Met Kinase-dependent Loss of the E3 Ligase Cbl in Gastric Cancer

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Background: Dysregulated Met signaling can promote tumorigenesis.

Results: Active Met kinase promotes loss of Cbl protein. This is rescued upon inhibition of Met kinase.

Conclusion: Met-dependent Cbl loss in gastric cancers releases other Cbl targets, such as the EGF receptor, from Cbl-mediated attenuation.

Significance: Uncoupling of Met and other RTKs from Cbl negative regulation in gastric cancers provides a mechanism for enhanced RTK signaling in cancer.

Receptor tyrosine kinases (RTKs) are key components of signaling cascades that promote cellular proliferation and differentiation. These processes are essential for normal human embryogenesis and homeostasis and, when dysregulated, can drive the unconstrained cell growth and invasiveness characteristic of many human cancers.

The Met RTK was initially identified as an oncogenic fusion protein (Tpr-Met) following treatment of the human osteogenic sarcoma (HOS) cell line with the carcinogen N’-nitro-nitrosoguanidine (1, 2). Met was subsequently identified as the receptor for hepatocyte growth factor (HGF) or scatter factor (3, 4). HGF-Met signaling promotes scatter and an invasive morphogenic response in epithelial cells in cell culture (5) and is required during embryogenesis for development of the placenta, liver, kidney, and neuronal and skeletal muscles (6). In the adult, the HGF-Met signaling axis is involved in wound healing and liver regeneration (7, 8). Tight regulation of Met signaling is required for many of these processes, and dysregulation of the Met signaling axis has been implicated in various human cancers.

Several mechanisms leading to dysregulation of Met in cancer have been identified. These include autocrine/paracrine activation, Met overexpression, genomic amplification, point mutation, and alternative splicing (9). MET amplification occurs frequently in gastric cancers (10) and to a lesser extent in non-small cell lung cancer and glioblastomas (11–14).

The loss of negative regulation represents an additional mechanism through which oncogenic activation of Met can occur (15). Negative regulation of Met is primarily mediated through the Cbl E3 ligase. The Cbl family of E3 ligases consists of three mammalian homologues: c-Cbl, Cbl-b, and Cbl-3 (16–18). These cytoplasmic proteins are conserved in their N-terminal halves and consist of a tyrosine kinase binding (TKB) domain, a linker region, and a RING domain, the latter of which is required for functional E3 ligase activity (reviewed in Ref. 19). The C-terminal portions are less well conserved and include a proline-rich region and a UBA domain (c-Cbl and Cbl-b) (19). The UBA domains of both c-Cbl and Cbl-b facilitate dimerization, but only the Cbl-b UBA domain is able to bind ubiquitin (20–22). The presence of key tyrosine residues as well as proline-rich regions allows Cbl proteins to function also as scaffolds capable of recruiting a number of SH2 and SH3 domain-containing proteins (19).

Both c-Cbl and Cbl-b act as E3 ligases and ubiquitinate their target substrates (reviewed in Ref. 23). The overlap of c-Cbl and Cbl-b function is evident, as CBL-/-- or CBLB-/-- mice are both viable, but mice deficient in both are embryonic lethal (23, 24). Similarly, in osteoclasts, the depletion of both proteins is required to disrupt the microtubule network and induce apo-
activity. In human tumors whereby Cbl loss is dependent on Met kinase such as EGFR. This represents a mechanism of RTK cross-talk the capability of dysregulating the signaling by other Cbl targets a loss of Cbl would not only enhance signaling by Met but has uncouple from Cbl-dependent negative regulation. Moreover, human gastric cancers leads to the loss of Cbl protein. This MARCH 9, 2012 • VOLUME 287 • NUMBER 11

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Antibody 148 was raised in rabbit against a C-terminal peptide of human Met (36). Met DL-21 antibody was purchased from Upstate Biotechnology (Lake Placid, NY). c-Cbl, Cbl-b, Src, and ubiquitin (P4D1) antibodies were acquired from Santa Cruz Biotechnology (Santa Cruz, CA). Met (AF276) and c-Cbl antibodies used for immunofluorescence were obtained from R&D Systems (Minneapolis, MN) and Epitomics Inc. (Burlingame, CA). Actin and tubulin antibodies were obtained from Sigma-Aldrich. Phospho-specific Met Tyr 1234/1235, EGFR Tyr 992, the general phosphotyrosine pTyr-100, K4B-specific polyubiquitin (D9D5), and total EGFR antibodies were purchased from Cell Signaling Technology (Mississauga, Ontario, Canada). HA.11 monoclonal and phospho-Src tyrosine 418 antibodies were obtained from Covance (Berkeley, CA) and Invitrogen, respectively. β-Catenin antibody was purchased from BD Biosciences.

HGF was a generous gift from Genentech (San Francisco, CA), and EGFR was purchased from Roche Diagnostics. Concanamycin, lactacystin, and PP2 were purchased from EMD Chemicals (Gibbstown, NJ) and utilized at final concentrations of 0.1, 10, and 10 μM, respectively. The Met inhibitor PHA-665752 was a kind gift from Pfizer (final concentration 0.1 μM).

Cell Culture and Transient Transfections—HEK 293, Oka-jima, and MKN45 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (FBS). Snu-5 and KATO II cell lines (a kind gift from Dr. Daniel Haber) were cultured in RPMI supplemented with 10% FBS. Transient transfections with HEK 293 cells were performed using Lipofectamine Plus reagent according to the manufacturer’s protocol (Invitrogen).

Immunoprecipitation and Western Blotting—HEK 293, Okajima, MKN45, Snu-5, and KATO II cells were harvested in TGH lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 1 mM sodium fluoride, 1 mM sodium vanadate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). HEK 293 transfections were harvested at 48 h post-transfection. Lysates were incubated with antibody for 1 h at 4 °C with gentle rotation followed by a 1-h incubation with protein A- or G-Sepharose beads. Captured proteins were collected by washing three times in TGH lysis buffer, eluted by boiling in SDS sample buffer, resolved by SDSPAGE, and transferred to a nitrocellulose membrane. Membranes were blocked in 3% bovine serum albumin in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 2.5 mM EDTA, and 0.1% Tween 20) for 1 h and incubated with primary antibodies in TBST overnight at 4 °C. Membranes were then incubated with secondary antibodies in TBST for 1 h. After three washes with TBST, the bound proteins were visualized with an ECL detection kit (Amersham Biosciences).

For Western blot analysis of c-Cbl and Cbl-b ubiquitination, cells were harvested in 150 μl of denaturing TSD lysis buffer (50 mM Tris-HCl, pH 7.5, 1% SDS, 5 mM DTT, 50 μM MG132, 50 mM N-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium fluoride, 1 mM sodium vanadate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Lysates were boiled at 99 °C for 5 min and then pelleted in a microcentrifuge at 10,000 rpm for 5 min. One hundred microliters of supernatant was then diluted to a final concentration of 0.1% SDS with TNESV buffer (50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 100 mM NaCl, 2 mM.
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EDTA, 10 mM N-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium fluoride, 1 mM sodium vanadate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Immunoprecipitations were performed as described above but with TNEVS buffer used in place of TGH lysis buffer.

For blots that required quantitation, membranes were blocked with Li-COR blocking buffer (Li-COR Biosciences) and incubated with primary antibodies as described above followed by incubation with infrared (IR)-conjugated secondary antibodies prior to detection and analysis on the Odyssey IR imaging system (Li-COR Biosciences).

Generation of Cbl-b Constructs—Site-directed mutagenesis was performed using the QuikChange kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions to create the Cbl-b constructs with the following primers (and their complementary primers): Cbl-b—(1–483) (5′-CCACCTGATCACCAGATGTC-3′); -(1–541) (5′-GGATGTCGACTC-3′); -(1–603) (5′-GGATGTCGACTC-3′); -(1–664) (5′-GGATGTCGACTC-3′); and -(1–821) (5′-GGATGTCGACTC-3′) as per the manufacturer’s instructions with the following primer sequences: (sense, 5′-ACCAAGATAGTTAG-3′; antisense, 5′-AGATCTTCCTCCCAGCTTTCTCCTCC-3′); (sense, 5′-CCCGTTGA-3′; antisense, 5′-AGATCTTCCTCCCAGCTTTCTCCTCC-3′); (sense, 5′-CTGGCACCCGTGGCTCCTGCCCTGGATCTAATTCAG-3′; antisense, 5′-GACTCGTTTGTA-3′); (sense, 5′-ACCAGGATAGTCTCCCCTTGCCCAGAGAAG-3′; antisense, 5′-CTGGATGTCGACTC-3′); (sense, 5′-CTGGATGTCGACTC-3′; antisense, 5′-GGATGTCGACTC-3′); (sense, 5′-GGATGTCGACTC-3′). Real-time PCR was performed using LightCycler 480 and LightCycler 480 SYBR Green I Master (Roche Applied Science) as per the manufacturer’s instructions. Measurements were taken in duplicate, three times for each set of samples, and three sets of samples (biological replicates) were measured. The level of c-Cbl or Cbl-b mRNA is expressed as the mean ± fold-difference normalized to the level of c-Cbl or Cbl-b mRNA in the DMSO control.

Confocal Immunofluorescence Microscopy—MKN45 cells were seeded at 1.6 × 10^5 on glass coverslips (Bellco Glass Inc., Vineland, NJ) in 24-well plates (Nalgene Nunc, Rochester, NY). Forty-eight hours later, cells were washed once with PBS and then fixed with 2% paraformaldehyde (Fisher Scientific) in PBS for 20 min followed by washing three times in PBS. Residual paraformaldehyde was removed with three 5-min washes with 100 mM glycine in PBS. Cells were permeabilized with 0.2% Triton X-100/PBS and blocked for 30 min with blocking buffer (2% bovine serum albumin, 0.2% Triton X-100, 0.05% Tween 20, and PBS). Coverslips were incubated with primary and secondary antibodies diluted in blocking buffer for 1 h and 40 min, respectively, at room temperature, and nuclei were counterstained with 4',6-diamidino-2-phenylindole. Coverslips were mounted with Immu-Mount (Thermo Shandon Inc., Pittsburgh, PA). Confocal images were taken using a Zeiss 510 Meta laser scanning confocal microscope (Carl Zeiss, Canada Ltd., Toronto, Ontario, Canada) with an ×100 objective. Image analysis was carried out using the LSM 5 image browser (Empix Imaging, Mississauga, Ontario, Canada).

Co-localization Studies—Quantification of co-localization between Met and c-Cbl or Met and Cbl-b was performed using MetaMorph software for object-based co-localization measurements. The results were logged into Excel for analysis.

RESULTS

Cbl Protein Levels Are Elevated following Inhibition of Met Kinase—To establish whether amplification of MET in the Okajima, MKN45, Snu-5, and KATO II gastric cancer cell lines leads to constitutive activation of Met protein (10), proteins from whole cell lysates were immunoblotted with Met and phospho-Met antibodies (Fig. 1, A and B). The phospho-Met antibody is specific for tyrosines 1234 and 1235 in the activation loop of the kinase domain. High levels of Met and phospho-Met were observed in all of the cell lines tested.

To examine the status of Cbl proteins, lysates were immunoblotted with antibodies raised to the C-terminal regions of c-Cbl and Cbl-b. Whereas the c-Cbl antibody is selective for immunoprecipitation and immunoblotting of c-Cbl (supplemental Fig. 1), some weak cross-reactivity of the Cbl-b antibody with c-Cbl was observed when c-Cbl was overexpressed in transient transfections (supplemental Fig. 1). However, in the gastric cancer cell lines used, the Cbl-b antibody detected only...
Cbl-b, as established by the difference in molecular mass of c-Cbl (110 kDa) when compared with Cbl-b (125 kDa).

Immunoblotting with c-Cbl and Cbl-b antibodies demonstrated low levels of c-Cbl protein in all gastric cancer cell lines tested as well as variable levels of Cbl-b (Fig. 1, A and B). Interestingly, when Met kinase activity was inhibited with a small molecule Met kinase inhibitor (0.1 μM PHA-665752) for 4, 8, or 16 h, a pronounced increase in c-Cbl protein levels was observed in all cell lines, and quantitative real-time PCR was performed to ascertain levels of c-Cbl and Cbl-b mRNA. PHA-665752 is a small molecule inhibitor that specifically targets Met, and inhibition of unrelated kinases occurs only at an IC₅₀ ≥ 2.5 μM (38). Quantitation of c-Cbl and Cbl-b protein levels from three experimental replicates revealed an inverse correlation between Met kinase activity and c-Cbl or Cbl-b levels in each cell line (supplemental Fig. 2).

To determine whether the change in Cbl protein levels, upon the addition of Met inhibitor, occurs through enhanced transcription, RNA from cells treated or not with Met inhibitor was isolated and reverse-transcribed, and the c-Cbl and Cbl-b levels were determined using quantitative real-time PCR. At 16 h post-treatment with Met inhibitor, a time at which c-Cbl protein levels were maximally elevated, no significant increases in c-Cbl mRNA were observed (Fig. 1, C and D), when compared with the DMSO control. Hence, differences in Cbl protein levels upon Met inhibitor treatment were not due to an increase in CBL transcription.

To confirm that the increase in Cbl protein level was Met-dependent, and not due to off-target effects of the inhibitor, knockdown of Met protein was executed with siRNA in KATO II cells (Fig. 1E). Upon depletion of Met protein, c-Cbl levels increased (Fig. 1E), confirming the dependence of Cbl protein loss on Met.

As c-Cbl and Cbl-b can heterodimerize (21, 22), we assessed whether this might play a role in their stability. To address whether Cbl-b influences c-Cbl stability and vice versa, c-Cbl or
Cbl-b protein levels were depleted with targeted siRNA. Neither Cbl-b nor c-Cbl knockdown significantly altered steady-state stability levels of the other protein (supplemental Fig. 3).

**Cbl Levels are Independent of Src Kinase Activity**—Overexpression of activated Src kinase has been shown to lead to a decrease in Cbl protein levels (39). Src phosphorylation has also been demonstrated downstream of activated Met kinase (40). To test whether Met-dependent Cbl loss requires Src, cells were treated with the Src inhibitor PP2 (10 μM), a Met inhibitor (0.1 μM PHA-665752), or both. In gastric cancer cells with amplified MET, Src kinase is constitutively active, as evident by the basal tyrosine phosphorylation of Src Tyr-418 (Fig. 2). Interestingly, treatment with the Met inhibitor did not significantly decrease Src tyrosine phosphorylation, and inhibition of Src with PP2, as evident by the loss of Tyr-418 phosphorylation, did not significantly alter Met tyrosine phosphorylation (Fig. 2). Thus, the basal Src activation observed in these gastric cancer cell lines appears independent of Met kinase activity. Importantly, the level of c-Cbl or Cbl-b protein did not decrease upon inhibition of Src kinase and was elevated only following inhibition of Met kinase (Fig. 2). Moreover, inhibition of both Met and Src kinases increased the amount of Cbl proteins to a level comparable, but not exceeding, that with Met inhibitor alone. Hence, together these data support the conclusion that in these gastric cancer cell lines, Cbl protein levels are dependent on the kinase activation status of Met and not Src.

**Transient Overexpression of Met Promotes Loss of Cbl-b**—To elucidate the requirements through which Met promotes loss of Cbl in gastric cancer cell lines, a transient transfection and structure-function approach was undertaken. Met was initially expressed at increasing concentrations with either HA-tagged c-Cbl or Cbl-b (Fig. 3A). In HEK 293 cells, overexpression of Met protein alone is sufficient to activate Met kinase (Fig. 3A) (36) and constitutive phosphorylation of Met. Increasing levels of Met expression resulted in a significant decrease in Cbl-b protein levels, whereas c-Cbl protein levels were largely unaffected under these conditions (Fig. 3A). Tpr-Met, the truncated, constitutively active, cytoplasmic variant of Met, also induced a dramatic loss in Cbl-b protein upon transient co-expression (Fig. 3B). As expected, the overexpression of either c-Cbl or Cbl-b promoted an efficient loss of total Met protein and a corresponding decrease in phospho-Met (pTyr-1234/1235) levels (Fig. 3A) (27). However, neither c-Cbl nor Cbl-b overexpression promotes loss of Tpr-Met, as Tpr-Met lacks Tyr-1003, the binding site for the Cbl TKB domain (27) (Fig. 3B).

Quantitation of c-Cbl and Cbl-b mRNA levels in the absence and presence of Met show that Cbl-b mRNA levels are not decreased upon transient co-expression with Met (supplemental Fig. 4A). Thus, this finding supports our data in gastric cancer cell lines, demonstrating that a decrease in Cbl protein levels in the presence of active Met protein is not due to alterations in the level mRNA. Co-expression of either c-Cbl or Cbl-b with increasing levels of EGFR or activated Src (Src Y527F) resulted in a decrease in both c-Cbl and Cbl-b protein levels (Fig. 4, C and D), corroborating previously published data (39, 41). Because transient Met expression promoted a significant and consistent loss of Cbl-b protein levels, structure-function studies were carried out with Cbl-b.

**Met-dependent Cbl-b Loss Requires Met Kinase Activity and Intact Cbl-b TKB and RING Domains**—As a means to validate the requirement for Met kinase activity in promoting Cbl loss, wild-type Met and a mutant unable to promote transfer of the γ-ATP (kinase-dead K1110A) constructs were expressed alone or with HA-tagged Cbl-b. Co-expression of wild-type Met and Cbl-b resulted in a loss of both Met and Cbl-b protein (Fig. 4A). However, this loss was not observed upon co-expression of Met K1110A with Cbl-b (Fig. 4A), demonstrating the dependence of Cbl-b protein loss on Met kinase activity.

To determine the regions or domains of Cbl-b required for Cbl-b loss, a panel of HA-tagged Cbl-b mutants (Fig. 4B) consisting of wild-type Cbl-b, a Cbl-b unable to bind Met through its TKB domain (G298E), an E3 ligase-dead mutant (Cbl-b C373A), and multiple truncation mutants that excise the UBA
domain (Cbl-b DUBA), proline-rich regions, and/or key tyrosine residues (Tyr-665/Tyr-709) (Cbl-b-(1–483), -(1–541), -(1–603), -(1–664), and -(1–821)) were examined for their stability when expressed in HEK 293 cells alone or with wild-type Met. With the exception of the G298E and C373A mutants, in the presence of Met, the protein levels of each Cbl-b construct were decreased as compared with their expression levels in the absence of Met (Fig. 4C). Cbl-b G298E, which abrogates TKB function, uncoupling Met-Cbl-b direct interaction, and Cbl-b C373A, which lacks functional E3 ligase activity in the RING domain, did not exhibit protein loss upon co-expression with Met, indicating a necessity for TKB and RING function in promoting Cbl-b loss downstream from Met (Fig. 4C). In support of this observation, when c-Cbl or Cbl-b were transiently expressed in HEK 293 cells with Met and FLAG-tagged ubiquitin, increased ubiquitination of Cbl-b, as compared with c-Cbl, was observed, an effect that was enhanced upon treatment with the proteasome inhibitor lactacystin prior to cell lysis (supplemental Fig. 6). The low level of c-Cbl ubiquitination following transient transfection substantiates the lack of c-Cbl protein loss, when compared with Cbl-b, upon overexpression with Met.

Cbl-b is in part recruited to Met indirectly through the Grb2 adaptor protein (27). The requirement for indirect association of Met and Cbl-b (via Grb2) for Met-dependent Cbl-b loss was also assessed. The C-terminal half of Cbl-b contains six putative Grb2 binding sites. Three of these sites were absent in Cbl-b-(1–664), and the remaining three sites were mutated through site-directed mutagenesis (Cbl-b-(1–664) 9PA). This construct, as demonstrated through GST-Grb2 pulldown (Fig. 4F), is dramatically impaired in its ability to bind Grb2 as compared with wild-type Cbl-b. The protein levels of Cbl-b-(1–664) 9PA decrease in the presence of Met (Fig. 4E), demonstrating that recruitment of Grb2 is dispensable for Cbl-b protein loss. To our knowledge, this is the first demonstration of these three Grb2 binding sites on Cbl-b. Altogether, this demonstrates that the C-terminal UBA domain on Cbl-b, as well as the proline-rich regions and tyrosines 665 and 709, is dispensable for Met-dependent Cbl-b protein loss after transient transfection. Furthermore, as Cbl-b ubiquitination was observed and Cbl-b E3 ligase activity is required, these data support the conclusion that that Cbl-b loss may occur through self-ubiquitination.

**Met-dependent Cbl Loss Requires Proteasome Activity**—Basal activation of Met kinase in the gastric cancer cell lines correlates with low levels of Cbl proteins. This suggests that Met signaling may target Cbl protein for degradation. To test whether Cbl loss was induced in response to Met activity, HEK 293 cells, which stably express a doxycycline-inducible Met, were transiently transfected with HA-Cbl-b and stimulated with HGF (supplemental Fig. 6A). By 2 h of HGF stimulation, Cbl-b levels decreased when compared with the unstimulated control (supplemental Fig. 6A). To determine whether Cbl-b protein was being degraded through the lysosome or the proteasome, inhibitors (concanamycin or lactacystin, respectively) for both were used. Cells were pretreated with concanamycin or lactacystin for 4 h prior to HGF stimulation. The addition of concanamycin did not affect Cbl-b loss with HGF stimulation, whereas the presence of lactacystin partially rescued Cbl-b levels (supplemental Fig. 6A). In Snu-5 and KATO II cells, lactacystin treatment resulted in an increase in c-Cbl levels, as shown in Fig. 5, A and B. Quantitation of three independent experiments demonstrates that upon Met inhibition, c-Cbl protein levels increased 2.5-fold (Fig. 5C). The same trend was observed in Okajima and MKN45 cells (supplemental Fig. 6B). Altogether, these data implicate a role for the proteasome in Cbl degradation downstream from kinase-active Met.

Considering that c-Cbl protein levels are increased upon inhibition of the proteasome, we assessed whether c-Cbl is ubiquitinated under basal conditions in gastric cancer cells. To this end, KATO II cells were treated overnight (for 16 h) with Met inhibitor followed by treatment with lactacystin for 8 h. Subsequent to cell lysis, c-Cbl proteins were immunoprecipitated under denaturing conditions to uncouple the associated proteins, and ubiquitination was detected using anti-ubiquitin
antibodies (P4D1 and antibodies raised to Lys-48-linked polyubiquitin (D9D5)). c-Cbl is ubiquitinated in the presence of active Met, which is diminished following the inhibition of Met, even though c-Cbl protein levels are elevated (Fig. 5, F and G). The comparable patterns of total ubiquitin and Lys-48-linked polyubiquitin indicate that c-Cbl is polyubiquitinated and contains Lys-48-linked chains (Fig. 5F). As Lys-48-linked chains are thought to promote proteasomal degradation, and c-Cbl ubiquitination is most apparent where Met is active and following treatment with lactacystin (Fig. 5F), we concluded that Met activation promotes c-Cbl polyubiquitination and subsequent degradation by the proteasome.

In gastric cancer cells, c-Cbl co-immunoprecipitates with Met and is tyrosine-phosphorylated, both of which events are decreased in the presence of Met inhibitor and are hence dependent on Met kinase activity (Fig. 5E). In comparison, although Cbl-b can co-immunoprecipitate with Met in gastric cancer cell lines, Cbl-b tyrosine phosphorylation was not
observed (supplemental Fig. 6C). In support of the enhanced association of c-Cbl with Met, c-Cbl co-localizes with Met both at the membrane and in punctate structures in MKN45 gastric cancer cells (supplemental Fig. 7B). Moreover, we observed 64% co-localization of c-Cbl protein with Met by immunofluorescence as compared with only 34% of the Cbl-b protein (supplemental Fig. 7A). Hence, the enhanced association of Met and c-Cbl compared with Cbl-b in gastric cancer cells, as observed by immunofluorescence, likely contributes to the enhanced ubiquitination and decreased stability of c-Cbl over Cbl-b protein levels.

EGFR Degradation and Cbl Recruitment Are Impaired in MET-amplified Gastric Cancer Cell Lines—Cbl proteins negatively regulate multiple receptor tyrosine kinases including EGFR (reviewed in Ref. 42). Hence, to investigate whether Met-dependent Cbl loss in gastric cancer cell lines augments the stability and/or signaling of other kinases, we assessed the stability of EGFR in MKN45 cells upon EGF stimulation in the presence or absence of Met inhibitor (Fig. 6A). Although stimulation with EGF promotes EGFR protein loss, EGFR loss is statistically significant only in the presence of the Met inhibitor, when Cbl levels are elevated, and not in control cells (Fig. 6B).

To establish whether the enhanced degradation of EGFR in MKN45 cells in the presence of EGF and the Met inhibitor corresponds with increased association between Cbl and EGFR, the ability of Cbl and EGFR to co-immunoprecipitate was investigated. Under basal conditions in which Met is active, c-Cbl co-immunoprecipitates with a phospho-protein corresponding to the molecular weight of Met, which disappears upon treatment with the Met inhibitor (Fig. 6C). Cbl-b also co-immunoprecipitates with active Met (Fig. 6D). Interestingly, upon stimulation with EGF, phospho-EGFR predominantly co-immunoprecipitates with Cbl-b (Fig. 6, C and D). Notably, this EGF-dependent association of Cbl-b and EGFR is significantly enhanced when Met kinase activity is inhibited (Fig. 6D). c-Cbl also co-immunoprecipitates with EGFR following stimulation with EGF, and although this is enhanced following inhibition of Met, this co-immunoprecipitation is consistently less that with Cbl-b (Fig. 6C). Hence, under conditions where Met is inhibited and Cbl protein levels are increased, Cbl is able to more effi-
ciently target EGFR for degradation due to enhanced association with EGFR and decreased competition for Cbl binding from Met.

**DISCUSSION**

Protein tyrosine kinases act as molecular switches to control a variety of cellular signals, and their dysregulation contributes to many human malignancies. Notably, members of the Cbl family can serve as negative regulators for many tyrosine kinases, and loss of Cbl-dependent negative regulation is recognized as a mechanism that contributes to tumorigenesis (43). Here we have demonstrated Met-dependent loss of Cbl protein as a mechanism to decrease Cbl-mediated negative regulation of RTKs in human cancer. Although other reports have detailed the loss of Cbl protein downstream from EGFR and Src kinases (39, 41, 44), this is the first report to demonstrate that Cbl protein loss is dependent on an activated Met kinase in human tumors.

The mechanism of Cbl loss downstream from Met is dependent on an intact Cbl TKB domain and RING domain. This is distinct from Src-dependent Cbl loss, which occurs independently of the Cbl RING domain (Table 1) (39). Furthermore, Src-dependent Cbl loss requires tyrosines Tyr-700, Tyr-731, and Tyr-774 in the C-terminal half of Cbl (39, 45), of which the equivalent tyrosines (Tyr-665 and Tyr-709) are dispensable for Met-dependent Cbl-b loss (Fig. 4). In support of this, we have demonstrated that Met-dependent c-Cbl or Cbl-b loss in multiple gastric tumor cell lines is independent of Src kinase activity. In a manner similar to Met, Cbl-b loss downstream from the

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**FIGURE 6. Met-dependent Cbl loss releases EGFR from Cbl-targeted degradation.** A, MKN45 cells were treated with Met inhibitor (36 h) and then stimulated or not with EGF (100 ng/ml) for 1 h. Proteins were immunoblotted with specific antibodies. B, EGFR protein levels were quantified from five representative experiments. EGFR levels were normalized to actin levels, and the means ± S.E. were plotted. *, statistical significance of p < 0.04 was determined using Student's t test. C and D, lysates from A were immunoprecipitated with antibodies specific to c-Cbl (C) or Cbl-b (D) and the co-immunoprecipitated proteins separated by SDS-PAGE, and immunoblotted with the designated antibodies. Sizes of the molecular weight markers are depicted on the left, and the arrows designate the migration of phospho-proteins corresponding to Met and EGFR.

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| Structural requirements for Cbl loss downstream from various tyrosine kinases |
| --- |
| **TKB** | **RING** | **C-terminal half** |
| Required | Not required | Required |
| Required | Required | Required |
| Required | Required | Not required |
| Required | Required | Required |
| Required | Required | Required |
| Required | Required | Required |
| **Proteasome or Lysosomal** |
| Both | Proteasome (inferred) | Proteasome |

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**TABLE 1**

| Structural Elements of Cbl required for downregulation: |
| --- |
| **TKB** | **RING** | **C-terminal half** |
| Required | Not required | Required |
| Required | Required | Required |
| Required | Required | Not required |
| Required | Required | Required |
| Required | Required | Required |
| Required | Required | Required |

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**EGFR**

Also requires both intact Cbl TKB and RING domains and not C-terminal proline-rich or UBA regions (41).

Inhibition of the proteasome rescues Cbl levels downstream from Met kinase. This supports the requirement for an intact Cbl E3 ligase function whereby Cbl is auto-ubiquitinated following Met activation. Consistent with this understanding, c-Cbl ubiquitination is elevated in gastric cancer cell lines where Met is activated. Similarly, ubiquitination of Cbl has been
observed downstream from several activated kinases including EGFR, Src, and CSF-1 receptor (39, 41, 46). Met kinase activity is required for Cbl loss (Figs. 1 and 4A). Tyrosine phosphorylation of Cbl is required for activation of its E3 ligase (47, 48), and mutants that either remove the tyrosine phosphorylation site in the linker region of Cbl or promote loss of ligase activity are increasingly found in tumors (49–53). Hence, the ubiquitination of Cbl, subsequent to constitutive Met kinase activation, may be a negative feedback mechanism regulating c-Cbl stability in human tumors. Other E3 proteins have been shown to self-ubiquitinate and target themselves for degradation, and regulation of Cbl activity, by mediating its stability, has been documented previously to occur (reviewed in Ref. 44).

We show a preferential loss of c-Cbl protein over Cbl-b downstream from Met in multiple gastric cancer cell lines. This difference in specificity is reflected by enhanced association of Met with c-Cbl in gastric cancer cell lines over Cbl-b and elevated ubiquitination of c-Cbl under these conditions. Alternatively, following transient co-transfections, we observed a preferential Met-dependent decrease in Cbl-b over c-Cbl protein levels, which correlated with enhanced ubiquitination of Cbl-b under these conditions. Hence, Met-dependent Cbl loss correlates with preferential association of Met with c-Cbl versus Cbl-b under different conditions. Although the basis for this is unclear, Cbl-b is preferentially targeted for ubiquitination and degradation in T cells in response to CD28 stimulation of TCR (reviewed in Ref. 23), whereas upon treatment of chronic myeloid leukemia patients with the Bcr/Abl inhibitor imatinib, c-Cbl expression and protein levels increase, and Cbl-b levels decrease (23, 54). Moreover, c-Cbl and Cbl-b show distinct temporal association with the EGFR following stimulation with EGF (26), supporting the idea that c-Cbl versus Cbl-b may be enriched in distinct subcellular compartments.

Constitutive Met activation following amplification in gastric cancers is not the result of an autocrine loop, as HGF mRNA is undetectable in these cell lines (10). Moreover, sequencing of Met has not yet revealed the presence of known activating mutations in MKN45, Snu-5, and KATO II gastric cancer cell lines where MET is amplified (10). Thus, the loss of Cbl protein and negative regulation of Met may be a contributing factor enhancing Met prolonged activation in the absence of ligand. Tpr-Met, an oncogenic Met variant, is the result of a genomic rearrangement, where a leucine zipper dimerization domain promotes enforced dimerization of the Met kinase in the absence of ligand, driving constitutive activation and phosphorylation (2, 55). However, another mechanism contributing to the transforming ability of Tpr-Met is the uncoupling from Cbl-mediated negative regulation (31). Tpr-Met is cytoplasmically located, lacks the juxtamembrane Tyr-1003, and is thus unable to associate with Cbl, enter the endocytic pathway, and be degraded efficiently (31). Restoration of the Cbl binding site, membrane localization, and Cbl expression are required for the down-regulation of Tpr-Met and suppression of transformation (31). In murine models, wild-type or mutated Met (M1250T) under the murine mammary tumor virus (MMTV) promoter are weakly transforming (56). However, mice expressing a Met receptor with both M1250T and Y1003F mutations, thereby diminishing the ability of Cbl to bind and ubiquitinate Met, exhibit a greater penetrance and a shorter latency (~100 days less) (56), demonstrating that the loss of Cbl-mediated negative regulation enhances Met oncogenic capabilities. Interestingly, the human gastric cancer cell line Hs746T expresses a mutated Met receptor (MetΔexon14) that lacks the direct Cbl binding site (Tyr-1003) in addition to amplification of MET (35). In these cells, inhibition of constitutively active Met does not impact c-Cbl or Cbl-b protein levels (data not shown), supporting the idea that Met-dependent
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Cbl loss requires association between the two proteins and highlighting that selection can occur for alternative mechanisms that uncouple Met from Cbl. Moreover, in lung cancers where tested, more than 80% of the tumors with c-CBL genomic changes also exhibit mutations in either MET or EGFR (52). Exogenous expression of c-Cbl in lung cancer cells can also inhibit tumor growth and metastasis in a xenograft mouse model (57). Thus, taken together, these findings support the idea that the Met-dependent loss of Cbl proteins exhibited in the four gastric cancer cell lines examined here (Okajima, MKN45, Snu-5, and KATO II) would lead to enhanced dysregulation of MET and, potentially, other Cbl targets.

Notably, in the four gastric cancer cell lines tested, Met-dependent suppression of Cbl levels augments the stability of other Cbl target proteins such as EGFR (Fig. 6). This has important implications for RTK cross-talk, whereby activation of Met can lead to tyrosine phosphorylation and activation of EGFR and vice versa (43). Hence, Met-dependent Cbl loss in gastric cancers thereby provides a mechanism through which Met activation can indirectly enhance EGFR signaling (Fig. 7). Met may also promote EGFR stability by virtue of its overexpression, thereby sequestering Cbl away from EGFR. Thus, in these cancer cells, Met recruitment of Cbl and targeted down-regulation may take advantage of a normal feedback mechanism to regulate Cbl, creating a platform of activated RTKs that together contribute to the potentiation of oncogenic signaling.

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