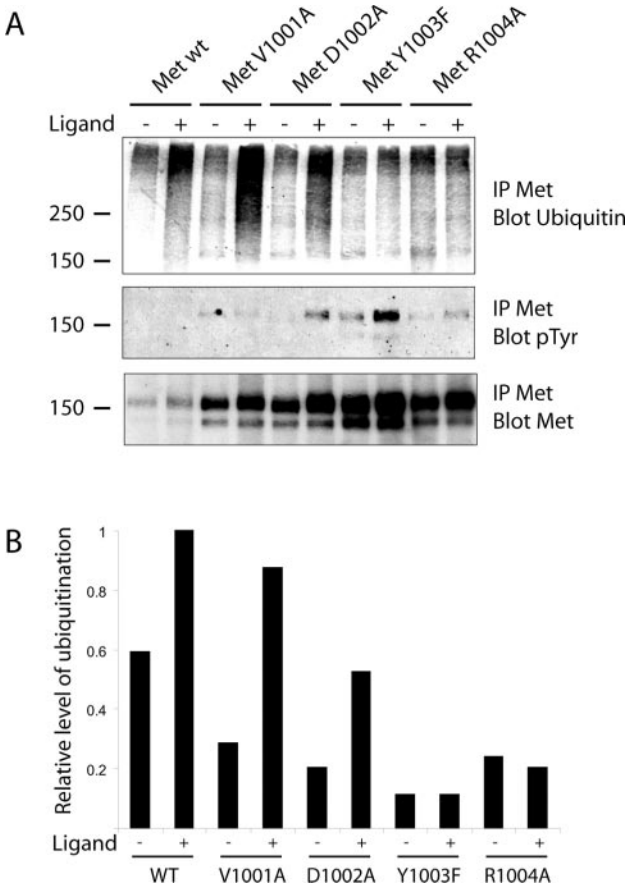

FIG. 5. **Mutation of the DpYR motif abrogates ligand-induced ubiquitination of the Met receptor in stable cell lines.** *A*, Rat-1 fibroblast cell lines expressing either Met WT (*wt*) or Met mutant receptors were stimulated for 5 min with CSF-1 and lysed immediately. The CSF-Met receptor proteins were immunoprecipitated (*IP*), resolved by SDS-PAGE and transferred, to a nitrocellulose membrane. The membrane was immunoblotted first with P4D1 ubiquitin antibodies, stripped, and reprobed with Met antibodies and then stripped again and reprobed with 4G10 phosphotyrosine antibodies. *B*, quantification of Met ubiquitination. The anti-ubiquitin blot and the anti-Met blot in *A* were quantified using the ImageJ, version 1.31 software from the National Institutes of Health. The values from the anti-ubiquitin blot were normalized for the Met protein levels in the absence of stimulation.

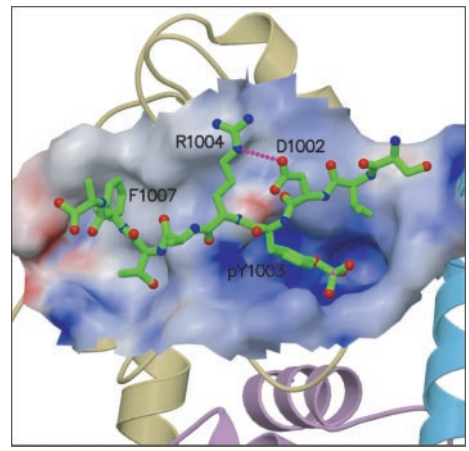

FIG. 6. **Role of salt bridge formation between Asp^{1002} and Arg^{1004} in the DpYR motif for the binding of the Met receptor to the TKB domain of c-Cbl.** The Met peptide S1000-P1008 (*green*) containing the DpYR motif was modeled based on the structure of the ZAP-70 pTyr^{292} peptide co-crystallized with c-Cbl (Protein Data Bank code 2CBL) (11). The side chains of both Asp^{1002} and Arg^{1004} extend out along the surface of the TKB domain of c-Cbl in the opposite direction as the side chain of pTyr^{1003} and can form a salt bridge (*magenta dashed line*). The electrostatic potential (*positive* in *blue* and *negative* in *red*) of the molecular surface of the TKB domain of c-Cbl was calculated with the program GRASP (40). It indicates that neither Asp^{1002} nor Arg^{1004} is associated with a highly charged surface of c-Cbl. In contrast, pTyr^{1003} binds to the positively charged phosphotyrosine binding pocket, which is composed of three domains: SH2 (*light yellow*); EF-hand (*light purple*); and 4H (*light blue*). The specific binding between the Met receptor and c-Cbl is supported further by the binding of Phe^{1007} to the hydrophobic pocket. The figure was created using MOLSCRIPT, POVSCRIPT+, and Raster3D (41–43).

rylation of Tyr^{1003} or that of other tyrosine residues required for full catalytic activity of the receptor (Tyr$^{1234-1235}$) as demonstrated by using the phosphorylation site-specific Met antibodies (Fig. 1*C*). These studies identify Asp and Arg as important for Cbl TKB domain interaction (Figs. 2 and 3). The 4H, EF-hand, and SH2-like domains of c-Cbl and Cbl-b are conserved highly. However, only the SH2-like domain is conserved in Cbl-3 with the 4H and EF-hand domains notably different. This finding suggests a role for the 4H and EF-hand domains in c-Cbl and Cbl-b binding to the Met receptor, which is in agreement with the c-Cbl·ZAP-70 crystal structure (11).

The c-Cbl TKB domain is structurally similar to an SH2 domain, although there is only 11% identity at the amino acid level. The phosphotyrosine binding pocket is well conserved, containing an invariant arginine residue that forms two hydrogen bonds with the phosphate group (11). However, the SH2 domain in c-Cbl lacks the secondary β-sheet and the loop that define the binding specificity of an SH2 domain with residues located downstream from the phosphotyrosine residue (30, 31).

The crystal structure of a peptide from ZAP-70 indicates that residues positioned at pTyr^{-2} and pTyr^{+3} directly interact with c-Cbl to assist the specific binding of pTyr^{292} in ZAP-70 to the phosphotyrosine binding pocket of c-Cbl (11). The modeled structure of the Met peptide predicts that it utilizes a slightly different mechanism than ZAP-70 to promote the specific binding of pTyr^{1003} to the TKB domain of c-Cbl (Fig. 6). The Met receptor is predicted to form a salt bridge between Asp^{1002} and Arg^{1004} in the DpYR motif to stabilize the peptide conformation most favorable to expose pTyr^{1003} of Met toward the phosphotyrosine binding pocket of c-Cbl (Fig. 6). Therefore, the substitution of either Asp^{1002} or Arg^{1004} in the DpYR motif would result in a loss of the salt bridge, which would be expected to alter the projected orientation of pTyr^{1003} toward the phosphotyrosine binding pocket of c-Cbl, decreasing the binding affinity of the TKB domain of c-Cbl to the Met receptor.

The crystal structure of the c-Cbl·ZAP-70 complex also revealed that a medium-sized hydrophobic residue (Φ) at position pTyr^{+4}, *i.e.* proline in the case of ZAP-70, binds to a hydrophobic pocket (11). In the case of the Met receptor, the phenylalanine residue at position pTyr^{+4} also lies in that hydrophobic pocket. However, the F1007A substitution does not seem to affect the association of the c-Cbl/Cbl-b TKB domain with Tyr^{1003} in the Met receptor (Fig. 2), even though alanine is less capable of forming hydrophobic interactions than phenylalanine.

The specific substitution of Tyr^{1003} in Met with a non-phosphorylatable phenylalanine residue uncouples the recruitment of the c-Cbl TKB domain, significantly diminishes ubiquitination of the receptor, and leads to enhanced receptor stability and oncogenic activation (10). An intact DpYR motif necessary for c-Cbl and Cbl-b TKB domain binding also is required for efficient ubiquitination of the Met receptor following stimulation (Figs. 4*A* and 5*A*). Moreover, the substitution of the aspartate or arginine residues with alanine is sufficient to endow the Met receptor with transforming activity (Fig. 4, *B* and *C*) where the transforming activity of these Met receptor mutants corresponds to their degree of ubiquitination. Whereas the Y1003F and R1004A Met receptor mutants show no detectable increase in ubiquitination following ligand stimulation and transform

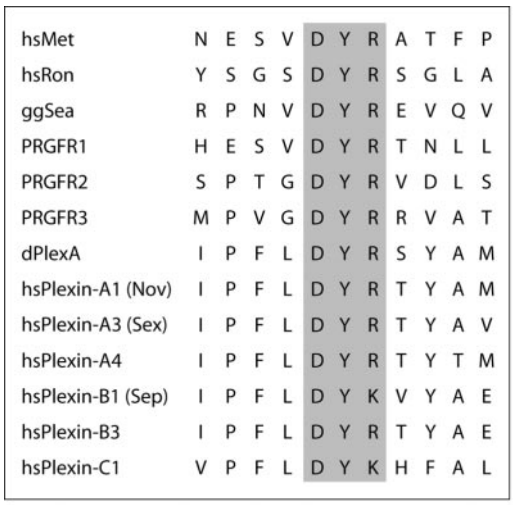

FIG. 7. **The DYR motif is conserved in the Met receptor family as well as in the Plexin receptor family.** The alignment of the DYR motif conserved in all of the members of the Met receptor family and in members of the Plexin receptor family is shown. Plexins are the receptors for semaphorins. In all cases, the DYR motif is located in the juxtamembrane region of the receptor. *hsMet*, human Met; *hsRon*, human Ron; *ggSea*, chicken Sea; *PRGFR*, Puffer fish plasminogen-related growth factor receptor; *dPlexA*, *Drosophila* PlexA; *hsPlexin*, human plexin.

with similar efficiency, the D1002A Met receptor mutant is ubiquitinated at low levels and shows a decreased efficiency of transformation when compared with the Y1003F and R1004A Met receptor mutants. The lower levels of ubiquitination of the DpYR Met mutant receptors in stable Rat-1 fibroblast cell lines correlate with their elevated steady-state protein levels and elevated base-line phosphotyrosine levels when compared with WT Met and Met V1001A mutant receptors (Fig. 5). Consistent with these observations, Madin-Darby canine kidney epithelial cells expressing D1002A and R1004A Trk-Met hybrid receptor mutants acquire a fibroblastoid phenotype in the absence of hepatocyte growth factor stimulation (32). These observations further support a role for the Asp^{1002} and Arg^{1004} residues in Cbl-mediated down-regulation of the Met receptor.

Juxtamembrane tyrosine residues play a role in the autoinhibition of the vascular endothelial growth factor receptor-1 (33), Eph (34, 35), and c-Kit (36) RTKs. The structural studies demonstrated that a helix containing the juxtamembrane unphosphorylated tyrosine residue adopts a conformation that distorts the small lobe of the kinase domain, preventing receptor activation. Upon receptor activation, the phosphorylation of the juxtamembrane tyrosine promotes a conformational change that allows full activation of the receptor. The substitution of this residue with a non-phosphorylatable phenylalanine residue prevents the activation of the Eph receptor (34, 35), whereas the deletion of these tyrosine residues in c-Kit and Eph receptors removes this inhibitory mechanism (35, 36). In the case of the Met receptor, the substitution of Tyr^{1003} in the juxtamembrane domain with a phenylalanine residue does not prevent the activation and tyrosine phosphorylation of the Met receptor (Fig. 1*C*). This finding suggests a distinct mechanism for the negative regulation of Met by the juxtamembrane Tyr^{1003} residue, consistent with the requirement for Tyr^{1003} and surrounding amino acids Asp^{1002} and Arg^{1004} for binding the c-Cbl TKB domain and c-Cbl-dependent ubiquitination and down-regulation of the Met receptor (Figs. 2 and 3) (10).

The DpYR motif is conserved within Met family members, Ron, and Sea as well as within Met orthologues in Puffer fish (Fig. 7), suggesting a conserved function for this motif in the Cbl recruitment and negative regulation of the Met receptor family. In support of this finding, in a similar manner to the Met receptor, this tyrosine in the Ron RTK (Tyr^{1017}) is required for the recruitment of the c-Cbl TKB domain and is essential for ubiquitination and degradation of Ron (37). Moreover, a DpYR motif is conserved in plexins, which are receptors for semaphorins that promote cell repulsion (38). Although mammalian plexins were identified through their homology with the extracellular domain of the Met receptor, the homology with the cytosolic domain of the Met receptor family has not been reported yet (39). The presence of a conserved DpYR motif in plexins raises the possibility that this motif may represent an unsuspected Cbl recruitment site in these receptors that modulates their stability.

There is no precedent for the association of a given phosphotyrosine binding domain, either phosphotyrosine binding or SH2, to unrelated consensus binding sites. This work demonstrates the versatility of the Cbl TKB domain to bind to different consensus sequences, which constitutes a unique feature and highlights the need to identify and characterize other Cbl TKB domain binding sites.

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A Conserved DpYR Motif in the Juxtamembrane Domain of the Met Receptor Family Forms an Atypical c-Cbl/Cbl-b Tyrosine Kinase Binding Domain Binding Site Required for Suppression of Oncogenic Activation

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